14-3-3 Mediates Phosphorylation-Dependent Inhibition of the Interaction between the Ubiquitin E3 Ligase Nedd4-2 and Epithelial Na^+ Channels

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ABSTRACT: Although recent studies show that the 14-3-3 protein is a negative regulator of ubiquitin E3 protein ligases, the molecular mechanism remains largely unknown. We previously demonstrated that 14-3-3 specifically binds one of the E3 enzymes, Nedd4-2 (a human gene product of KIAA0439, termed hNedd4-2), which can be phosphorylated by serum glucocorticoid-inducible protein kinase 1 (SGK1); this binding protects the phosphorylated/inactive hNedd4-2 from phosphatase-catalyzed dephosphorylation [Ichimura, T., et al. (2005) *J. Biol. Chem. 280,* 13187−13194]. Here we report an additional mechanism of 14-3-3-mediated regulation of hNedd4-2. Using surface plasmon resonance spectrometry, we show that 14-3-3 inhibits the interaction between the WW domains of hNedd4-2 and the PY motif of the epithelial Na^+ channel, ENaC. The inhibition was dose-dependent and was dependent on SGK1-catalyzed phosphorylation of Ser468 located between the WW domains. Importantly, a mutant of hNedd4-2, which can be phosphorylated by SGK1 but cannot bind 14-3-3, reduced SGK1-mediated stimulation of the ENaC-induced current in *Xenopus laevis* oocytes. In addition, 14-3-3 had similar effects on hNedd4-2 that had been phosphorylated by cAMP-dependent protein kinase (PKA). Our results, together with the recent finding on 14-3-3/parkin interactions [Sato, S., et al. (2006) *EMBO J.* 25, 211−221], suggest that 14-3-3 suppresses ubiquitin E3 ligase activities by inhibiting the formation of the enzyme/substrate complex.

The 14-3-3 protein comprises a family of acidic, dimeric proteins with subunit molecular masses of ∼30 kDa distributed widely among eukaryotic cells (for reviews, see refs *1*−*4*). This protein family has been linked to numerous biological activities, including the activation of enzymes involved in monoamine synthesis such as tyrosine and tryptophan hydroxylases (*5, 6*), the regulation of protein products of proto-oncogenes and oncogenes such as Raf-1 and Bcr-Abl protein kinases (*7*−*11*), and the retention of ligands in pro-apoptotic pathways (such as those involving Bad and FKHRL1) in the cytoplasm (*12, 13*). Recent proteomic studies (*14*−*18*) suggest that eukaryotic cells contain several hundred additional proteins whose physiological functions might be regulated by 14-3-3. In many cases, binding between 14-3-3 and its target proteins is triggered by phosphorylation of the targets, particularly at specific Ser/Thr residues (*19*). Thus, the 14-3-3 family is believed to be a key regulator of a wide variety of cell signaling pathways mediated by protein phosphorylation.

Nedd4-2[1] is a member of the Nedd4 family of ubiquitin E3 protein ligases that are implicated in diverse cellular pathways. Members of the Nedd4 family, including Nedd4-2, share a common modular structure containing a series of tryptophan-rich sequences (called WW domains) that interact with a proline-tyrosine motif (called PY motif) in substrate molecules (for a review, see ref *20*). One of the physiological substrates of Nedd4-2 is the epithelial Na^+ channel, ENaC. In this case, Nedd4-2 binds ENaC by direct interaction of its WW domains with PY motifs in the C-terminal tail of each of the ENaC subunits (α, *β*, and *γ*), thereby catalyzing the ubiquitination of the ENaC channel (*21*−*24*). This ubiquitination accelerates the rate of ENaC degradation and

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[1] Abbreviations: Nedd4-2, neural precursor cell-expressed, developmentally downregulated gene 4 isoform 2; hNedd4-2, human Nedd4-2 isoform (KIAA0439); E3, ubiquitin-protein isopeptide ligase; ENaC, epithelial Na^+ channel; SGK1, serum glucocorticoid-inducible kinase 1; PKA, cAMP-dependent protein kinase; PY motif, PP*X*Y sequence; GST, glutathione *S*-transferase; FRT, Fischer rat thyroid; MS, mass spectrometry.

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reduces the copy number of the channel at the cell surface (*25*), ultimately resulting in a drastic decrease in Na^+ currents in *Xenopus laevis* oocytes or Fischer rat thyroid (FRT) cells (*22*−*24*, *26*). This regulation is believed to be important physiologically, because Liddle's syndrome, an inherited disorder of human hypertension (*27*), is linked to mutations in ENaC that invariably cause either deletion or structural alteration of the β and γPY motifs (*28*, *29*). Recently, Nedd4-2 was shown to be a substrate of serum glucocorticoid-inducible protein kinase 1 (SGK1), and, importantly, phosphorylation of Nedd4-2 reduces its affinity for ENaC, thereby reducing ubiquitin-mediated degradation of ENaC both in *Xenopus* oocytes (*30*) and in FRT cells (*31*, *32*). Several reports also suggest that Nedd4-2 and SGK1 cooperatively regulate the activity of ENaC, whereas other studies suggest that SGK1 regulates ENaC independently of Nedd4-2 (*33*).

We previously used mass spectrometry based proteomic technologies combined with a novel tandem affinity purification approach to identify Nedd4-2 (a human KIAA0439 gene product; termed hNedd4-2) as a SGK1-dependent binding target of 14-3-3 (η-isoform; ref *18*). We also demonstrated that the 14-3-3η/hNedd4-2 complex was present under physiological conditions in vivo (*18*). This finding provided the first evidence that 14-3-3 targets a ubiquitin E3 ligase and directly regulates its function. Indeed, the interaction inhibited the dephosphorylation of hNedd4-2, reduced the rate of Nedd4-2-catalyzed ubiquitination of ENaC subunits, and maintained the SGK1-stimulated increase in ENaC-mediated Na^+ currents. The importance of this interaction was also confirmed by a recent publication with *Xenopus* Nedd4-2 (*34*). However, the detailed mechanism by which 14-3-3 modulates Nedd4-2 function is unknown.

In the present study, we used surface plasmon resonance (SPR) spectrometry to examine the effect of 14-3-3 binding on the interaction between the WW domains of hNedd4-2 and the PY motif of γENaC. Our results indicate that 14-3-3 negatively modulates Nedd4-2 function by preventing the access of phosphorylated Nedd4-2 to substrates.

EXPERIMENTAL PROCEDURES

Plasmids and Proteins. To create a vector encoding GST-WW, which included the WW domain of hNedd4-2, the WW domain (WW1−4) insert of pGEX4T-3 (*20*) was amplified by PCR using the primers 5′-CATGCCATGGTGTCCCCTATACTAGGTTATTGG-3′ and 5′-GGCCTCGAGAATAGCTGGGTTCTGCAGTC-3′ and then ligated into the bacterial expression vector PET15b (Novagen). GST-WW was expressed in *Escherichia coli* BL21 using 1 mM isopropyl β-D-thiogalactoside and incubation overnight at 30 °C and was subsequently purified by affinity chromatography using glutathione-Sepharose beads (Amersham Biosciences). GST-14-3-3η was expressed in *E. coli* JM109 and purified as described previously (*35*). The mutants S382A, S468A, and P470A were generated by site-directed mutagenesis using the following mutagenic primers: 5′-GCTGTCACCGACGCAGTTGCAG-3′ (for S382A), 5′-GCCTCGCCAACAGTAACTTTATC-3′ (for S468A), and 5′-GCAACAGTAACTTTATCTGCCC-3′ (for P470A). The S382A/S468A double-mutant construct was generated by site-directed mutagenesis by templating the S382A plasmid using the mutagenic primer 5′-GCCTCGCCAACAGTAACTTTATC-3′. The NP470A construct was produced by site-directed mutagenesis by templating the KIAA0439 plasmid (*18*) using the mutagenic primer 5′-GCAACAGTAACTTTATCTGCCC-3′.

Surface Plasmon Resonance Analysis. All studies were performed on a Biacore 2000 instrument. The ENaC peptides (the PY peptides of biotinylated rat γENaC) containing the following two sequences were purchased from Tan Laboratories, L.C.: biotin-VPGTPPPRYNTLRLD (the PY peptides of rat γENaC) and biotin-VPGTPPPRANTLRLD (the PY peptides carrying the mutation Y628A). The ENaC peptides were immobilized on the surface of a streptavidin-coated sensor chip (SA, Biacore) by applying 50-μL aliquots of the peptide solutions (100 μg/mL in running buffer composed of 10 mM HEPES, pH 7.4, 3 mM EDTA, 150 mM NaCl, and 0.005% (w/v) Tween 20). The concentration of the immobilized peptides was adjusted to yield 150−180 resonance units (RU) for each experiment. Samples were injected at 25 °C at a flow rate of 20 μL/min, and binding was recorded by monitoring the increase in RU for 3 min. Binding surfaces were regenerated (to remove bound analyte) by injection of 100 mM NaOH for 1 min. The equilibrium dissociation constant (K_d) was calculated by K_d = 1/association constant (K_a), in which K_a was determined from the plot of RUeq/[analyte] vs RUeq at different analyte concentrations by Scatchard plot analysis (RUeq, the steady-state resonance unit).

In Vitro Phosphorylation and Pull-Down Assay. Phosphorylation of GST-WW and mutants thereof by SGK1 was carried out at 30 °C for 30 min in 25-μL reactions containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM ATP, 0.1 μg of SGK1 (Δ1−59, S422D; Upstate Biochemical), and 5 μg of GST-WW or GST-WW mutants. Phosphorylation of the GST constructs by PKA was performed under the above conditions except that 0.2 μg of the catalytic subunit of bovine PKA (*36*) was used instead of SGK1. To quantitate the level of phosphorylation, the GST constructs were phosphorylated under the above conditions in the presence of 0.4 μCi of [γ-^{32}P]ATP and then analyzed by SDS−PAGE followed by autoradiography. The incorporation of ^{32}P was measured by scintillation counting of excised protein bands. For pull-down assays, phosphorylation of the GST constructs by SGK1 was carried out at 30 °C for 30 min in 10-μL reactions containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM ATP, 0.04 μg of SGK1, and 2 μg of GST-WW or mutants thereof. Phosphorylation of GST constructs by PKA was performed under the above conditions using 0.2 μg of the catalytic subunit of PKA. The GST-fusion proteins were incubated with 0.5 μg of 14-3-3η for 5 min at 30 °C and then pulled down with glutathione-Sepharose beads. After washing, the 14-3-3η bound to GST-WW immobilized on the Sepharose beads was analyzed by SDS−PAGE followed by immunoblotting using an antibody directed against 14-3-3η (Immuno-Biological Laboratories).

Electrophysiological Measurements. Electrophysiological studies in *X. laevis* oocytes were performed as described previously (*37*, *38*). In brief, cRNAs (three human ENaC subunits, 0.1 ng each; SGK1 or PKA, 0.2 ng; wild-type hNedd4-2 or NP470A mutant, 0.01 ng) were injected into oocytes. Electrophysiological measurements were recorded 24−48 h after injection using a two-electrode voltage clamp technique. The recording solution had an ionic composition

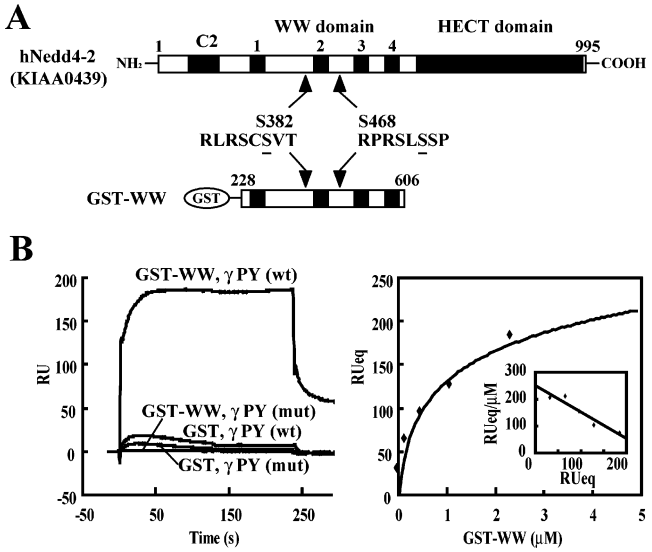

FIGURE 1: Characterization of the recombinant WW protein. (A) Schematic illustration of hNedd4-2 (KIAA0439) and GST-WW. The two SGK1 phosphorylation sites and the WW and HECT domains are shown. (B) Left: Biacore sensorgram of GST-WW binding to γPY peptide. Wild-type and mutated (Y628A) PY peptides were immobilized on the SA sensor chip, and GST-WW (15 μg) and GST (control, 15 μg) were passed through at a flow rate of 20 μL/min. Binding was recorded by monitoring the increase in resonance units (RU) for 3 min. Right: Kinetic analysis of the association between GST-WW and γPY peptide. Steady-state resonance (RUeq) was plotted at each GST-WW concentration (0−2.5 μM, corresponding to 0−15 μg of protein). Inset: Scatchard plot derived from the data in B for GST-WW. The line was fitted by the method of least squares.

of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5. All electrophysiological recordings were performed at 25 ± 1 °C at a holding potential of −60 mV.

Other Methods. The amounts of recombinant proteins were measured via densitometric quantitation of Coomassie Blue stained protein bands from SDS−PAGE.

RESULTS AND DISCUSSION

Characterization of Recombinant hNedd4-2 WW1−4. To obtain hNedd4-2, we initially attempted to produce the full-length protein using a bacterial expression system. The majority of the expressed protein was insoluble, however, and did not renature significantly in aqueous solution. Thus, we used the WW-domain protein, a truncated form of hNedd4-2 containing the entire WW region (WW1−4; Figure 1A), which could be purified in a soluble form as a GST fusion protein (termed GST-WW). We determined whether the purified GST-WW binds ENaC using SPR spectrometry to measure the real-time interaction of GST-WW with a synthetic γPY peptide identical to the PY motif of γENaC (see Experimental Procedures). As shown in Figure 1B (left panel), GST-WW bound to the immobilized γPY peptide with a very rapid on/off rate. An analogous peptide containing the mutation Y628A abolished the binding, indicating that the interaction is specific (Figure 1B, left panel). This mutation leads to an impaired interaction between Nedd4-1 and ENaC in human embryonic kidney Hek293 cells (*21*, *39*). However, the association and dissociation rates exhibited complex kinetics and did not fit a simple 1:1 binding ratio (data not shown), probably because the GST-fusion protein contained multiple WW domains. The steady-state resonance was therefore estimated by carrying out multiple measurements at various concentrations of analytes (Figure 1B, right panel). These results showed that the binding was dose-dependent, and the association constant (K_a) calculated from the slope of the corresponding Scatchard plot line was 0.99×10^6 M (inset). The K_a value was not influenced by the amount of immobilized γPY peptide (data not shown). The equilibrium dissociation constant (K_d) calculated from $1/K_a$ was 1.0 μM, suggesting that the binding between GST-WW and γPY was rather strong. This value is similar to those measured for the binding of γPY to mouse and rat Nedd4-2 GST-WW constructs (*40*, *41*).

14-3-3 Inhibits the Interaction between GST-WW and γPY in the Presence of SGK. The GST-WW protein was maximally phosphorylated by SGK1 to a level of ~2 mol of phosphate/mol of protein (see Figure 3A, lane 1), and its binding affinity for γPY was analyzed as described above in the presence or absence of the GST-14-3-3η fusion. As shown in Figure 2A, the binding affinity of GST-WW for γPY was inhibited by ~40% by phosphorylation alone, as expected from a previous far-western analysis indicating that SGK1 decreased Nedd4-2 binding to ENaC (*31*). Furthermore, we found that GST-14-3-3η enhanced the SGK1-catalyzed inhibition of the WW/γPY interaction by 2-fold, for a total inhibition of ~80% (Figure 2A). We performed several experiments to confirm the specificity of this observation. First, we found that the effect of GST-14-3-3η was saturable, with maximal inhibition occurring at ~0.5 μM (Figure 2B). This value was equivalent to a binding stoichiometry of 1:0.7 (mol/mol) for the GST-WW and GST-14-3-3η dimer, suggesting that maximal inhibition occurred at a molar ratio of ~1:1. Second, 14-3-3η lacking a GST-tag inhibited the WW/γPY interaction as efficiently as GST-14-3-3η, whereas the GST moiety alone or V180D, a dominant-negative point mutant of 14-3-3η (*18*), had no activity (Figure 2C). Third, we could not detect the activity of 14-3-3η in the absence of SGK1, indicating that the inhibition was dependent on the phosphorylation of GST-WW (Figure 2D).

We previously showed that SGK1 phosphorylates hNedd4-2 primarily at two serine residues (Ser382 and Ser468) and that the phosphorylation of one of the sites, Ser468, is essential for the interaction with 14-3-3 (ref *18*, see also Figure 3B, lanes 2 and 3). To examine whether the phosphorylation of this residue is also responsible for the observed inhibition described above, three phosphorylation-deficient mutants of GST-WW were prepared by replacing Ser382 or/and Ser468 by Ala (termed S382A, S468A, and S382A/S468A). Each mutant was analyzed by SPR spectrometry for the ability to bind the immobilized γPY in the presence or absence of GST-14-3-3η. As shown in Figure 2E−G, although all mutants retained their binding affinity for γPY, the inhibitory effect of GST-14-3-3η was observed with S382A but not with S468A or S382A/S468A, each lacking Ser468. Thus, 14-3-3-mediated inhibition of the WW/γPY interaction is dependent on phosphorylation of Ser468.

The sequence surrounding Ser468 is consistent with the consensus 14-3-3 binding motif Rxx*pS*xP (where *pS* is phosphoSer, ref *19*). Because the high-affinity binding of this motif to 14-3-3 often requires a Pro residue two residues

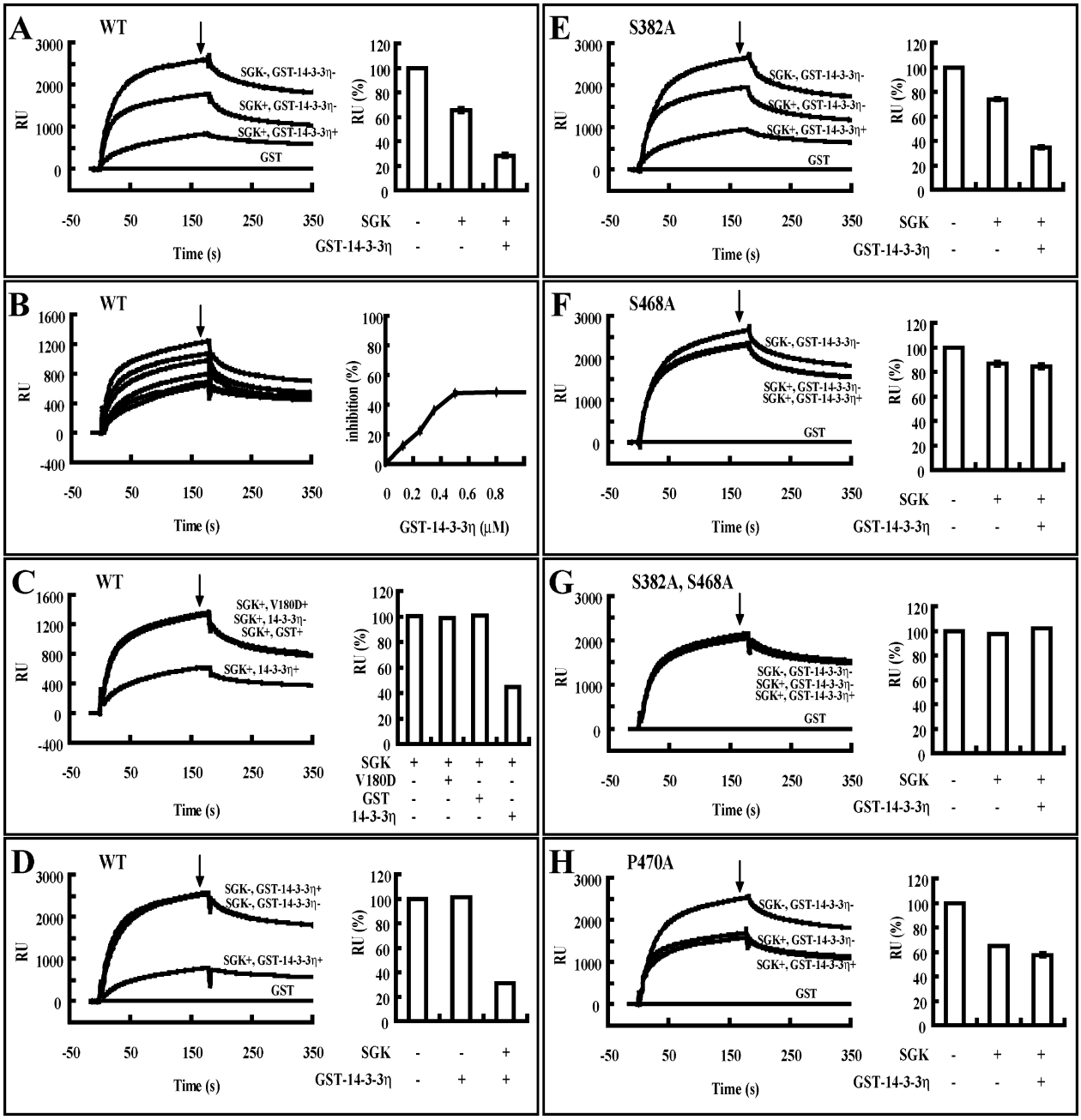

FIGURE 2: Inhibition of the association between GST-WW and γPY by 14-3-3 and SGK1. (A) Left: GST-WW (5 μg) was incubated with or without SGK1 (0.1 μg) in the presence or absence of GST-14-3-3η (5 μg), and the mixtures were analyzed as in Figure 1B using a sensor chip immobilized with the γPY peptide. A control experiment was performed with GST (5 μg). Right: The extent of association at 170 s, indicated by an arrow in the left panel, is presented graphically. Results represent the ratios compared with the SGK1(−)/GST-14-3-3η (−) experiment; the mean ± SD values of three experiments are shown. (B) Left: GST-WW (5 μg) was incubated with SGK1 (0.1 μg) and various concentrations (0−0.8 μM, corresponding to 0−8 μg) of GST-14-3-3η and then analyzed as in Figure 2A. Right: The extent of association at 170 s, indicated by an arrow in the left panel, is plotted compared with the amount of GST-14-3-3η. Results represent the mean ± SD of three experiments. (C and D) GST-WW was incubated with the indicated proteins (each 0.5 μM) and then analyzed as in Figure 2A. (E−H) Mutated GST-WWs (S382A for E, S468A for F, S382A/S468A for G, and P470A for H, each 5 μg) were incubated with the indicated proteins and then analyzed as in Figure 2A.

downstream from the phosphoSer (*19*), an additional GST-WW mutant was prepared by replacing Pro470 by Ala (P470A) under the assumption that such a mutation may not affect the basal level of SGK1-catalyzed phosphorylation but may reduce the binding affinity for 14-3-3. As expected, the P470A mutant was phosphorylated by SGK1 to the same extent as the wild-type protein but bound to 14-3-3η at a significantly reduced level (Figure 3A,B, lane 5). SPR spectrometry clearly demonstrated that, although the binding of P470A to γPY was also inhibited by phosphorylation alone, the effect of 14-3-3 on the WW/PY interaction was not detected in this mutant (Figure 2H), suggesting that 14-3-3 directly binds the phosphorylated WW protein to inhibit the interaction with γPY.

The GST-WW and γPY Is Also Inhibited Cooperatively by 14-3-3 and PKA. A recent study showed that PKA also phosphorylates and inhibits Nedd4-2 (*42*). This may be particularly important because PKA and SGK1 may regulate Nedd4-2 in different pathways or have a different time course of action during cell signaling processes. For example, PKA

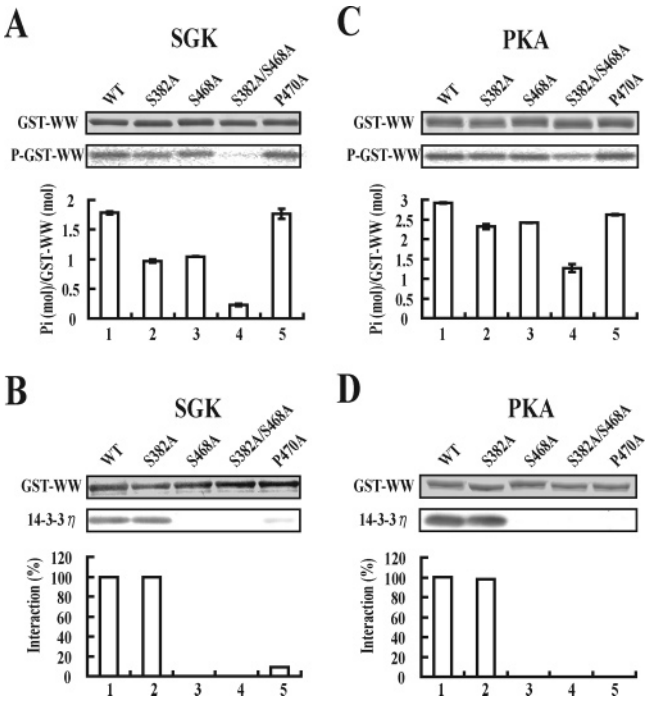

FIGURE 3: Characterization of mutated WW proteins in terms of phosphorylation and 14-3-3 binding. (A) The indicated GST fusion proteins (5 μg each) were phosphorylated with SGK1 and [γ-^{32}P]-ATP and then analyzed by SDS−PAGE followed by Coomassie staining (top), autoradiography (middle), and scintillation counting (bottom). Results shown in the bar diagrams represent mean ± SD values of two experiments. (B) The same fusion proteins shown in panel A (2 μg each) were phosphorylated with SGK1, incubated with 14-3-3η (0.5 μg), and glutathione-agarose beads were added to the mixture to pull down the protein complex. Bound 14-3-3 was then analyzed by SDS−PAGE followed by Coomassie staining (top) and immunoblotting with anti-14-3-3η (middle). The intensity of each immunostained band was also quantified by densitometry and is presented graphically (bottom). Results are expressed as the ratio to the control experiment (WT). (C and D) Experiments were carried out under the above conditions except that SGK1 was replaced with PKA.

is activated in response to vasopressin through a cAMP-dependent mechanism, whereas SGK1 is stimulated by insulin or steroid hormones via a PI3K-dependent signal transduction pathway (*42*, *43*). Moreover, vasopressin/PKA could phosphorylate Nedd4-2 over a short period (5−15 min), whereas SGK1 might maintain Nedd4-2 in an inactive phosphorylated form over several hours (*42*). Thus, although these two kinases may act in concert upon stimulation by different hormones, they may modulate Nedd4-2 nonsynergistically. To test whether 14-3-3 could also participate in the PKA-dependent regulatory mechanism of Nedd4-2, purified GST-WW and mutants thereof were phosphorylated by the catalytic subunit of PKA (Figure 3C), and the interaction with 14-3-3η was analyzed by a pull-down assay. GST-WW clearly associated with 14-3-3η in a phosphorylation-dependent and Ser468/Pro470-dependent manner (Figure 3D). SPR spectrometry showed that GST-14-3-3η interfered with the association between GST-WW and γPY in the presence of PKA in all experiments described above, as was the case for SGK1 (Figure 4). These findings indicate that 14-3-3 inhibits the interaction between GST-WW and γPY not only by an SGK1-dependent but also by a PKA-dependent mechanism. Co-immunoprecipitation assays using an antibody specific to Nedd4 further showed that the formation of the endogenous 14-3-3η/Nedd4-2 complex was indeed enhanced in mammalian PC12 cells after the cells were exposed to insulin or forskolin, potent activators of SGK1 and PKA, respectively (ref *18* and Figure 5).

A hNedd4-2 Mutant Deficient in 14-3-3 Binding Reduces Both SGK1- and PKA-Stimulated ENaC Currents. To determine the functional consequence of the phosphorylation-dependent interaction between 14-3-3 and the WW domain of hNedd4-2, we examined whether the interaction affects the ENaC-dependent Na^+ current in *X. laevis* oocytes. We showed previously that the V180D dominant-negative mutant of 14-3-3η completely abolished the stimulatory effect of SGK1 on the ENaC current (*18*). However, the possibility remained that the observed effect of the V180D mutant might reflect actions toward other potential 14-3-3 targets that influence the ENaC current. To exclude this possibility, we used the hNedd4-2 mutant P470A described above, which could be phosphorylated via SGK1 or PKA but had a reduced affinity for binding to 14-3-3 (Figures 2H and 4H). This mutant, termed NP470A, was expressed in oocytes along with ENaC subunits (α, β, and γ) and SGK1 or PKA, and amiloride-sensitive ENaC-mediated Na^+ currents were measured using a two-electrode voltage clamp technique (Figure 6). As described previously (*30*, *42*), the Na^+ current was markedly reduced in cells expressing wild-type (WT) hNedd4-2, whereas the current was recovered by coexpression of SGK1 or PKA (Figure 6A,B). Interestingly, the expression of the NP470A mutant also reduced the ENaC-mediated Na^+ current of the cells to almost the same level as the wild-type protein; however, the Na^+ current of those cells did not fully recover even in the presence of SGK1 or PKA (Figure 6A,B). These results suggest that the interaction with 14-3-3 has the potential to significantly inhibit hNedd4-2 activity with regard to the SGK1- or PKA-dependent control of ENaC currents.

Our SPR spectrometry results show that PKA represses the interaction between GST-WW and γPY more significantly than SGK1, regardless of the presence or absence of 14-3-3η (compare Figure 4A with Figure 2A). This is probably due to additional phosphorylation of GST-WW that is specifically mediated by PKA. Indeed, PKA phosphorylated GST-WW maximally to a level of ~3 mol of phosphate/mol of protein (Figure 3C, lane 1), which is one molar excess over SGK1-mediated phosphorylation (~2 mol of phosphate/mol of protein; Figure 3A, lane 1; see also Figure 3A,C, lane 4). This PKA-specific phosphorylation site and its physiological relevance are currently unknown. We assume, however, that this hNedd4-2 site might not be a natural substrate in vivo, because PKA modulated the hNedd4-2-mediated inhibition of Na^+ transport at a level similar to that of SGK1 in oocytes (Figure 6). This assumption is also supported by the recent finding that mutation of the SGK1 phosphorylation sites in human Nedd4-2 (Ser221 and Ser327 (*42*), where Ser221 and Ser327 are counterparts of Ser382 and Ser468 in hNedd4-2, respectively) nearly abolished the stimulus effect by PKA on ENaC currents in FRT cells.

Our data suggest that 14-3-3 is a common cofactor for both SGK1- and PKA-dependent regulation of hNedd4-2. Based on this and previous observations (*30*, *31*, *42*), we propose a potential role for 14-3-3 in the regulation of hNedd4-2 (Figure 7). In response to hormones such as

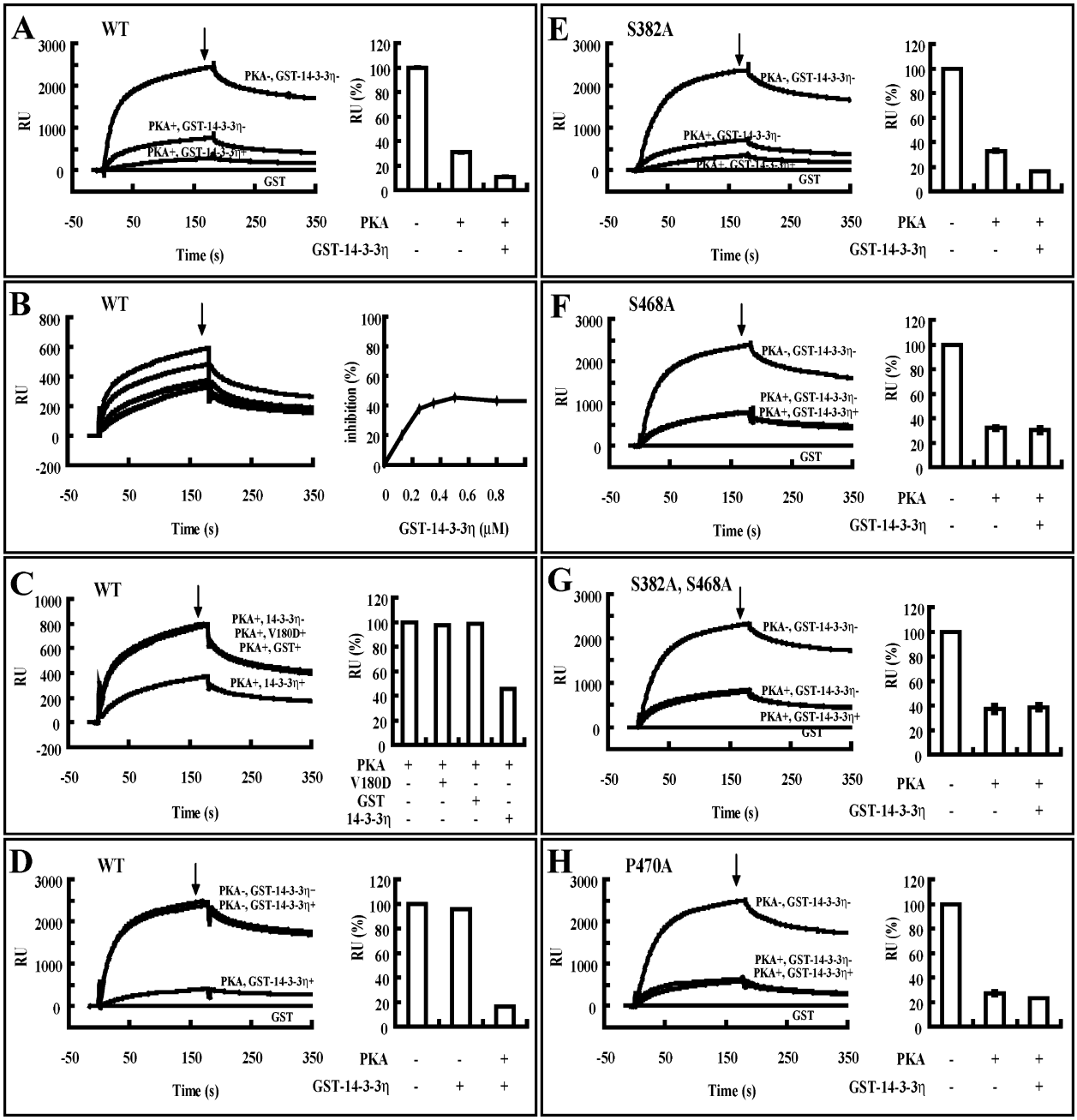

FIGURE 4: Inhibition of the association between GST-WW and γPY by 14-3-3 and PKA. Experiments were carried out under the same conditions shown in Figure 2 except that PKA (0.2 μg) was used instead of SGK1. Results shown in the bar diagram represent the mean ± SD values of two experiments.

aldosterone, insulin, or vasopressin, SGK1 and PKA become activated and catalyze the phosphorylation of hNedd4-2 at multiple sites including Ser468. This phosphorylation reduces the affinity of hNedd4-2 for ENaC, but is insufficient to fully inhibit this interaction until 14-3-3 binds to phosphorylated hNedd4-2 at a site that includes Ser468. 14-3-3 maintains hNedd4-2 in a phosphorylated form (*18*) and shuts down the binding of phosphorylated hNedd4-2 to the substrate, ENaC. This leads to an increase in the cell surface density of ENaC by inhibiting its degradation via the ubiquitin−proteasome system, causing an increase in the Na^+ current. However, it remains unclear as to whether 14-3-3 acts to mask the hNedd4-2 WW domains or to induce a conformational change in the WW domains. Future structural studies

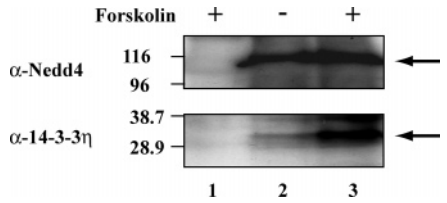

FIGURE 5: Forskolin enhances the interaction between Nedd4-2 and 14-3-3η in PC12 cells. PC12 cells were maintained as described previously (*45*), incubated in serum-free medium for 16 h, and then stimulated with 50 μM forskolin as indicated. After immunoprecipitation with anti-Nedd4 antiserum (20 μL, lanes 2 and 3) or preimmune serum (20 μL, lane 1), the immunocomplexes were analyzed by immunoblotting with anti-Nedd4 (upper panel) or anti-14-3-3η (lower panel). Arrows indicate the positions of endogenous Nedd4-2 and 14-3-3η.

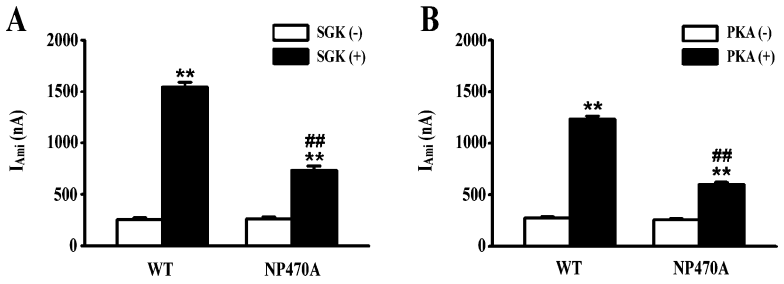

FIGURE 6: 14-3-3 is implicated in both SGK1- and PKA-dependent control of Na^+ transport. *Xenopus* oocytes were injected with cRNAs encoding each ENaC subunit (α, β, and γ), wild-type hNedd4-2 or NP470A mutant, and SGK1 (A) or PKA (B). Amiloride-sensitive Na^+ currents were measured using a two-electrode voltage clamp technique as described under Experimental Procedures. Data for each column experiment were obtained using seven oocytes. The statistical significance of the difference is expressed as $p < 0.01$ versus SGK1 (−) or PKA (−) (**) and $p < 0.01$ versus WT (##).

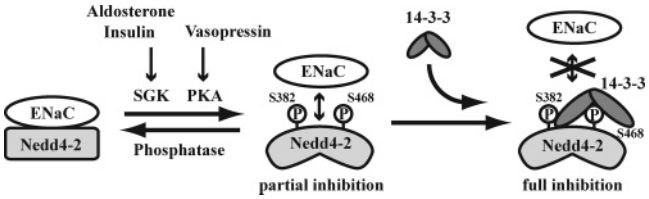

FIGURE 7: Schematic illustration of the proposed role for 14-3-3 in protein kinase dependent regulation of Nedd4-2.

Table 1: List of E3 Ubiquitin Protein Ligases So Far Identified as 14-3-3-Binding Proteins in Comprehensive Proteomic Studies

protein	GI	class	ref[a]
CBL	29731	RING finger	3, 5
CBLB	29789319	RING finger	5
CBLL1	13376204	RING finger	2
DTX2	24308253	RING finger	4, 5
HECTD1	32698702	HECT domain	3
ITCH	27477109	HECT domain	4
MYCBP2	7662380	RING finger	2, 3, 5
PHF3	34874598	RING finger	5
PRPF19	7657381	RING finger	3
RNF20	34868389	RING finger	5
RNF40	23618895	RING finger	5
SH3MD2	34878107	RING finger	5
SMURF1	34870386	HECT domain	5
SYTL4	17989356	RING finger	5
TRIM32	27714689	RING finger	4, 5
ZNF9	4827071	RING finger	1
ZNRF2	34855912	RING finger	5

[a] 1, Rubio et al. (*14*); 2, Meek et al. (*15*); 3, Jin et al. (*16*); 4, Benzinger et al. (*17*); 5, our unpublished data.

will address the detailed molecular mechanism of 14-3-3-mediated regulation of hNedd4-2.

A recent study identified another E3 ubiquitin ligase, parkin, as a target of 14-3-3 and demonstrated that the 14-3-3/parkin complex inhibits the formation of the parkin/substrate (synphilin) complex (*44*). Given that 14-3-3 binds many other ubiquitin E3 ligases, as revealed by proteomic studies (Table 1), this protein family may help regulate a wide variety of biological processes that are coupled with protein degradation via the ubiquitin−proteasome system.

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