A single WW domain is the predominant mediator of the interaction between the human ubiquitin-protein ligase Nedd4 and the human epithelial sodium channel

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The activity of the epithelial Na⁺ channel (ENaC) is required for the maintenance of salt and water balance in the body. Channel activity is regulated by the ubiquitin-protein ligase Nedd4 ['neuronal precursor cell-expressed developmentally down-regulated (gene 4)'] that interacts with the channel via its WW domains. Mutations in channel subunits that disrupt this interaction cause Liddle's syndrome, a severe inherited form of hypertension. In previous studies we showed that WW domains 2, 3 and 4 of human Nedd4 bound to the human ENaC (hENaC) subunits, whereas WW domain 1 did not. Here we extend this observation to determine the binding affinities of the human Nedd4 WW domains for hENaC C-terminal peptides. We show that WW domains 2, 3 and 4 bind with differing affinities to Na⁺

channel subunit peptides. WW domain 3 has the highest affinity and we predict that WW domain 3 contributes most of the binding because a construct containing the three WW domains bound no better than WW domain 3 alone. Further, a single amino acid change (Arg¹⁶⁵ → Thr) in WW domain 1 enables binding to the α subunit of the channel to occur, with an affinity comparable with that of WW domain 4. Differential binding propensities between the various WW domains and Na⁺ channel subunit peptides are explained on the basis of quantitative structural modelling of the complexes and their isolated components.

Key words: BIAcore, ENaC, surface plasmon resonance.

INTRODUCTION

The epithelial Na⁺ channel (ENaC) complex is located in the apical membranes of epithelial cells in the kidney, lung, colon and sweat gland, where it functions to regulate the movement of Na⁺ ions across the epithelial cell layer [1]. The Na⁺ channel is made up of three subunits: α , β and γ . Mutations in the epithelial Na⁺ channel subunit genes have severe effects. Loss-of-function mutations in any of the subunit genes cause salt wasting and a pseudo-hypoaldosteronism type I phenotype, both in humans [2] and in mouse models [3,4]. Liddle's syndrome, a severe inherited form of hypertension, is caused by a gain-of-function mutation in the cytoplasmic C-terminal region of either the β or γ human ENaC (hENaC) subunit gene (β hENaC and γ hENaC) [5,6]. *In vitro* expression of hENaC proteins containing Liddle's mutations leads to increased channel activity [7,8]. The regulatory process that has been disrupted in Liddle's syndrome is most likely to be the lack of retrieval and degradation of channels from the plasma membrane [9,10], potentially through disruption of the ubiquitination process. To support this hypothesis it has been demonstrated that the α and γ subunits of the channel become ubiquitinated, and that mutation of the N-terminal lysines required for covalent modification by ubiquitin leads to increased channel activity [11]. Ubiquitination of substrate proteins requires a series of enzymes including a substrate-

specific E3 ubiquitin-protein ligase. We, and others, have shown that Nedd4 ['neuronal precursor cell-expressed developmentally down-regulated (gene 4)'], an E3 ubiquitin-protein ligase, binds to and regulates ENaC activity [9,10,12,13]. Co-expression of human Nedd4 (hNedd4) with the Na⁺ channel in *Xenopus* oocytes potentially inhibits channel activity [12]. This is consistent with the hypothesis that Nedd4 facilitates addition of ubiquitin to Na⁺ channel subunits, leading to endocytosis and degradation of the channel proteins.

The hNedd4 protein contains four WW domains, small interaction modules containing 38–40 amino acids, including two conserved tryptophans, which mediate binding to the Na⁺ channel. WW domains have been grouped into four classes according to their ligand preference [14]. The WW domains of Nedd4, along with those of dystrophin and YAP65, are grouped together as class-I WW domains and they bind to ligands containing a PPXY motif (PY motif). Structural analysis of WW domains shows that they form a compact anti-parallel three-stranded β -sheet structure, which forms hydrophobic pockets into which the proline residues of the ligand specifically fit [15–17].

The WW-domain-binding motif, or PY motif, is present in the C-terminal region of each ENaC subunit. Previously, using *in vitro* binding assays, we showed that WW domains 2, 3 and 4 (WW2, WW3 and WW4) of hNedd4 individually bound to each

Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; ENaC, epithelial Na⁺ channel; hENaC, human ENaC; α hENaC, β hENaC and γ hENaC, α , β and γ subunits of hENaC; GST, glutathione S-transferase; PY motif, PPXY motif; Nedd4, neuronal precursor cell-expressed developmentally down-regulated (gene 4); hNedd4, human Nedd4; SPR, surface plasmon resonance; SH3, Src homology 3; WW1, 2, 3 or 4, WW domain 1, 2, 3 or 4; RMS, root mean square.

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of the hENaC subunits [12]. The PY motif is required for Nedd4 binding since binding was abolished in a β subunit containing a mutation within this motif (Tyr⁶²⁰ → Ala) [12]. The mutated β subunit also showed increased channel activity [7], and this motif is removed or mutated in Liddle's syndrome.

To determine whether the hNedd4 WW2, WW3 and WW4 domains bound with equal affinity to the Na⁺ channel subunits, we carried out surface plasmon resonance (SPR) binding analysis using immobilized Na⁺ channel subunit C-terminal peptides containing the PY motif, and purified hNedd4 WW-domain proteins. WW domain 1 (WW1) from human and mouse Nedd4 does not bind to channel subunits [12,18]; however, WW1 of rat Nedd4 does bind the channel subunits [13]. After comparing the WW1 amino acid sequences, we also investigated the binding efficiency of a human WW1 mutant (WW1-Arg¹⁶⁵ → Thr), designed to mimic the WW1 domain from rat Nedd4.

EXPERIMENTAL

Cloning, expression and purification of WW-domain fusion proteins

Individual WW domains of hNedd4 were cloned into the pGEX2TK vector to produce glutathione S-transferase (GST) fusion proteins as described previously [12]. The WW1 construct with the Arg¹⁶⁵ → Thr mutation was prepared using overlap-extension PCR. All the GST fusion proteins were expressed and purified as described previously [12]. To release the WW domains from the GST moiety, 50 units of thrombin protease (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) and 950 μ l of PBS were added for each 1 ml of glutathione-Sepharose bed volume. Cleavage took place at 22 °C for 4 h. A six-histidine-tagged construct containing WW2, WW3 and WW4 (spanning amino acids 290–466 of hNedd4) was prepared in the pProEX HTb vector (Invitrogen, Groningen, The Netherlands), and this fusion protein was expressed and purified following the manufacturer's protocol. The proteins were finally purified to apparent homogeneity (as judged by Coomassie Brilliant Blue-stained SDS/PAGE), and exchanged simultaneously into HBS-EP buffer [10 mM HEPES/150 mM NaCl/3 mM EDTA/0.005% P20 (non-ionic surfactant polysorbate 20; BIA core AB, Uppsala, Sweden; #BR-1000-54), pH 8.0] by size-exclusion chromatography using a Superdex-75 column (Amersham Pharmacia Biotech). Protein concentration was measured using absorbance at 280 nm [19].

Measurement of WW-domain binding to hENaC peptides using SPR

Three peptides were synthesized containing the PY motif of the C-terminal cytosolic tail of the hENaC subunits: the α subunit of hENaC (α hENaC), NH₂-TAPPPAYATLG-CONH₂; β hENaC, NH₂-GTPPPNYDSLRL-CONH₂; γ hENaC, Biotin-GTPPPKY-NTLR-CONH₂ (Auspep, Parkville, Victoria, Australia). For SPR experiments using a BIAcore 2000 system (BIAcore AB) [20,21], the α and β peptides were attached either via the N-terminal amino group or via a C-terminal arginine side chain to a carboxylated surface (sensor chip CM5) using standard amine immobilization chemistry. Briefly, the carboxy surface was activated with 1-(3-diethylaminopropyl)-3-ethylcarbodi-imide and *N*-hydroxysuccinimide. Peptides were dissolved in 100 mM sodium borate buffer, pH 8.6, to give a final concentration of 300–1000 μ g/ml. This solution was injected over the activated carboxylated surface at a flow rate of 5 μ l/min, and unreacted surface esters were subsequently blocked with 1 M ethanolamine. The amount of peptide immobilized was controlled by manually

varying the contact time. The γ hENaC peptide was dissolved in HBS-EP to give a final concentration of 2.2 μ g/ml and immobilized via its N-terminal biotin to the streptavidin on the surface of sensor chip SA. Interaction studies were carried out by injecting WW-domain-containing proteins over this surface at 20 μ l/min, using HBS-EP as a running buffer. The signal from a blank flowcell, which had been activated and blocked as above, but to which no peptide had been immobilized, was subtracted from all SPR measurements to correct for bulk solvent effects. The affinity constant, K_D , was estimated by evaluation of steady-state affinity data from a series of different concentrations of WW domains, using the formula $R_{eq} = CR_{max}/(Cn + 1/K_D)$, where R_{eq} is the steady-state binding response, C is the protein concentration, R_{max} is the theoretical maximum binding capacity of the surface and n is the steric-interference factor, which specifies how many binding sites are blocked, on average, by the binding of one analyte molecule.

Molecular modelling of hNedd4 WW domains bound to hENaC peptides

Homology models of the individual WW domains were obtained via the Swiss-Model Protein Modelling Server [22], based on the 'prototype WW domain' (PDB accession number 1EOM; [23]) and refined using the Swiss-Pdbviewer [24] and Maestro[®] version 2.1Beta (Schrödinger, Portland, OR, U.S.A.). Na⁺ channel peptides were introduced into the model based on the location of the β -dystroglycan peptide binding to dystrophin (PDB accession number 1EG4); the β -dystroglycan peptide was truncated and residues were mutated to give the required hENaC peptides. The β hENaC peptide was also modified to include the C-terminal helical motif reported by Kanelis et al. [17]. The WW-domain-hENaC peptide complexes were solvated using the generalized Born/solvent-accessible surface-area model [25] and subjected to sequential unrestrained stochastic dynamics (10 ps at 400, 300, 200 and 100 K). Representative structures were sampled during the 400 and 100 K dynamics simulations and minimized to 0.005 kJ · mol⁻¹ · Å⁻¹ using a Polak-Ribiere conjugate gradients algorithm under the Amber94 forcefield [26]. The lowest-energy structures obtained were used in all subsequent calculations. Lowest-energy structures of the isolated WW domains and hENaC peptides were obtained in the same fashion. Binding energies for the complexation of the WW domains by the hENaC peptides were calculated from the energy differences between the isolated and complexed species.

RESULTS

Binding of individual hNedd4 WW domains to hENaC

The hNedd4 protein contains four WW domains, which share 29% amino acid sequence identity overall, whereas the mouse and rat Nedd4 proteins contain only three WW domains. Comparison of the sequences of individual human WW domains with their mouse and rat equivalents suggests that WW3 of the hNedd4 protein is unique (Figures 1A and 1B). Binding experiments *in vitro* showed that WW2, WW3 and WW4 of hNedd4 all bound to full-length hENaC proteins. However, WW1 does not bind to any of the hENaC subunits [12,18,27].

We have used SPR to characterize the individual contributions made by the four WW domains of hNedd4 to its binding to the hENaC subunits. Peptides corresponding to the proline-rich PY motifs contained in the C-terminal cytosolic domains of each of the three hENaC subunits, previously identified as the target sites for Nedd4 WW-domain binding, were immobilized on to sensor chips. Figure 1(C) shows the regions of hENaC subunits used

Figure 1 Nedd4 WW-domain comparison and ENaC WW-domain-binding regions

(A) Multiple sequence alignment of the WW domains of Nedd4 from human (h), rat (r) and mouse (m). (B) Tree of WW domains drawn by the neighbour-joining method, using percentage amino acid identity. (C) Multiple sequence alignment of the C-terminal PY-motif region from human, rat and mouse ENaC subunits. Figure drawn using Jalview (available from <http://www.compbio.dundee.ac.uk/Software/JalView/jalview.html>).

and a comparison with the equivalent region in both rat and mouse ENaC subunits. Low surface densities (≈ 50 – 250 response units, equivalent to ≈ 0.04 – 0.20 pmol $\cdot$ mm $^{-2}$) were used in order to minimize the effects of mass transport on the measurements. The peptides were immobilized using strategies that ensured they were attached to the chip surface only via one terminus to reduce ambiguities arising from different coupling conformations.

Trial experiments to measure WW-domain binding affinities were performed using GST–WW-domain fusion proteins. However, sensorgrams obtained using intact fusion proteins were poorly reproducible and were uninterpretable using kinetic models, probably due to variable sample heterogeneity caused by fusion-protein dimerization in solution (results not shown). We therefore carried out detailed experimental analysis using WW domains cleaved from their GST fusion partners and subsequently purified by size-exclusion chromatography.

Individual hNedd4 WW domains, purified away from the GST component, bind to the peptides on the sensor chips with very high on and off rates, giving a ‘square wave’ binding curve, indicative of a low binding affinity (Figure 2A). Measurement of K_D by the evaluation of steady-state binding measurements for a WW-domain concentration series (Figure 2B) gave K_D values in the micromolar range (Table 1). To ensure an accurate estimation of these high K_D values, the maximum concentration of WW domain included in the concentration series was kept as high as practically possible. In the case of the weakest binding proteins (e.g. WW1–Arg 165 $\rightarrow$ Thr and WW4), the maximum protein concentration used was as close as possible to twice the estimated K_D . Where binding was so weak that it was not practically possible to measure well above the K_D (e.g. WW1), the calculated K_D can only be considered a minimum value.

To eliminate the possibility that the observed binding was non-specific, we performed the following control experiments. First, binding curves were recorded for a concentration series of an

unrelated protein of similar molecular mass, bovine pancreatic trypsin inhibitor (BPTI). The BPTI injections showed a slight concentration-dependent increase in SPR signal, but gave calculated apparent K_D values in the millimolar range (Table 1). To confirm that this millimolar binding represented a non-specific binding event, we performed competition experiments, where free hENaC C-terminal peptide was mixed with WW-domain protein before being injected over the chip. The binding of WW domains was decreased in the presence of competing peptide, but the weak binding of BPTI remained constant, confirming the specific nature of the WW binding, and the non-specific nature of the BPTI binding (Figure 2C).

It was clear that WW3 bound with the highest affinity to all three Na⁺ channel peptides. The binding of WW2 and WW4 was significantly less than that of WW3 although WW2 appeared to bind with at least twice the affinity of WW4. WW1 of hNedd4 bound extremely weakly to the α hENaC peptide, which is consistent with our previous results [12]. Competition experiments, as described above, indicated that the observed weak binding was specific; however, it was too weak to measure accurately. The estimated K_D of the WW1– α hENaC interaction was certainly greater than 500 μ M.

The rank orders of binding affinities of the individual hNedd4 WW domains for the α hENaC and β hENaC peptides were the same: WW3 $\gg$ WW2 $>$ WW4. The calculated binding affinities for the γ peptide were significantly weaker than those of the α and β peptides; however, WW3 also bound with the highest affinity to the γ peptide.

Binding of WW2, WW3 and WW4 to hENaC

We investigated whether a construct containing three WW domains could bind co-operatively to the Na⁺ channel peptides. A construct containing hNedd4 WW2, WW3 and WW4

Figure 2 Response curves showing binding of hNedd4 WW domains to ENaC peptide

(A) Typical BIAcore response curves produced by injecting isolated WW3 domain over immobilized β hENaC C-terminal peptide, shown in triplicate. (B) Estimation of K_D from steady-state equilibrium binding measurements. The equilibrium SPR response, R_{eq} , was measured in triplicate for each concentration of WW3 shown in (A), in each case using the response curve between $t = 120$ s and $t = 180$ s. The solid line represents the best fit to the data using the equation described in the Experimental section ($\chi^2 = 0.34$). (C) Inhibition of WW2 binding to immobilized β hENaC C-terminal peptide by competition with excess free peptide. RU, response units.

Table 1 Dissociation constants of isolated hNedd4 WW domains to target C-terminal peptides from hENaC subunits

K_D values (in μ M) are shown as means $\pm$ S.E.M., derived from determinations performed in triplicate. Except where noted, the steric-hindrance factor, n , was 1. ND, not determined; RU, response units; WW1RT, WW1-Arg¹⁶⁵ $\rightarrow$ Thr; WW234, WW2, WW3 and WW4; BPTI, bovine pancreatic trypsin inhibitor.

Protein	Immobilized C-terminal peptide ...	Dissociation constant, K_D (μ M)			
		α Subunit (50 RU)	β Subunit (50 RU)	β Subunit (150 RU)	γ Subunit (250 RU)
WW1		> 500	ND	ND	ND
WW1RT		255 $\pm$ 12	ND	ND	ND
WW2		101 $\pm$ 5	113 $\pm$ 9	109 $\pm$ 3	> 5400
WW3		4.6 $\pm$ 0.4	11.1 $\pm$ 0.3	ND	> 221
WW4		148 $\pm$ 16	660 $\pm$ 50	ND	> 794
WW234		13.8 $\pm$ 1.3	64.8 $\pm$ 4.7	34.9 $\pm$ 2.2, $n = 1$ 69.9 $\pm$ 4.3, $n = 2$	> 100
BPTI		> 20 000	> 4000	ND	ND

Figure 3 Molecular models of hNedd4 WW domains binding α ENaC peptide

Predicted binding conformations of hNedd4 WW1 (A), WW1-Arg¹⁶⁵ → Thr (B), WW2 (C), WW3 (D) and WW4 (E) bound to the α ENaC C-terminal peptide. The WW-domain surfaces are coloured by electrostatic potential: blue, positive; red, negative; white, neutral. The peptides are oriented with their amidic C-termini uppermost.

(WW234), which all bound individually to the hENaC peptides, did not bind with higher affinity than the WW3 domain alone (Table 1). Increased affinity for the WW234 construct would have been expected if co-operative behaviour occurred between the WW domains. This result suggests that WW3 is the predominant mediator of the binding of the protein containing three WW domains, and that binding of a single WW domain, bringing the other WW domains into close proximity of further binding sites, does not enhance their binding, at least under the conditions used in this study. We also tested the effect of ligand surface density on the apparent K_D value of WW234 (Table 1). Increasing the surface density of the immobilized ligand 3-fold did not change the measured K_D of a single domain (WW2), but did decrease the apparent K_D of the three-domain construct if the steric-interference factor, n , was assumed to still be unity. If n was assumed to be 2 (i.e. each molecule of WW234 binds on average to two immobilized ligands at any one time) then the apparent K_D of WW234 did not change. This result suggests that significant co-operative binding does not occur. However, the density of Na⁺ channel peptides on the sensor chip is not saturating, and we cannot readily test whether the availability of

nearby peptides is limiting further WW-domain binding in the WW234 construct. In addition, *in vivo*, the orientation of the hENaC subunits in the Na⁺ channel complex may facilitate the binding of more than one hNedd4 WW domain, but our observations would argue against any strong co-operative behaviour.

A single amino acid change permits binding of WW1 to hENaC

A multiple sequence alignment of the WW1 domains from rat, mouse and human Nedd4 (Figure 1A) shows that a threonine at position 26 of the aligned domains in rat Nedd4, which binds the Na⁺ channel [13], corresponds to an arginine in both mouse and human WW1, neither of which bind to the Na⁺ channel. This is in addition to a proximal basic lysine residue at position 30, which is unique to, and conserved within, the WW1 family. To determine whether a change of the arginine would alter the binding of human WW1 we made an Arg¹⁶⁵ → Thr mutation in hNedd4 (numbering is based on the hNedd4 amino acid sequence described in [12]) and tested this protein for its binding affinity to the α hENaC peptide. The results (Table 1) show that WW1-

Table 2 Energy changes (ΔE) for the binding of hENaC C-terminal peptides to hNedd4 WW domains

Contributions to the overall binding energy are given for the α -subunit C-terminal peptide only. All energies are relative to the values for the WW1 α -subunit C-terminus simulation. ND, not determined; WW1RT, WW1-Arg¹⁶⁵ $\rightarrow$ Thr.

C-terminal peptide	Energy term	ΔE (kJ $\cdot$ mol ⁻¹)			
		WW1RT	WW2	WW3	WW4
α Subunit	ΔE Conformational distortion	4.9	-45.8	-12	-24.2
	ΔE van-der-Waals energy	-13.3	2.9	-53.6	14.6
	ΔE Electrostatic energy	219.0	-197.5	70.3	165.9
	ΔE Solvation energy	-231.1	173.7	-81	-172.1
	Binding energy = total ΔE	-20.5	-66.7	-76.3	-15.8
β Subunit	Binding energy = total ΔE	ND	-44.6	-74.8	-37.6
γ Subunit	Binding energy = total ΔE	ND	8.9	-17.3	-9.3

Arg¹⁶⁵ $\rightarrow$ Thr is able to bind the α hENaC C-terminal peptide, indicating that the presence of Arg¹⁶⁵ contributes to the inability of hNedd4 WW1 to bind to the Na⁺ channel.

Molecular-modelling analysis of the hNedd4-ENaC binding interactions

Modelling studies were carried out to determine the origins of the rank order of binding of the hNedd4 WW domains to hENaC and, in particular, to explain the differences in binding of the WW1 and WW1-Arg¹⁶⁵ $\rightarrow$ Thr domains to the α hENaC peptide. The models show the characteristic grooves formed by WW-domain aromatic residues into which the prolines of the ENaC peptides fit, and unidirectional binding of the peptide (Figure 3). The backbones of all minimized hNedd4 WW domains superimposed upon the backbones of the original prototype [23] and dystrophin WW-domain structures were fitted with a root mean square (RMS) deviation of 1.4 Å or less (1 Å $\equiv$ 0.1 nm).

The energy contributions to the binding process, including changes in conformation, van-der-Waals, electrostatic and solvation energies on binding of the α hENaC peptide amides, were determined. Values are reported in Table 2 for all systems where binding was established, and are reported relative to those obtained for the equivalent modelled binding interaction with WW1. The order of binding is in broad agreement with that determined experimentally from the BIAcore SPR analysis, i.e. in relative terms WW3 is predicted to bind most efficiently, whereas WW2, WW4 and WW1-Arg¹⁶⁵ $\rightarrow$ Thr are predicted to bind less well. It should be remembered that the calculated energies are essentially enthalpies, and do not consider entropic contributions to the free energy. This is in contrast to the experimentally determined binding affinities, and may explain some of the differences between observed and calculated binding.

From the α hENaC peptide-binding data, it is clear that there is no single governing factor determining the binding of the WW domains to the Na⁺ channel C-terminal peptides. Surface electrostatic potential plays a significant role, being responsible for both the electrostatic interaction energy between WW domains and their ligated peptides, and the compensating desolvation energies. The overall impact of these two energies is always one of stabilizing the WW-domain-peptide interaction. The strength of binding of WW3 arises from the intimacy of its contact with the α hENaC peptide. This is shown by the van-der-Waals energy change for this system, which is $\approx$ 40 kJ $\cdot$ mol⁻¹ lower than for any other WW-domain interaction.

The reason for the Arg¹⁶⁵ $\rightarrow$ Thr mutation acting as a binding switch was investigated using RMS deviations between superimposed atoms for the native and WW1-Arg¹⁶⁵ $\rightarrow$ Thr domains,

quantitatively from the binding-energy data (Table 2) and qualitatively from the surface electrostatic potentials of the two domains in their bound states. There were no gross differences in the conformations of WW1 and WW1-Arg¹⁶⁵ $\rightarrow$ Thr: all atoms of the two WW domains superimposed well with an RMS deviation of 0.56 Å. The α hENaC peptides from the two complexes also superimposed well, with an RMS deviation of 0.66 Å, although the structural alignment was poorer at the C-terminal end of the peptides. The calculated absolute values of solvation-energy changes for WW1 and WW1-Arg¹⁶⁵ $\rightarrow$ Thr upon α hENaC binding were 134.7 and -96.4 kJ $\cdot$ mol⁻¹, respectively. This is consistent with the WW-domain binding surface being polar in the native WW1 and considerably more hydrophobic in WW1-Arg¹⁶⁵ $\rightarrow$ Thr. The Arg¹⁶⁵ guanidinium group is unlikely to have a strong direct influence on the α hENaC peptide, as the shortest separation between the two is 8.1 Å. In order to visualize the effect of the replacement of the Arg¹⁶⁵ guanidinium group by the Thr¹⁶⁵ hydroxy group, the electrostatic potentials were mapped on to the solvent exclusion surface of the two WW domains, as shown in Figure 3. In order to emphasize the differences due to the presence or absence of the positively charged Arg¹⁶⁵, the surface electrostatic potential isosurfaces were calculated using contours at +1.8 NC⁻¹ (blue) and -4.8 NC⁻¹ (red).

The results from the modelling of the β hENaC subunit binding to WW domains were consistent with those for the α hENaC subunit. Calculations were attempted with both a dystrophin-like extended β hENaC C-terminus and the C-terminal helical motif reported by Kanelis et al. [17] (results not shown). Only results from models with the helical motif correlated well with experimental values.

Binding of the C-terminal peptide from the γ hENaC subunit to WW domains was also analysed by molecular modelling. The results were in accordance with the low affinities produced by the SPR analysis and may be rationalized on the basis of the presence of the charged lysine and arginine residues in the γ peptide. In contrast, the C-terminal peptides of the α and β subunits are electrically neutral.

DISCUSSION

The interaction between the hENaC and hNedd4 is important for regulation of Na⁺ transport because when the Nedd4-binding motif is disrupted, hypertension is the result [6]. However, the details of the interaction, such as how many WW domains of hNedd4 bind channel subunits *in vivo*, and whether other proteins are recruited into a complex with hENaC and hNedd4, are not

known. Our results show that the hNedd4 WW domains bind with differing affinities to hENaC peptides. We show that the rank order of hNedd4 WW-domain binding to α hENaC and β hENaC is WW3 $\gg$ WW2 > WW4. WW3 binds to Na⁺ channel peptides with a dissociation constant that is an order of magnitude lower than that of the other WW domains, i.e. its association constant is an order of magnitude higher. Binding of a protein containing three of the WW domains (WW234) did not increase the binding affinity with hENaC peptides compared with WW3 alone. These results suggest that WW3 may be fully responsible for the hENaC–hNedd4 interaction. In support of our results, Snyder et al. [27] showed, using an *in vitro* binding assay, that WW3 is necessary and sufficient for binding α hENaC. Further, experiments *in vivo* showed that an intact WW3 domain in hNedd4 is critical for the inhibition of Na⁺ channel activity [27], although WW2 and WW4 are required for full inhibition. Multiple mouse Nedd4 WW domains were also required for regulation of Na⁺ current in mouse mandibular duct cells [18].

WW3 of hNedd4 binds with the highest affinity to ENaC peptides but is not present in the related mouse and rat Nedd4 proteins [12,13]. Therefore, unless mouse and rat Nedd4 or their corresponding ENaC protein subunits have compensating changes, it would be anticipated that the Nedd4 WW domains of these rodent species would have a reduced role in ENaC regulation. This is supported by recent evidence that mouse Nedd4, unlike hNedd4, does not regulate ENaC activity [28]. A Nedd4-related protein, Nedd4-2, from human and mouse also down-regulates ENaC activity [28,29]. Determination of the binding affinities of the four WW domains contained in the new protein for hENaC subunits may also prove to be important in understanding hENaC regulation by ubiquitination.

Binding affinities in the micromolar range were obtained for the WW domains of Nedd4 binding to Na⁺ channel peptides. Other studies have confirmed that, although WW-domain binding to their ligands is highly specific, it is of low affinity [30]. This is also consistent with the micromolar binding affinities of Src homology 3 (SH3) domains to their proline-rich ligands [14,30]. Other studies, using SPR, have been used to determine the affinity of SH3 domains with their ligands [31,32]. Russo et al. [31] report similar K_D values for the SH3–ligand interaction determined by both SPR and fluorescence spectroscopy. When this work was carried out, SPR had not to our knowledge been used to study WW-domain interactions. Our results show that this system gives comparable results to those obtained using other assay systems. Kanelis et al. [17] determined K_D values in the micromolar range for rat Nedd4 WW domains binding to rat ENaC peptides. However, none of the rat Nedd4 WW domains bound as tightly to ENaC as the hNedd4 WW3 domain. While this article was undergoing editorial review, Asher et al. [33] reported the measurement of the binding affinities of rat Nedd4 WW domains to rat ENaC peptides by SPR. Their measured affinities ($K_D = 0.67$ – $1.2 \mu\text{M}$) are significantly stronger than those reported here. This discrepancy may be due to Asher et al. using intact GST fusion proteins, rather than using WW domains cleaved from the GST moiety as reported here. The presence of the GST moiety may contribute to the apparently high affinities due to GST dimer formation [34,35]. We used WW domains cleaved from their GST fusion partners in this study to avoid potential artifacts of this nature.

Although showing a rank order of binding to hNedd4 WW domains similar to that of α hENaC and β hENaC, the γ hENaC peptide showed lower affinities in our experimental system. This may be due to the coupling chemistries used in attaching the γ peptide to the sensor chip (biotin–streptavidin, compared with amine binding for the α and β peptides), or due to the presence

of two charged amino acids in the γ peptide, although these residues are conserved in rat γ ENaC (Figure 1C). The variation between binding of the rat and human γ ENaC peptides may be explained by differences in the experimental system used, pH conditions, length of the peptide or the C-terminal moiety of the peptide.

Using available three-dimensional structures for WW-domain binding to peptide we were able to model the predicted structures for the hNedd4 WW domains binding to the three hENaC peptides, and derive the predicted binding energies for each interaction. The results from these analyses were in agreement with the experimentally determined order of hNedd4 WW-domain binding.

Using SPR analysis we show that the binding of WW1 to the α hENaC is so weak as to be only just detectable. This result confirms previous binding studies [12,27]. A single amino acid change (Arg¹⁶⁵ $\rightarrow$ Thr) in hNedd4 WW1 was sufficient to allow binding of hNedd4 WW1 to α hENaC, suggesting that we have identified a key difference between rat and human Nedd4, which explains in part why rat Nedd4 WW1 binds ENaC subunits whereas hNedd4 WW1 does not.

Using molecular modelling, we have begun to determine the reason for the lack of WW1 binding to the Na⁺ channel, and we show that the arginine at position 165 of the WW1 domain confers a polar binding surface, which may contribute to preventing appreciable binding of this domain to hENaC. When a neutral threonine replaces the protonated arginine the binding surface becomes more hydrophobic and binding of hENaC-derived peptides becomes detectable; however, the binding is still 2-fold less than that of WW2 or WW4 and more than 50-fold less than WW3. This result suggests that other amino acids, in addition to Arg¹⁶⁵, also contribute to the lack of binding by WW1. A strong candidate is Lys¹⁶⁹ in WW1. This hypothesis is supported by the observation that WW4, which also contains an arginine at the equivalent position to Arg¹⁶⁵ in WW1, binds ENaC. However, it also contains a compensating negatively charged Glu¹⁶⁹ residue. Nedd4 WW1 does bind to other proteins ([36], and S. J. Coddington-Lawson and F. J. McDonald, unpublished work). Lack of binding of hNedd4 WW1 to the Na⁺ channel may allow the recruitment of other proteins into a larger complex to perhaps mediate ubiquitination, internalization or some other form of regulation.

In conclusion, our data suggest that the unique WW3 domain in hNedd4 mediates most of the binding to hENaC. In addition, we identify a key amino acid in hNedd4 WW1 that prevents binding to ENaC, which explains, in part, the inability of WW1 to bind hENaC. Unravelling the details of the interaction of hENaC and hNedd4 will contribute to our understanding of the regulation of hENaC by hNedd4 under normal and hypertensive conditions.

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