

Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na⁺ channel cell surface expression

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The epithelial Na⁺ channel (ENaC) plays an essential role in the regulation of whole body Na⁺ balance and blood pressure. The cell surface expression of this channel, a complex of three subunits (α , β and γ ENaC), has been shown to be regulated by hormones such as aldosterone and vasopressin and by intracellular signaling, including ubiquitylation and/or phosphorylation. However, the molecular mechanisms involving phosphorylation in the regulation of ENaC are unclear. Here we show by expression studies in *Xenopus laevis* oocytes that the aldosterone-induced Sgk1 kinase interacts with the ubiquitin protein ligase Nedd4-2 in a PY motif-dependent manner and phosphorylates Nedd4-2 on Ser444 and, to a lesser extent, Ser338. Such phosphorylation reduces the interaction between Nedd4-2 and ENaC, leading to elevated ENaC cell surface expression. These data show that phosphorylation of an enzyme involved in the ubiquitylation cascade (Nedd4-2) controls cell surface density of ENaC and propose a paradigm for the control of ion channels. Moreover, they suggest a novel and complete signaling cascade for aldosterone-dependent regulation of ENaC.

Keywords: aldosterone/ion channel/Na⁺ transport/phosphorylation/ubiquitylation

Introduction

Na⁺ homeostasis, which is crucial for the maintenance of blood volume and pressure, is primarily regulated in the kidney, particularly in the distal areas of the nephron including the cortical collecting duct (CCD). Specialized epithelial cells (principal cells) express the amiloride-sensitive epithelial Na⁺ channel (ENaC) at the apical side (Garty and Palmer, 1997), allowing entry of Na⁺ into the

cell and a Na⁺,K⁺-ATPase, extruding Na⁺ in exchange for K⁺, at the basolateral side. The concerted action of these two membrane proteins results in transepithelial Na⁺ absorption from the urinary to the sanguine compartment. In this context, ENaC activity, the rate-limiting step of Na⁺ transport, is under complex hormonal regulation, including aldosterone, vasopressin and insulin. However, the molecular mechanisms of this regulation are poorly understood (Verrey *et al.*, 2000).

ENaC comprises three homologous subunits (α , β and γ) that are each composed of two transmembrane domains, an extracellular loop and short N- and C-termini (Canessa *et al.*, 1993, 1994). Significantly, each subunit contains a PY motif (xPPxY) in the C-terminal region, which we and others have shown to interact with the WW domains of the ubiquitin protein ligases Nedd4-1 and Nedd4-2 (Staub *et al.*, 1996; Kanelis *et al.*, 1998, 2001; Abriel *et al.*, 1999; Harvey *et al.*, 1999; Farr *et al.*, 2000; Kamynina *et al.*, 2001a,b; Snyder *et al.*, 2001). Because ubiquitylation (the covalent attachment of ubiquitin polypeptides to target proteins) of plasma membrane proteins is recognized as a mechanism to target such proteins for internalization (reviewed in Hicke, 1997, 1999; Rotin *et al.*, 2000), we postulated that Nedd4 isoforms may control cell surface expression of ENaC via a ubiquitylation-dependent mechanism (Staub *et al.*, 1996). This model is supported by the findings that the α and γ ENaC subunits are subjected to ubiquitylation (which controls cell surface expression; Staub *et al.*, 1997), and that Nedd4-2 and, to a weaker extent Nedd4-1, regulate ENaC activity (Dinudom *et al.*, 1998; Goulet *et al.*, 1998; Abriel *et al.*, 1999; Harvey *et al.*, 1999, 2001; Farr *et al.*, 2000; Kamynina *et al.*, 2001a,b; Snyder *et al.*, 2001). However, it is not known whether such ubiquitylation is under physiological control. The importance of the ENaC PY motifs is corroborated by findings that Liddle's syndrome, an inherited form of human hypertension (Liddle *et al.*, 1963; Botero-Velez *et al.*, 1994), is linked to mutations in ENaC that invariably cause either the deletion or the alteration of the β or γ PY motif (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a,b; Tamura *et al.*, 1996; Jeunemaitre *et al.*, 1997; J.Inoue *et al.*, 1998; T.Inoue *et al.*, 1998; Gao *et al.*, 2001; Yamashita *et al.*, 2001). When channels containing Liddle mutations are expressed in heterologous cell systems (e.g. *Xenopus laevis* oocytes), higher amiloride-sensitive Na⁺ currents (a measure of ENaC activity) are observed (Schild *et al.*, 1995, 1996; Snyder *et al.*, 1995), which are due to increased cell surface expression, open probability (Firsov *et al.*, 1996) and reduced Na⁺ feedback inhibition (Kellenberger *et al.*, 1998).

Recently, it has been reported that the expression of Sgk1 kinase (serum- and glucocorticoid-regulated kinase; Webster *et al.*, 1993), a member of the PKB/Akt family of serine/threonine kinases, is induced by aldosterone in cells

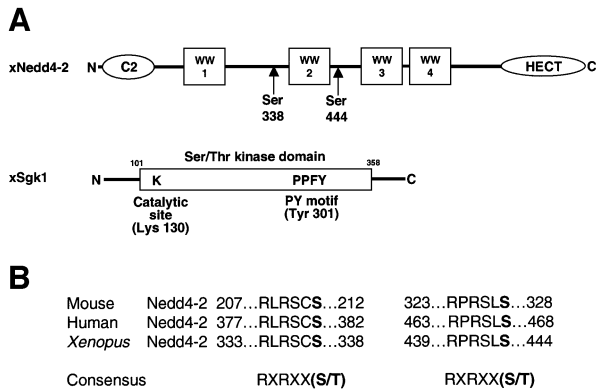


Fig. 1. Schematic view of Nedd4-2 and Sgk1. (A) Scheme of *Xenopus* Nedd4-2 with the consensus phosphorylation sites and *Xenopus* Sgk1 with the indication of the catalytic domain, the catalytically essential Lys130 and the PY motif. (B) Conserved consensus phosphorylation sites in mouse, human and *Xenopus* Nedd4-2.

of the CCD and, importantly, stimulates ENaC activity when co-expressed in *Xenopus* oocytes (Chen *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999). These findings suggested that Sgk1 may represent an important mediator for aldosterone-dependant ENaC regulation. It was further demonstrated in oocytes that Sgk1 increases the cell surface expression of ENaC (Alvarez de la Rosa *et al.*, 1999; Loffing *et al.*, 2001), may be regulated by insulin via PI-3 kinase-dependent phosphorylation and interacts *in vitro* with the C-termini of α and β ENaC (Wang *et al.*, 2001). However, the target(s) of Sgk1 and the molecular mechanism(s) of its action on ENaC are not known.

In this study, we have determined that the Sgk1 sequence contains a PY motif and Nedd4-2 comprises two conserved consensus sites for phosphorylation by Sgk1 [RXRXX(S/T); Kobayashi and Cohen, 1999; Park *et al.*, 1999], suggesting that (i) Sgk1 may interact with Nedd4-2 in a PY motif–WW domain-dependent mode and (ii) Nedd4-2 may be a target of Sgk1. Indeed, we found in *Xenopus* oocytes that Sgk1 phosphorylates Nedd4-2 in a PY motif-dependent manner, Nedd4-2 phosphorylation is required for Sgk1 action on cell surface density of ENaC and Sgk1 reduces the interaction between Nedd4-2 and ENaC. These data therefore show a novel mechanism of regulated ubiquitylation of an ion channel and suggest a complete signaling pathway between aldosterone and its main downstream target in the distal nephron, ENaC.

Results

Sgk1 stimulates phosphorylation of Nedd4-2

The recent reports that the aldosterone-inducible Sgk1 kinase controls ENaC activity (Chen *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999) and the identification of consensus phosphorylation sites for Sgk1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999) prompted us to look for potential phosphorylation sites in Nedd4-2, which is a regulator of ENaC. Indeed, we found two such conserved consensus motifs at Ser338 and Ser444 in *Xenopus* Nedd4-2 (Figure 1), suggesting that Nedd4-2 may be a target of Sgk1. To test this hypothesis, we expressed Nedd4-2 in *Xenopus* oocytes with or without

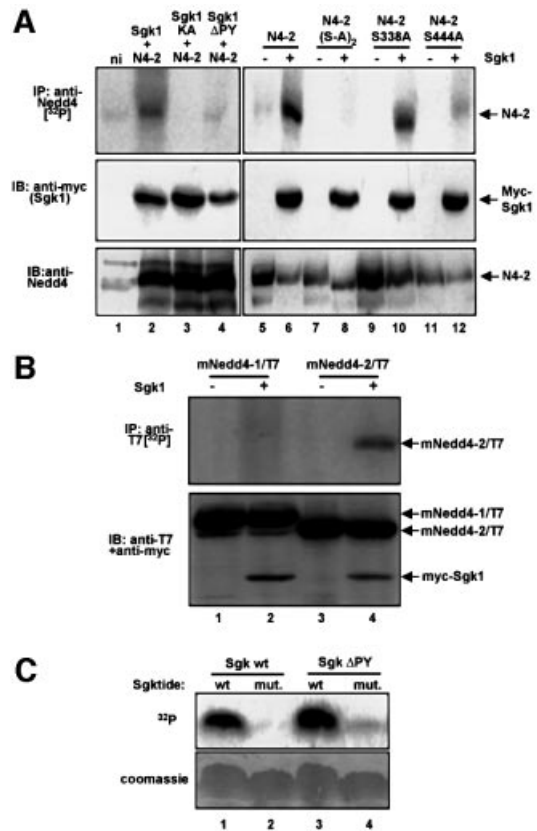


Fig. 2. Sgk1 phosphorylates Nedd4-2, but not Nedd4-1. (A) Oocytes expressing either wild-type Nedd4-2 (N4-2) or the phosphorylation mutants Nedd4-2-S338A-S444A [N4-2(S-A)₂], Nedd4-2-S338A, Nedd4-2-S444A and myc-Sgk1 [wild-type, catalytically inactive (KA, K130A) or PY motif-mutated (Δ PY, Y301A)] were incubated with [³²P]ortho-phosphate and treated as follows: (top), immunoprecipitation from lysates with anti-Nedd4-2 antibodies and autoradiography; middle, western blot on lysates with anti-myc antibody (recognizing Sgk1); bottom, western blot on lysates with anti-Nedd4-2 antibodies. (B) Mouse Nedd4-1 (mNedd4-1/T7) or Nedd4-2 (mNedd4-2/T7), both epitope-tagged with a T7 epitope (Novagen), were expressed with or without Sgk1 (as indicated) and phosphorylation was followed as described in (A), except that the Nedd4 proteins were immunoprecipitated with anti-T7 antibody (top). Expression of mNedd4-1, mNedd4-2 and Sgk1 was followed by western blot analysis on lysates, using both anti-T7 (Nedd4-1 or Nedd4-2) and anti-myc (Sgk1) antibodies. (C) Phosphorylation of a synthetic peptide substrate (Sgktide) by Sgk1. Wild-type and mutant myc-Sgk1 (lacking a functional PY motif) were expressed in *Xenopus* oocytes and immunoprecipitated with anti-myc antibodies. The immunoprecipitated kinases were assayed in a kinase assay with Sgktide or a mutant peptide lacking the phosphorylation site as described in Materials and methods. Phosphorylated peptides were then analyzed by separation on a tricine acrylamide gel followed by autoradiography. Both wild-type and mutant Sgk1 were able to phosphorylate Sgktide, but not its mutant (top). Bottom, Coomassie Blue staining.

c-myc-tagged Sgk1, incubated the oocytes with [³²P]ortho-phosphate and immunoprecipitated Nedd4-2 from a cellular lysate. We found that Nedd4-2 was strongly phosphorylated when Sgk1 was co-expressed (Figure 2A, lanes 2 and 6, top). Weak phosphorylation, possibly due to endogenous Sgk1 activity, was observed when Sgk1 was omitted (lane 5) or when a catalytically inactive Sgk1 was used (lane 3; Sgk1-KA, K130A). We then mutated, either together or separately, the two putative phosphorylation sites on Nedd4-2. This led to complete inhibition of Nedd4-2 phosphorylation when

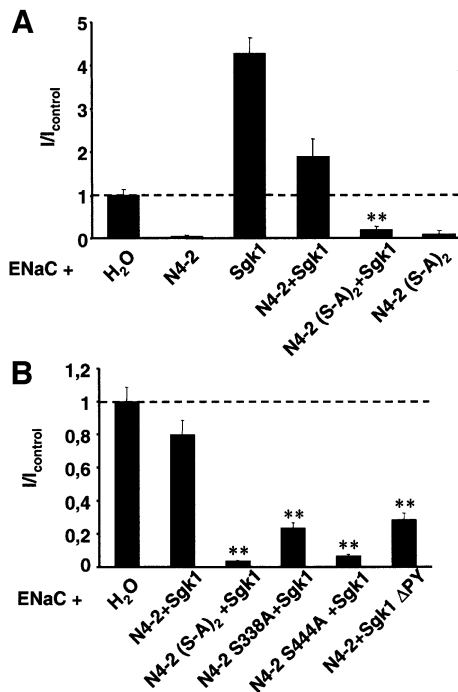


Fig. 3. Sgk1-dependent regulation of ENaC requires Nedd4-2 phosphorylation sites and the PY motifs on Sgk1. (A) Oocytes were injected with cRNA encoding Nedd4-2 or the Nedd4-2 phosphorylation mutant, Sgk1 cRNA and ENaC (as indicated). Amiloride-sensitive Na⁺ currents were measured and normalized to control oocytes (expressing only ENaC). $n = 15$ oocytes from three animals; ** $p < 0.01$ level of significance versus N4-2 + Sgk1. (B) Same as (A), but cRNA encoding either wild-type or mutant Sgk1 (Sgk1ΔPY), wild-type or phosphorylation site mutant Nedd4-2 and ENaC (as indicated) were injected. ** $p < 0.01$ versus control. The observed variation of N4-2 + Sgk1 between (A) and (B) is due to batch-to-batch variation.

both sites were mutated [Figure 2A, lane 8; N4-2(S-A)₂, S338AS444A], weak reduction when Ser338 was mutated (lane 10; Nedd4-2-S338A) and a much stronger reduction when Ser444 was changed to Ala (lane 12; Nedd4-2-S444A). As mentioned, Sgk1 also contains a PY motif, which may serve as an interaction site with the Nedd4-2 WW domains. Indeed, mutation of the PY motif abrogated Nedd4-2 phosphorylation almost completely (Figure 2A, lane 4; Sgk1ΔPY), although the same mutant was still able to phosphorylate a synthetic peptide substrate (Sgktide; Park *et al.*, 1999), demonstrating that mutation of the PY motif does not impair catalytic activity (Figure 2C). As shown in Figure 2B, Sgk1 did not efficiently phosphorylate the paralog Nedd4-1, which contains no consensus sequences for Sgk1-dependent phosphorylation and does not seem to be primarily involved in ENaC regulation (Kamynina *et al.*, 2001a). Hence, Sgk1 induces phosphorylation of Nedd4-2 (but not Nedd4-1) via a PY motif-dependent mechanism, essentially on Ser444 and to a lesser extent on Ser338.

Sgk1 regulates ENaC via Nedd4-2 phosphorylation

If phosphorylation of Nedd4-2 is the mechanism by which Sgk1 stimulates ENaC activity, mutation of the phosphorylation sites on Nedd4-2 would be expected to abrogate the stimulatory effect of Sgk1. To test this hypothesis, we expressed ENaC together with various

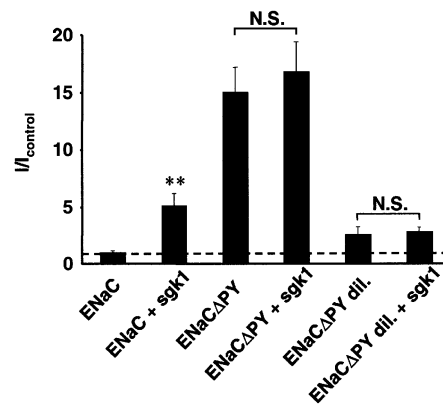


Fig. 4. The PY motifs of ENaC are required for stimulation of ENaC by Sgk1. Currents of oocytes expressing either wild-type ENaC or mutant ENaC lacking all PY motifs (ENaCΔPY), with and without Sgk1. ENaCΔPYdil containing 10 times less cRNA encoding ENaC was injected in order to get lower basal amiloride-sensitive currents. $n = 18$ oocytes from three animals; ** $p > 0.01$ versus ENaC.

Sgk1 and Nedd4-2 mutants in *Xenopus* oocytes (Figure 3). As reported previously, wild-type Nedd4-2 strongly reduced ENaC currents, whereas Sgk1 stimulated them (Figure 3A). When Sgk1 was expressed in excess over Nedd4-2, the inhibitory effect of Nedd4-2 on ENaC was reduced, leading to currents that were sometimes even higher than in the control oocytes (Figure 3A; N4-2 + Sgk1). However, Sgk1 did not increase ENaC activity when co-expressed with Nedd4-2 lacking both phosphorylation sites [Figure 3A, compare N4-2(S-A)₂ with N4-2(S-A)₂ + Sgk1]. In a separate experiment, we determined the importance of the individual phosphorylation sites for ENaC regulation. We found that when Ser338 alone was mutated (Figure 3B; Nedd4-2 S338A + Sgk1), Sgk1's effect was markedly reduced, whereas when Ser444 was mutated, Sgk1's effect was blunted (Nedd4-2 S444A + Sgk1). Thus, phosphorylation of Nedd4-2 on Ser444 is essential for the regulation of ENaC by Sgk1, while phosphorylation of Ser338 may also play a role. Finally, mutation of the Sgk1 PY motif reduced the ability of Sgk1 to interfere with Nedd4-2-dependent inhibition of ENaC (Figure 3B), consistent with an Sgk1-Nedd4-2 interaction involving the Sgk1 PY motif.

Because Nedd4-2 interacts not only via its WW domains with the PY motifs of Sgk1 but also with the PY motifs of ENaC, thereby exerting its effect on ENaC (Kamynina *et al.*, 2001a), one would expect that removal or mutation of the ENaC PY motifs abrogates Sgk1-dependent ENaC stimulation. Indeed, we found that ENaC channels lacking all functional PY motifs were not stimulated by Sgk1 (Figure 4, ENaCΔPY). Removal of PY motifs resulted in enhanced activity of ENaC, due in part to the described additional effects of the PY motif on open probability (Firsov *et al.*, 1996), raising the possibility that the system was unable to support additional Na⁺ transport, which may explain the lack of stimulation by Sgk1. However, Sgk1 remained without effect, even when 10 times less ENaCΔPY cRNA was injected, yielding low basal currents comparable to wild-type ENaC (Figure 4; compare ENaCΔPYdil with ENaCΔPYdil + Sgk1).

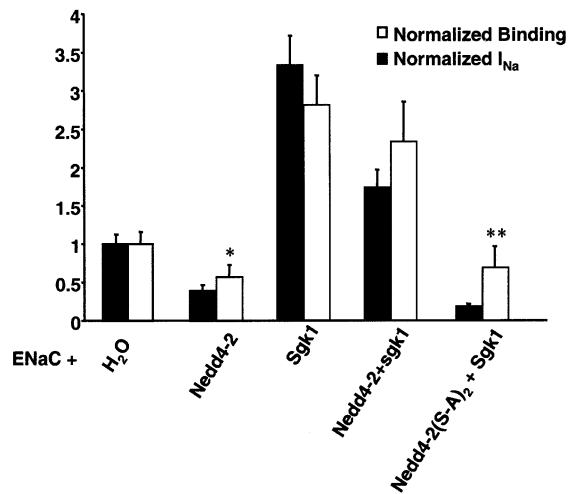


Fig. 5. Sgk1-dependent phosphorylation of Nedd4-2 controls ENaC cell surface expression. Oocytes were co-injected with cRNA encoding FLAG-tagged ENaC together with either H₂O, wild-type or mutant Nedd4-2 lacking both phosphorylation sites [Nedd4(S-A)₂] and Sgk1, as indicated. Amiloride-sensitive Na⁺ currents (filled bars) and binding of iodinated anti-FLAG antibodies (non-filled bars) to quantitate the number of channels at the cell surface were measured in the same oocytes, as described previously (Firsov *et al.*, 1996; Abriel *et al.*, 1999). Current and binding values were normalized to control values (ENaC + H₂O). *n* = 18 oocytes from three animals; **p* < 0.05 versus control, ***p* < 0.01 versus Nedd4-2 + Sgk1.

Therefore, the PY motifs of ENaC are required for the stimulation of ENaC-related currents by Sgk1.

Sgk1-dependent phosphorylation of Nedd4-2 controls cell surface expression of ENaC

We then investigated whether Sgk1-dependent phosphorylation of Nedd4-2 affects cell surface density of ENaC. We expressed ENaC, which was FLAG-tagged at the extracellular loops of the β and γ subunits, together with Sgk1 and various forms of Nedd4-2. This allowed quantification of ENaC surface expression by binding of ¹²⁵I-labeled FLAG antibodies (Firsov *et al.*, 1996). As shown previously, both Nedd4-2 (negatively; Abriel *et al.*, 1999) and Sgk1 (positively; Alvarez de la Rosa *et al.*, 1999; Loffing *et al.*, 2001) influenced amiloride-sensitive Na⁺ currents (Figure 5, filled bars) and antibody binding proportionally (non-filled bars), confirming that they mostly affect the expression of channels at the cell surface and not the intrinsic properties of ENaC. When Nedd4-2 and Sgk1 were co-expressed, channel density was high, which is compatible with the idea that Sgk1 interferes with Nedd4-2-dependent suppression of ENaC. In contrast, mutations of the phosphorylation sites on Nedd4-2 lead to a low number of ENaC channels even in the presence of Sgk1 [Nedd4-2(S-A)₂ + Sgk1], corroborating that phosphorylation of Nedd4-2 is required for Sgk1-dependent control of ENaC levels at the plasma membrane. We further confirmed this effect by immunocytochemical detection of FLAG-tagged ENaC on cryosections of *Xenopus* oocytes (Figure 6). Consistent with the binding experiments, co-expression of ENaC with Nedd4-2 and Sgk1 (+ Nedd4-2 + Sgk1) increased ENaC cell

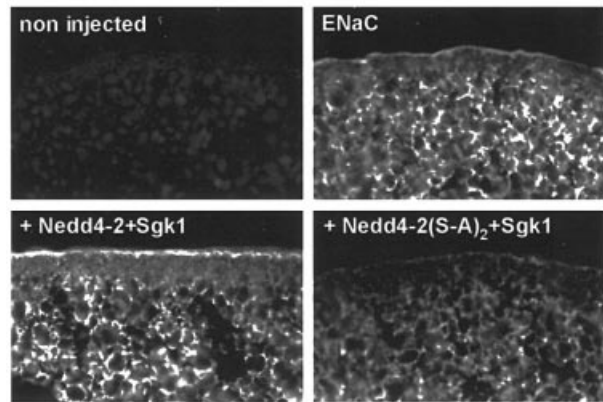


Fig. 6. Immunostaining of ENaC in oocytes expressing either wild-type or phosphorylation mutant Nedd4-2 and Sgk1. FLAG-tagged ENaC was followed by immunofluorescence with anti-FLAG antibodies on cryosections in either uninjected oocytes or oocytes expressing ENaC alone, ENaC plus wt-Nedd4-2 plus Sgk1 (+ Nedd4-2 + Sgk1) or ENaC plus Nedd4-2 S338A-S444A plus Sgk1 [+ Nedd4(S-A)₂ + Sgk1].

surface abundance, whereas co-expression of ENaC with Sgk1 and the phosphorylation mutant Nedd4-2 [+Nedd4-2(S-A)₂ + Sgk1] decreased it.

Sgk1 interferes with the interaction between Nedd4-2 and ENaC

We wished to know whether Sgk1-dependent phosphorylation of Nedd4-2 influenced ENaC cell surface expression. Sgk1 may interfere either with Nedd4-2–ENaC interaction or with other parameters, such as enzymatic Nedd4-2 activity. To determine whether the Nedd4-2–ENaC interaction was regulated by Sgk1, we expressed FLAG-tagged ENaC subunits together with mutant Nedd4-2 (Nedd4-2-CS) and various Sgk1 mutants. We chose to use the catalytically inactive Nedd4-2-CS (Abriel *et al.*, 1999) in order to avoid interference of ENaC ubiquitylation with the Nedd4-2–ENaC interaction. Cells were labeled overnight with [³⁵S]methionine and immunoprecipitations with anti-FLAG antibodies (recognizing FLAG-tagged ENaC) were performed. The immunoprecipitated material was analyzed by SDS–PAGE and autoradiography or western blotting. As can be seen, all three ENaC subunits were immunoprecipitated (Figure 7A, arrows). When Nedd4-2-CS was co-expressed, an additional band was observed (Figure 7A, arrowhead), which was identified as the Nedd4-2 mutant protein using anti-Nedd4-2 antibodies (Figure 7B, arrowhead). When Sgk1 was added, the intensity of the Nedd4-2 band was reduced (Figure 7A, lane 3) and dropped below the detection limit by the anti-Nedd4-2 antibody (Figure 7B, lane 3). When either catalytically inactive Sgk1 (Sgk1-KA, lane 4) or Sgk1 lacking the PY motif (Sgk1- Δ PY, lane 5) were expressed, the quantity of co-immunoprecipitated Nedd4-2-CS was not affected. Therefore, these data suggest that Sgk1 interferes with the interaction of Nedd4-2 and ENaC in a phosphorylation- and PY motif-dependent manner. This reduced interaction is likely to be the cause of the observed stimulation of ENaC surface expression by Sgk1.

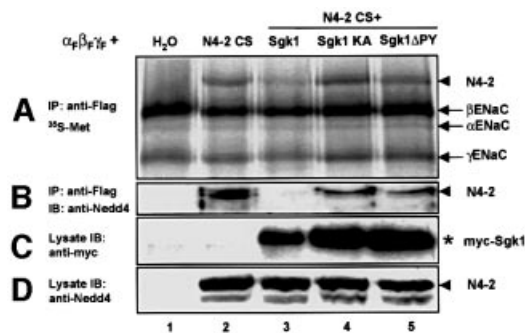


Fig. 7. Sgk1 interferes with ENaC–Nedd4-2 interaction. Oocytes were injected with cRNA encoding FLAG-tagged ENaC, Nedd4-2-CS and Sgk1, as indicated. After overnight incubation with [³⁵S]methionine, membrane fractions were lysed and immunoprecipitations performed with anti-FLAG antibodies. (A) Immunoprecipitated material analyzed by SDS-PAGE autoradiography and (B) western blotting using an anti-Nedd4-2 antiserum. Lysates were analyzed by western blotting using (C) anti-c-myc (i.e. Sgk1) and (D) anti-Nedd4-2 antibodies.

Discussion

In this study, we demonstrate that Sgk1-dependent phosphorylation of Nedd4-2 regulates ENaC cell surface expression and suggest a novel mechanism for the hormonal regulation of ENaC by aldosterone. Phosphorylation events have long been recognized to play a role in the regulation of ENaC (Sariban-Sohraby *et al.*, 1988; Ling and Eaton, 1989; Matsumoto *et al.*, 1993; Shimkets *et al.*, 1998; Blazer-Yost *et al.*, 1999; Chigaev *et al.*, 2001), but the molecular mechanisms (identity of the substrates, phosphorylation sites and involved kinases) remained vague. The recent discoveries that aldosterone-induced expression of Sgk1 kinase correlates with induction of transepithelial Na⁺ transport and that this kinase stimulates the ENaC activity at the cell membrane (Alvarez de la Rosa *et al.*, 1999; Chen *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999; Loffing *et al.*, 2001) pointed to Sgk1 as an important player in ENaC regulation, but the molecular mechanisms remained elusive.

The present data reveal the mechanism of Sgk1-dependent ENaC regulation. As indicated in Figure 1, Nedd4-2 contains two conserved consensus sites for phosphorylation by Sgk1 kinase [RXRXX(S/T); Kobayashi and Cohen, 1999; Park *et al.*, 1999]. Indeed, we demonstrate by co-expression of Sgk1 and Nedd4-2 that primarily Ser444 and, to a lesser extent, Ser338 are phosphorylated by Sgk1 kinase and that this phosphorylation depends on intrinsic Sgk1 kinase activity (Figure 2A). In *Xenopus* oocytes, when both phosphorylation sites are mutated, no phosphorylation of Nedd4-2 is observed. Phosphorylation of Nedd4-2 by the over-expressed Sgk1 kinase in the oocytes appears to be specific, as the paralog Nedd4-1, which does not contain a consensus for Sgk1 phosphorylation, is not primarily involved in ENaC regulation and is not phosphorylated by Sgk1. In addition, co-expression of Sgk1 together with ENaC in *Xenopus* oocytes does not induce phosphorylation of ENaC subunits (P.J.Plant and O.Staub, unpublished observations), which is in accordance with other reports finding no evidence for ENaC phosphorylation (Alvarez de la Rosa *et al.*, 1999; Chigaev *et al.*, 2001).

Several lines of evidence suggest that Sgk1 and Nedd4-2 interact via the PY motif on Sgk1 and, consequently, on the WW domains on Nedd4-2: (i) Nedd4-2 phosphorylation is largely reduced when Sgk1 is mutated on the tyrosine of the PY motif, (ii) mutation of the Sgk1 PY motif leads to a reduced effect of Sgk1 on Nedd4-2-dependent inhibition of ENaC activity, (iii) the PY mutant of Sgk1 does not interfere with ENaC–Nedd4-2 interaction, (iv) the PY mutant retains catalytic activity toward a synthetic peptide substrate and (v) in a two-hybrid screen using Sgk1 as a bait, Nedd4-1, which contains highly similar WW domains to Nedd4-2, was pulled out as a weak interacting protein with Sgk1 (E.Kamynina and O.Staub, unpublished observations). The fact that we are unable to co-immunoprecipitate the two proteins when co-expressed in oocytes suggests that the interaction between Sgk1 and Nedd4-2 is of a transient nature or, alternatively, this interaction may be indirect.

We found that the PY motifs of ENaC are required for Sgk1 to stimulate ENaC activity, which would be expected if the effect of Sgk1 is via Nedd4-2. These results apparently differ from earlier published data in which additional effects of Liddle mutations and Sgk1 were observed (Alvarez de la Rosa *et al.*, 1999; Shigaev *et al.*, 2000). However, the differences may be explained by the fact that only one subunit (β) was mutated (Shigaev *et al.*, 2000) and that both studies employed heterologous (mouse) Sgk1 in *Xenopus* oocytes (Alvarez de la Rosa *et al.*, 1999; Shigaev *et al.*, 2000). Alternatively, Sgk1 may act through other signaling pathways, in addition to phosphorylation of Nedd4-2.

Sgk1-dependent phosphorylation is likely to interfere with the ENaC–Nedd4-2 interaction, as suggested by the co-immunoprecipitation experiments, which show that less Nedd4-2 is co-immunoprecipitated with ENaC when Sgk1 is present. These effects depend on the catalytic activity of Sgk1 (the inactive SgkK130A does not interfere with ENaC–Nedd4-2 binding), which excludes the idea that the observed reduction of co-immunoprecipitated Nedd4-2 protein is simply due to a displacement of Nedd4-2 by Sgk1 via WW domain–PY motif interaction. Intriguingly, the phosphorylation sites are not localized in the vicinity of the WW domains (the interacting domains with ENaC). In fact, the predominantly used Ser444 is situated between WW domains two and three. Therefore, it is likely that phosphorylation at this site does not directly disturb interactions with ENaC, but instead induces a conformational change that renders the protein less competent for interaction with ENaC.

Cell surface expression of plasma membrane proteins is controlled by ubiquitylation (Bonifacino and Weissman, 1998; Hicke, 1999; Rotin *et al.*, 2000), primarily by regulating the rate of endocytosis, although alternative mechanisms (i.e. control of sorting at the level of the Golgi apparatus) have been proposed (Helliwell *et al.*, 2001). As a previous report suggested that Sgk1 does not affect the retrieval of ENaC from the plasma membrane (Alvarez de la Rosa *et al.*, 1999), it will be of further interest to determine whether the Sgk1–Nedd4-2 effect is primarily on insertion, retrieval or both. Our data show by two different approaches that Sgk1-dependent phosphorylation of Nedd4-2 and, consequently, regulated ubiquitylation affects channel number at the cell surface. To our

knowledge, this is the first reported case where phosphorylation of a ubiquitin protein ligase controls cell surface expression of an ion channel protein. In view of the increasing number of channels and membrane proteins containing PY motifs that are regulated by the family of Nedd4/Nedd4-like proteins [e.g. cardiac voltage-gated Na⁺ channel (Abriel *et al.*, 2000) or chloride channel ClC-5 (Schwake *et al.*, 2001)], it is possible that the proposed regulatory mechanism represents a common mode of channel regulation.

In conclusion, our data suggest that Sgk1 binds via its PY motif to the WW domains of Nedd4-2. This interaction leads to the phosphorylation of Ser444 and, to a lesser extent, Ser338, which in turn reduces the affinity of Nedd4-2 towards ENaC. Consequently, ENaC becomes less ubiquitylated, leading to the accumulation of ENaC channels at the cell surface. This accumulation may be due to either increased insertion of ENaC at the cell surface or reduced internalization, or both. This novel mode of regulation of ENaC by the aldosterone-induced Sgk1 kinase suggests, for the first time, an entire signaling pathway from aldosterone to ENaC, the major target of aldosterone regulation in the distal nephron.

Materials and methods

cDNA constructs

Xenopus Nedd4-2 constructs (Abriel *et al.*, 1999) were mutated to Ala on Ser338 or Ser444 or both together. Mouse Nedd4-1 and Nedd4-2 (Kamynina *et al.*, 2001a) were labeled with a T7 epitope (Novagen) at their C-terminus. *Xenopus* Sgk1 (Chen *et al.*, 1999) was labeled with a c-myc epitope (AEEQKLISEEDL) at its N-terminus. To create the catalytically inactive mutant K130A, Lys130 was mutated to Ala in the c-myc construct and Tyr301 changed to Ala to create an Sgk1 construct lacking a functional PY motif (Sgk1-ΔPY). The following *Xenopus* ENaC constructs were used: wild-type α, β and γ xENaC as described (Puoti *et al.*, 1995) or wild-type α, β and γ xENaC subunits each containing a FLAG epitope (DYKDDDDK). In α, the FLAG epitope replaced amino acids 133–140 (VQGWIPNN), in β it was inserted between Phe143 and Thr144 and in γ it replaced amino acids 140–147 (KRDVGNNV). xENaC subunits lacking PY motifs (ENaCΔPY) were generated by mutating Tyr599 in αxENaC to Ala, Arg583 in βxENaC to a stop codon and Tyr637 in γxENaC to Ala. cDNAs encoding rENaC subunits (Figures 3B, 5 and 6) were used as described (Canessa *et al.*, 1994). All cDNAs were subcloned into the pSDeasy plasmid (Puoti *et al.*, 1997).

Expression in *Xenopus* oocytes and electrophysiological measurements

Plasmids encoding ENaC, Sgk1 and Nedd4-2 proteins were linearized and transcribed, the cRNA injected into *Xenopus* oocytes and electrophysiological measurements performed after overnight incubation as described previously (Puoti *et al.*, 1995; Abriel *et al.*, 1999; Chen *et al.*, 1999). The following quantities of cRNA were injected: ENaC, 3 ng for each subunit; Sgk1, 7 ng; Nedd4-2, 6.6 ng (Figures 2 and 7) and 1.5 ng (Figures 3, 5 and 6).

Cell surface binding assay and immunofluorescence studies

The binding assay and the immunofluorescence studies using anti-FLAG antibodies were performed as described (Firsov *et al.*, 1996; Abriel *et al.*, 1999) in modified Barth's solution (MBS) containing 10 mM Na⁺.

Biochemical analysis of *Xenopus* oocytes

For the phosphorylation experiment, injected oocytes were kept overnight in MBS (Schild *et al.*, 1996), labeled for 3 h in MBS (5 μl/oocyte) containing 4 μCi/μl of [³²P]ortho-phosphate, washed three times with MBS and lysed in Triton X-100 homogenization buffer (20 μl/oocyte, 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 100 mM NaF, 10 mM Na pyrophosphate and 10 mM Na-ortho-vanadate). After two centrifugations at 4°C for 10 min at

20 000 g, the supernatant was recovered and immunoprecipitations with anti-*Xenopus* Nedd4-2 antibodies (Abriel *et al.*, 1999) were performed. The immunoprecipitated material was analyzed by SDS-PAGE (7%) followed by autoradiography. Western blot analysis and the co-immunoprecipitation analysis were performed as described by Kamynina *et al.* (2001a), using either anti-c-myc (Santa Cruz Biotechnology), anti-FLAG (Sigma; M2) or anti-*Xenopus* Nedd4-2 antibodies (Abriel *et al.*, 1999).

In vitro kinase assay

Oocytes injected with cRNA encoding myc-Sgk1 or myc-Sgk1 Y301A were kept overnight in MBS and lysed in Triton X-100 homogenization buffer without phosphatase inhibitors. After centrifugation at 4°C for 10 min at 20 000 g, the supernatant was recovered and immunoprecipitations of wild-type or mutant myc-Sgk1 with anti-myc antibodies were performed. The immune complexes were washed five times with Triton X-100 homogenization buffer without phosphatase inhibitors, followed by washing with kinase assay buffer [50 mM Tris-HCl pH 7.5 and 0.1% (v/v) 2-mercaptoethanol]. The kinase assay was carried out as described (Park *et al.*, 1999). Briefly, 30 μl of beads containing the immunoprecipitated kinase were incubated in a final volume of 50 μl for 1 h at 30°C with 1 mM Sgk1tide (KKRNRRLSVA) or 1 mM of mutated Sgk1tide (KKRNRRLAVA) as peptide substrates, 10 mM MgCl₂ and 100 μM [³²P]ATP (1000–2000 c.p.m./pmol). Phosphorylated peptides were separated on 16% tricine acrylamide gel and analyzed by autoradiography.

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