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Characterization of the interactions between Nedd4-2, ENaC, and *sgk*-1 using surface plasmon resonance

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Abstract

Previous studies have characterized interactions between the ubiquitin ligase Nedd4-1 and the epithelial Na^+ channel (ENaC). Such interactions control the channel cell surface expression and activity. Recently, evidence has been provided that a related protein, termed Nedd4-2, is likely to be the true physiological regulator of the channel. Unlike Nedd4-1, Nedd4-2 also interacts with the aldosterone-induced channel activating kinase *sgk*-1. The current study uses surface plasmon resonance to quantify the binding of the four WW domains of Nedd4-2 to synthetic peptides corresponding to the PY motifs of ENaC and *sgk*-1. The measurements demonstrate that WW3 and WW4 are the only Nedd4-2 domains interacting with both ENaC and *sgk*-1 and that their binding constants are in the 1–6 μM range.

Keywords: ENaC; Nedd4; *sgk*; WW domain; Epithelial Na channel; Surface plasmon resonance

1. Introduction

Active Na^+ reabsorption in kidney collecting duct, distal colon, lung, and exocrine glands is mediated by an apical amiloride-blockable Na^+ channel [1–3]. The channel is composed of three homologous subunits, denoted α, β, and γENaC (epithelial Na^+ channel). Its central role in maintaining salt and water balance has been conclusively demonstrated by identifying genetic diseases associated with mutations in ENaC subunits, as well as by the phenotypic analysis of ENaC knockout mice (for review, see Refs. [1–3]). ENaC's cell surface expression is determined by interactions between PY motifs in the C-tails of all three subunits and the WW domains of the ubiquitin ligase Nedd4 (neural precursor cells expressed developmentally down-regulated). Such protein–protein interactions lead to channel ubiquitination, internalization, and degradation [4–8].

The role of Nedd4 as a channel regulator was originally elucidated from its identification as an ENaC-interacting protein in a two hybrid screen [5]. Later, a closely related protein termed Nedd4-2 (also known as KIAA0439, LdI-1, Nedd4La, Nedd18, or Nedd4L) has been cloned and shown to down-regulate ENaC in a similar way [9–11]. Recently, evidence has been provided that Nedd4-2 and not the previously characterized Nedd4 (now termed Nedd4-1) is the physiological regulator of ENaC. First, Nedd4-2 has more profound effects on channel activity when co-expressed in *Xenopus* oocytes, and unlike Nedd4-1 is co-immunoprecipitated with ENaC [9,10]. Second, Nedd4-2 (but not Nedd4-1) is a substrate to the aldosterone-induced kinase *sgk*-1 (serum and glucocorticoid dependent kinase). Phosphorylation of Nedd4-2 is likely to mediate at least part of the hormonal effect on Na^+ transport [12,13]. These studies also provided evidence that Nedd4-2 is capable of binding *sgk*-1 by interacting with the PY motif of this protein kinase.

Previous studies have used co-immunoprecipitation and in vitro binding assays to demonstrate direct interactions between the three (mouse and rat) or four (human and *Xenopus*) WW domains of Nedd4-1 and the PY motifs of ENaC [14–16]. These studies have established that β and γENaC primarily interact with WW3. WW2 and WW4 (in human) bind the channel to a lesser degree, and WW1 does not bind at all. Such domain specificity has not been well characterized in Nedd4-2, whose WW motifs are 25–35% different from those of Nedd4-1. Moreover, the above studies provided qualitative demonstration of specific asso-

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ciation between the two proteins, but gave no quantitative information on its kinetic parameters and binding constants. This information can be determined by surface plasmon resonance [17]. In this method, a peptide or protein is immobilized on a gold sensor chip and serves as a substrate. A second protein (analyte) is passed over the chip and its binding is monitored as a change in refractive index. The method enables a 'real-time' detection of complex formation and dissociation, as well as evaluation of the binding affinities. Such measurements have been recently reported for the binding of Nedd4-1 to β and γENaC PY peptide [18–20]. The current study describes such measurements for Nedd4-2 and also quantifies interactions between the WW domains of this ubiquitin ligase and the PY motif of *sgk*-1.

2. Methods and materials

cDNA fragments containing one or more WW domains of mouse Nedd4-2 (mNedd4-2) were amplified by PCR from an est clone (accession number BI559036). The protein regions amplified and the primers used are listed in Table 1. *sgk*-1 lacking the first 60 amino acids, which lower expression levels [21], was amplified from mouse embryo est clone (accession number AA389214). The amplified fragments were subcloned into the *Eco*RI/*Xho*I site of pGEX-4T-1 (Nedd4-2) or the *Bam*HI/*Eco*RI site of pGEX-2T (*sgk*-1), and verified by sequencing. Recombinant proteins were expressed in a protease-deficient *E. coli* strain and purified on glutathione beads as described previously [22]. Binding of the recombinant protein to the ENaC domain peptide has been measured in a BIAcore sensor as detailed before [18,19]. Both wild-type and mutated biotinylated peptides corresponding to the PY domains of β and γENaC were used as substrates on a streptavidin-coated sensor chip.(c.f. Table 1 in Ref. [19]). Other experiments have used the biotinylated *sgk*-1 PY peptide E M L Y G L P P F Y S R N T A E (residues 289–304 of mouse *sgk*-1) and the equivalent peptide in which Y298 was mutated to A. The analytes (recombinant GST–WW proteins) were dissolved in HBS buffer composed of: 10 mM HEPES pH 7.4, 140 mM NaCl, 3.4 mM EDTA, and 0.005% P20. In one set of experiments, NaCl was replaced by the equivalent amount of KCl. In another, EDTA was omitted and the buffer contained 1 mM $CaCl_2$ instead. Analytes were injected at a flow rate of 20 μl/min, and association was monitored simultaneously in four channels containing different peptide substrates or biotin alone. Readings in the biotin channel were used as background and subtracted from values recorded in the other three channels. Next, HBS buffer alone was injected to monitor the dissociation of the analyte–substrate complex. The chip was regenerated by the injection of 10 μl 0.05% SDS in HBS and then extensively washed in HBS buffer.

Table 1
Domains and primers used for recombinant mouse WW proteins[a]

Domain	Sequence	Primer	
WW1	^{60}DSA–NNI^{116}	sense	5′-GGAATTCGACTCAGC-TTCCCAGCACC-3′
		antisense	5-TTCTCGAGATGTTATT-GTCTGATTCCG-3′
WW2	^{237}STP–GSA^{309}	sense	5′-CGAATTCTCCACGCC-ATCAGTGGCC-3′
		antisense	5′ AACTCGAGGCTGATC-CGGAGGCACCG-3′
WW3	^{364}PYN–LNP^{424}	sense	5′-GGAATTCCCTTACAA-CTCCCCCAAACC-3′
		antisense	5′-CCCTCGAGGGGTTTA-AAGATGCTTTTGACC-3′
WW4	^{419}KAS–YSR^{475}	sense	5′-GGAATTCAAAGCATC-TTTAAACCCC-3′
		antisense	5′-CCCTCGAGTCTGGA-GTACGGAACAGCC-3′
WW1+2	^{60}DSA–GSA^{309}	sense	as WW1
		antisense	as WW2
WW2+3	^{237}STP–LNP^{424}	sense	as WW2
		antisense	as WW3
WW3+4	^{364}PYN–YSR^{475}	sense	as WW3
		antisense	as WW4
WW1–3	^{60}DSA–LNP^{424}	sense	as WW1
		antisense	as WW3
WW1–4	^{60}DSA–YSR^{475}	sense	as WW1
		antisense	as WW4

[a] Amino acid numbers refer to database entry AF277232.

3. Results

Fig. 1 depicts a typical experiment showing the interaction of C-tail peptides with different recombinant WW proteins. β and γ PY peptides were found to bind similarly to the various WW domains. Binding was observed only for fusion proteins, which include WW3 (WW3, WW2+3, and WW3+4) or WW4, while others (WW1, WW2, and WW1+2) gave negligibly small signals. The binding observed for WW1–4 or WW1–3 was lower than that seen for WW3 alone (data not shown). It should also be noted that since the BIAcore signal is proportional to the analyte mass, WW3+4 binding is not much higher than that of WW3. Mutating γY628 into A inhibited the binding by nearly 40-fold, indicating that the interaction is specific. Thus, in agreement with previous studies with human Nedd4-1 [15,16], the major Nedd4-2 domain interacting with the PY ENaC peptide is WW3. Residue S328 located between WW2 and 3 is one of the two *sgk*-1 phosphorylation sites affecting the channel Nedd4-2 interaction [12,13]. Mutation of this residue in GST–WW2+3 into E had no significant effect on the association to PY peptides (not shown).

Association and dissociation followed complex kinetics, and for most fusion proteins, at least two phases could be detected. The only exception was WW2+3 whose association could be well fitted to a simple Langmuir binding. However, the dissociation phase of this analyte is best

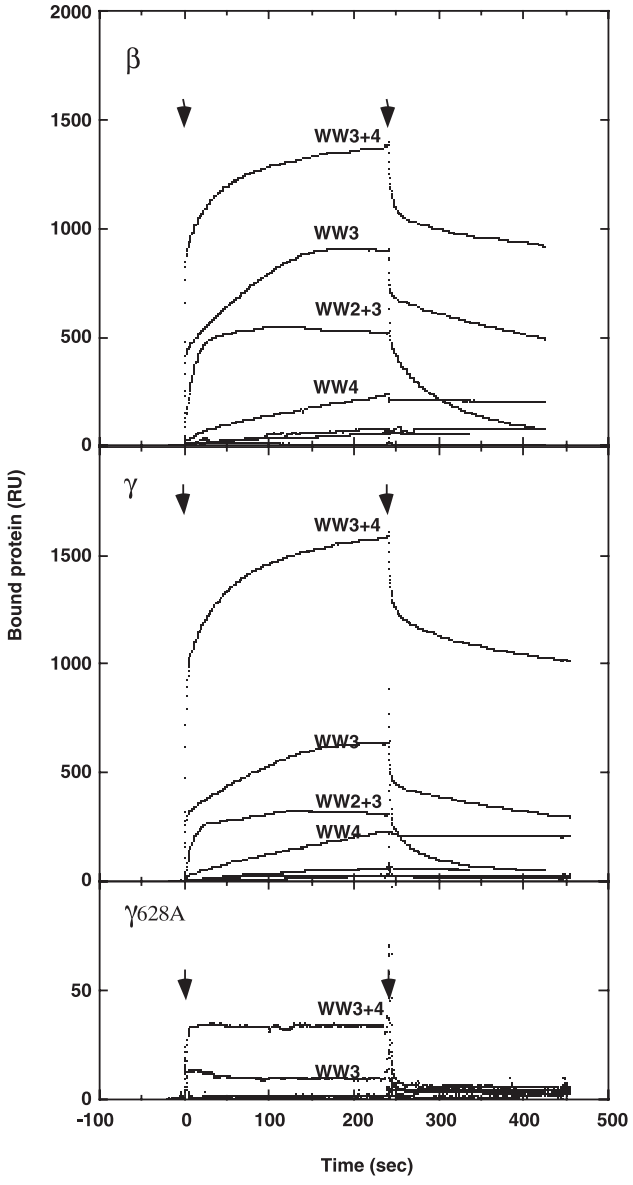

Fig. 1. Four micromolars of different recombinant proteins were applied to a sensor chip having immobilized β, γ, and γ628A peptides (first arrow, $t=0$). The proteins used were: WW1, WW2, WW3, WW4, WW1+2, WW2+3, and WW3+4. Binding was monitored for 4 min and then the analyte was replaced by HBS buffer (2nd arrow). Association and dissociation of the different WW containing recombinant proteins to β (upper panel), γ (middle panel), and γY628A (lower panel) are depicted in RU.

described as the sum of two exponents, possibly due to the presence of two WW domains on this fusion protein. Equilibrium constants of the above interactions were therefore estimated by carrying out measurements at increasing analyte concentrations and fitting the steady state binding levels to Michaelis–Menten type kinetics. Fig. 2 summarizes such data for both β and γ peptides, and the equilibrium constants obtained from the best fits are listed in Table 2. The values obtained were all in the micromolar range, and the binding to β was characterized by a somewhat higher affinity than the binding to γ. The above measurements have been carried out using intact GST–WW recombinant proteins. To exclude any major effects of the GST moiety, the recombinant protein was digested by thrombin, and the WW3 fragment was purified and assayed for binding to β PY peptide (insert in Fig. 2A). The results obtained were similar to those seen for the intact protein,

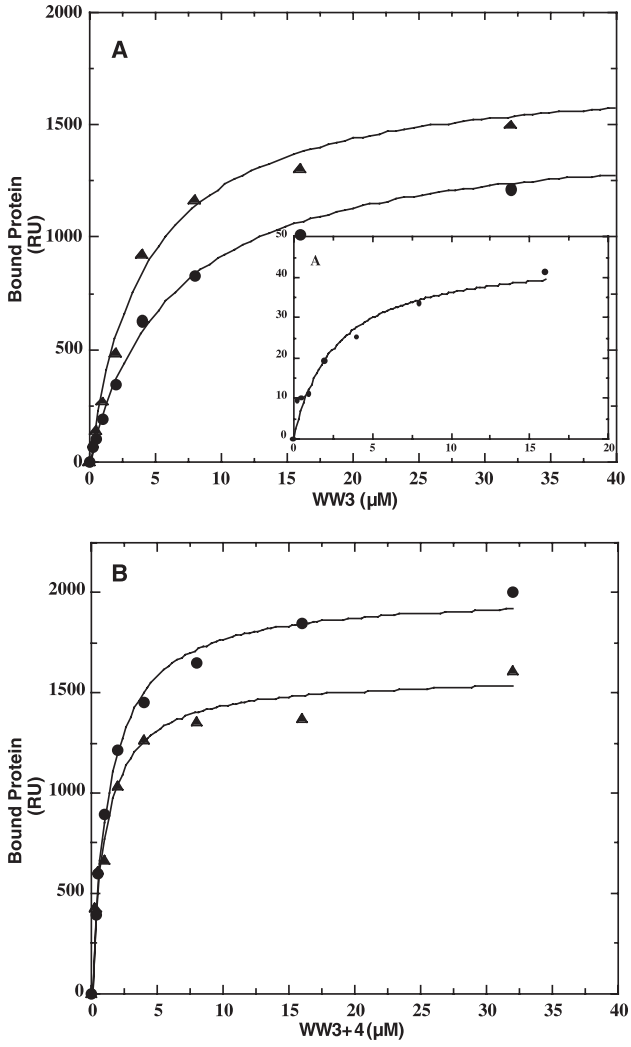

Fig. 2. Binding was measured at the indicated concentrations of WW3 (A) or WW3+4 (B). The binding in RU is plotted against the analyte concentration for β (triangles) and γ (circles). The solid lines are the best fits of the experimental data for the equation $R=R_{max}/(1+K_d/C)$, where R is the steady state signal observed at concentration C, R_{max} is the maximal signal for saturable binding, and K_d is the equilibrium constant. Insert: Binding of WW3 lacking the GST moiety to the γ peptide.

Table 2
Equilibrium binding constants for different analytes[a]

Analyte	β K_d (μM)	γ K_d (μM)
GST–WW3	4.20 ± 0.4	6.1 ± 0.5
GST–WW3+4	1.01 ± 0.2	1.3 ± 0.2
GST–WW2+3	1.00 ± 0.2	3.9 ± 1.0

[a] K_d values were calculated as described in the legend to Fig. 2 for different substrates and analytes.

e.g. a K_d value of 2.6 ± 0.7 vs. 4.2 ± 0.5 μM. However, the enzymatic digestion also resulted in considerable degradation of the WW domains themselves, making data quantification problematic. In addition, the signal measured in this way was much smaller (e.g. 5-fold) due to the large decrease in analyte mass.

An alternative way to calculate the equilibrium constants is by pre-equilibrating a given amount of analyte with increasing substrate concentrations and applying the mixture to a sensor chip containing the immobilized substrate. In this case, the equilibration is done in solution and the BIAcore measurement is used to determine the relative amount of free analyte. Such measurements are illustrated in Fig. 3. The best fitted line had a K_d value of 4.6 ± 1.1 μM for the equilibrium in solution between WW3 and γ peptide. This is in good agreement with the value of 6.1 ± 0.5 μM measured for the binding to the immobilized peptide.

Previously, we have shown that βT613 and γT623 may play a role in the regulation of ENaC by Nedd4 [19]. These residues can be phosphorylated by ERK and the phosphorylated PY peptides bind WW domains with higher affinity than the nonphosphorylated ones. These results were obtained with Nedd4-1, so it was important to test this for Nedd4-2 as well. Accordingly, binding of WW3 and WW3 + 4 to immobilized phosphorylated and nonphosphorylated γ was compared. Indeed, the presence of phospho-threonine at position 623 lowered K_d by 3-fold (Fig. 4); that is, K_d values of 0.9 and 2.8 μM were measured for phospho-γ and γ, respectively.

ENaC can be downregulated by cell Na^+ and this effect depends on the integrity of the PY motifs [23,24]. Thus, it is

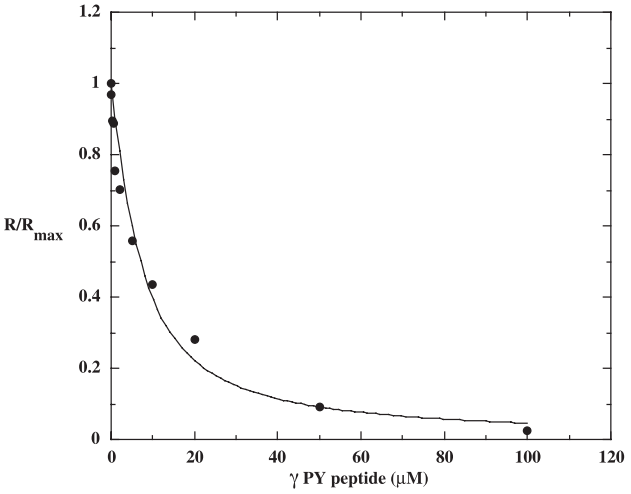

Fig. 3. WW3 (5 μM) was pre-incubated with increasing concentrations of nonbiotinylated γ peptide, and the mixture was applied to a sensor chip having immobilized γ peptide. The steady state signal R was expressed as R/R_{max}, where R_{max} is the steady state signal at zero competing peptide. Data were fitted to the equation $R/R_{max}=(B-A-K_d)/2B+((A+B+K_d)^2-A/B)^{1/2}$, where A is the solution concentration of the peptide, B is the analyte concentration (5 μM), and K_d is the solution equilibrium constant. The best fit was obtained for $K_d = 4.6 \pm 1.1$ μM (continuous line).

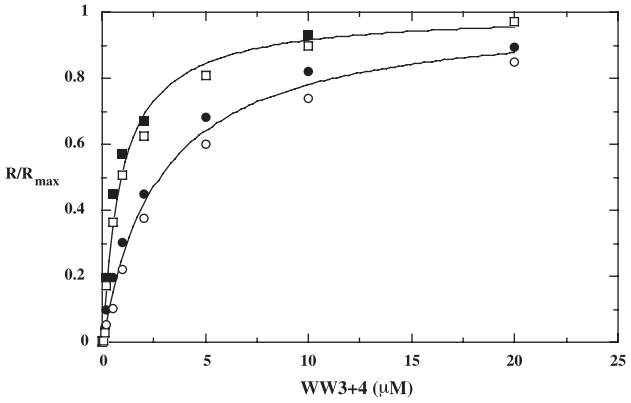

Fig. 4. WW3 + 4 was applied to a sensor chip having immobilized γ peptide (circles) and γ peptide with phospho-threonine at position 623 (squares). Two different experiments are presented (closed and open symbols). The continuous lines are the best-fitted curves for equilibrium constants of 2.8 μM (nonphosphorylated) and 0.9 μM (phosphorylated).

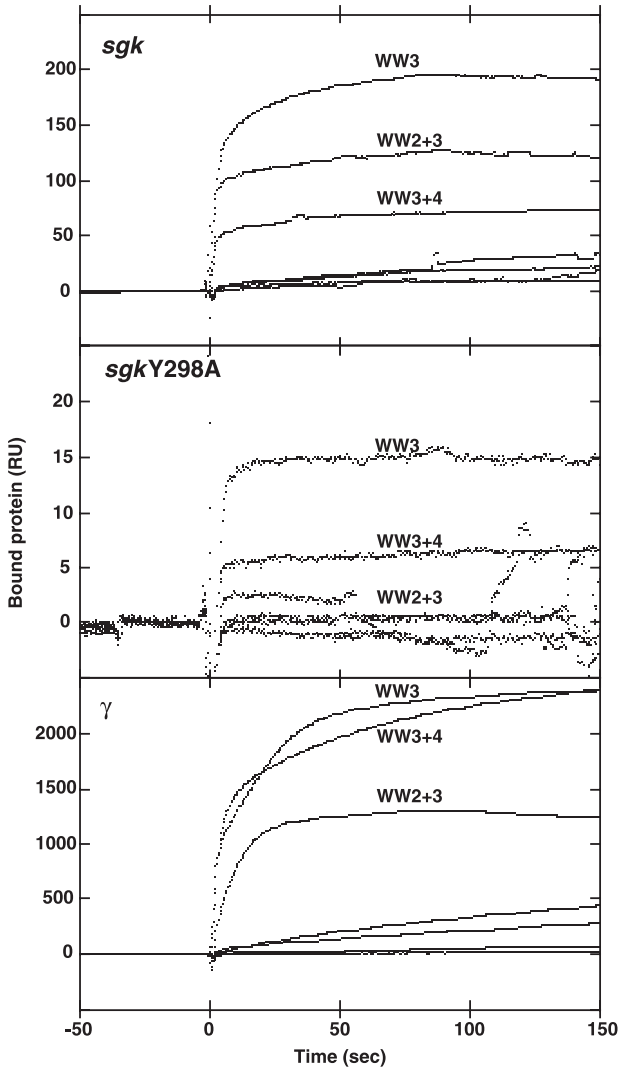

Fig. 5. *sgk*-1 PY, *sgk*-1 PY298A, and γ PY peptides were immobilized on a sensor chip. WW1, WW2, WW3, WW4, WW1 + 2, WW2 + 3, and WW3 + 4 were applied at a concentration of 5 μM.

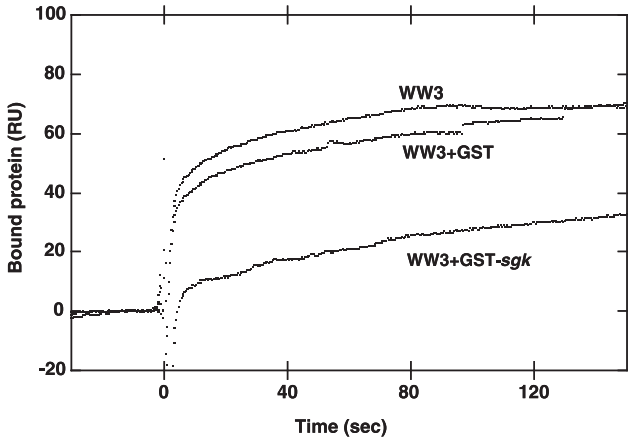

Fig. 6. WW3 (2 μM) was pre-equilibrated with 2 μM GST, GST-*sgk*, or diluent. Binding of these analytes to *sgk* PY peptide has been monitored.

possible that the ionic composition of the cell affects the WW/PY interactions. Replacing 140 mM NaCl in the analyte buffer by the same concentration of KCl caused no difference in the kinetics or extent of the association/dissociation of WW3/PY peptide. There was also no effect by the replacement of EDTA by 1 mM Ca^{2+} (data not shown).

The Nedd4-2 phosphorylating kinase *sgk*-1 also has a PY motif. Mutating this motif inhibits the phosphorylation of Nedd4-2 as well as the activation of ENaC, suggesting a role in the *sgk*-1/Nedd4-2 interaction [12,13]. To assess characteristics of such binding, BIAcore experiments have been carried out using wild-type and mutated *sgk-1* PY peptides. The *sgk*-1 peptide bound the Nedd4-2 WW domains with maximal binding for WW3 (Fig. 5). The signal was, however, ~10-fold lower than that measured in parallel for the γENaC PY peptide. Fitting data for increasing WW3 concentrations to Michaelis–Menten kinetics indicates that the low signal is not the result of a higher K_d but reflects a ~10-fold lower R_{max}. Specificity of the above binding was established by two experiments. (a) Mutating Y298 on the *sgk*-1 peptide into A largely inhibited WW association. (b) Pre-incubation of WW3 with GST-*sgk* inhibited association to the immobilized *sgk* PY peptide, while a similar incubation with GST alone had no effect (Fig. 6).

4. Discussion

Interactions between the PY motifs of ENaC subunits and the WW domains of Nedd4-1 has been extensively characterized before [5,14,15]. Recently, however, evidence has been provided that the physiological regulation of the channel is mediated by another, related protein termed Nedd4-2 [9,10,12,13]. The WW domains of Nedd4-2 share only 65–75% identity with Nedd4-1. In addition, this protein lacks the C2 domain and has an additional WW domain (at least in rodents). Thus, it was important to examine whether its binding characteristics are similar to those measured before for Nedd4-1. Our results indicate that the only Nedd4-2 WW domains that significantly bind to β and γ PY peptide are WW3 and WW4, where WW3 plays the major role in such binding. This is in agreement with the functional effects of such domains in *Xenopus* Nedd4 [9]. This study reported a relief of ENaC inhibition by mutations in WW3 and 4 but not in WW1 or WW2. In this case, however, WW4 was more effective than WW3 [9]. While this manuscript was in preparation, another study comparing the binding of different Nedd4-2 WW domains by a far-Western analysis has been published [25]. It reported that WW3 and WW4 are the only two domains that bind ENaC. The bindings of the two fusion proteins to α and β are similar, but for γ, WW3 binds much better than WW4.

Binding affinities have been calculated by fitting steady state values to simple binding kinetics. The K_d values obtained were all in the range of a few micromolars. This is in agreement with the values measured by us for Nedd4-1 [18,19] but lower than those reported by others [20,26]. It has been proposed that the stronger binding observed in our studies may be due to the use of intact GST fusion proteins and the possible formation of GST dimers [20]. However, in this study, we have demonstrated that cleaving the GST moiety does not significantly increase K_d. In addition, experiments in which GST has been added as a second analyte, after GST–WW binding had reached a steady state, did not result in an increased signal, precluding significant contribution of GST dimerization. The differences in K_d can therefore be accounted for by the different peptide sequences used in the various studies. The current data also confirm that phosphorylation of γT623 increases the binding affinity of Nedd4-2, and this could therefore be another channel regulatory mechanism [19].

We have also attempted to use binding of recombinant proteins to immobilized peptides for characterizing *sgk*-1 Nedd4-2 interactions. Specific binding of WW domains to *sgk* PY peptide was observed; however, the signal detected was much smaller than that seen for ENaC PY peptides. As discussed above, the lower binding to the *sgk* reflects a lower R_{max} and not a higher K_d. For a given analyte, R_{max} is determined only by the number of binding sites available on the chip, i.e. the amount of peptide immobilized, its packing, and secondary structures that may limit analyte association. Immobilization of the γ and *sgk* PY peptides gave rise to similar signals (± 10%), suggesting that equal amounts of the two peptides were immobilized. Thus, the most likely interpretation is that a secondary structure or a particular arrangement of this peptide on the sensor chip tends to limit binding. The low signals observed prevented detailed kinetic analysis of the WW/*sgk* interaction. Nevertheless, the data indicates that WW binding to the *sgk* PY peptide follows the same characteristics observed for ENaC, i.e. significant binding is seen only for WW3 or a fusion protein in which one of the two domains is WW3. This is the first

description of the WW specificity of *sgk* binding. It indicates that the two WW domains not participating in the interaction with ENaC are also not being used to bind *sgk*-1.

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