beta1Pix exchange factor stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in kidney epithelial cells

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Our previous work has established that the metabolic sensor AMP-activated protein kinase (AMPK) inhibits the epithelial Na⁺ channel (ENaC) by promoting its binding to neural precursor cell–expressed, developmentally down-regulated 4–2, E3 ubiquitin protein ligase (Nedd4-2). Here, using MS analysis and in vitro phosphorylation, we show that AMPK phosphorylates Nedd4-2 at the Ser-444 (Xenopus Nedd4-2) site critical for Nedd4-2 stability. We further demonstrate that the Pak-interacting exchange factor βPix is required for AMPK-mediated inhibition of ENaC-dependent currents in both CHO and murine kidney cortical collecting duct (CCD) cells. Short hairpin RNA–mediated knockdown of βPix expression in CCD cells attenuated the inhibitory effect of AMPK activators on ENaC currents. Moreover, overexpression of a βPix dimerization–deficient mutant unable to bind 14–3–3 proteins (Δ602–611) increased ENaC currents in CCD cells, whereas overexpression of WT βPix had the opposite effect. Using additional immunoblotting and co-immunoprecipitation experiments, we found that treatment with AMPK activators promoted the binding of βPix to 14–3–3 proteins in CCD cells. However, the association between Nedd4-2 and 14–3–3 proteins was not consistently affected by AMPK activation. βPix knockdown, or overexpression of WT βPix or the βPix-Δ602–611 mutant. Moreover, we found that βPix is important for phosphorylation of the aforementioned Nedd4-2 site critical for its stability. Overall, these findings elucidate novel molecular mechanisms by which AMPK regulates ENaC. Specifically, they indicate that AMPK promotes the assembly of βPix, 14–3–3 proteins, and Nedd4-2 into a complex that inhibits ENaC by enhancing Nedd4-2 binding to ENaC and its degradation.

ENaC² is apically expressed in many salt-reabsorbing epithelia (1). In the aldosterone-sensitive distal nephron of the kidney and colon epithelia, ENaC is a critical regulator of sodium balance, blood volume, and blood pressure (2). ENaC also plays an important role in fluid reabsorption at the air–liquid interface in distal lung airways, which determines the rate of mucociliary transport (2, 3). ENaC appears to form a heterotrimERIC channel composed of three subunits (α, β, and γ), each of which contains two transmembrane domains, cytosolic amino and carboxyl termini, and an extracellular loop (4, 5). Abnormal ENaC activity is a characteristic feature in patients with type 1 pseudohypoaldosteronism, Liddle syndrome, and cystic fibrosis (6).

ENaC expression and activity at the apical membrane are regulated by Nedd4-2, a member of the E6–associated protein C Terminus (HECT) family of E3 ubiquitin ligases, which, in turn, is regulated by several kinases, including serum- and glucocorticoid-induced kinase 1 (SGK-1) and PKA (7–9). Nedd4-2 directly binds to PY motifs on the C termini of ENaC subunits, promoting ENaC ubiquitination, internalization, and degradation. SGK1 and PKA activate ENaC largely through a mechanism involving phosphorylation of xNedd4-2 predominantly at residues Ser-338, Thr-363, and Ser-444, which appears to enhance the association of Nedd4-2 with 14–3–3 scaffolding proteins and thereby prevent Nedd4-2-mediated degradation of ENaC (10–14).

β₁-Pix exchange factor stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in kidney epithelial cells

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AMPK is a Ser/Thr kinase that exists as a heterotrimer comprised of a catalytic α-subunit and regulatory β- and γ-subunits. AMPK is known as a key homeostatic regulator of energy balance at the cellular and systemic levels. A reduction in the energy charge of the cell (increased AMP or ADP or reduced ATP) or increased calcium, osmotic, or oxidative stress elicits AMPK activation during conditions of metabolic and other cellular stresses (15, 16). Phosphorylation of Thr-172 within the α subunit accounts for most of the activation of AMPK by upstream kinases, such as liver kinase B1 (LKB1) or Ca²⁺/calmodulin-dependent kinase-β (17, 18). Besides being an energy sensor, AMPK also plays an important role in apoptosis, cell growth, gene transcription, and protein synthesis (19). In recent years, reports regarding the roles of AMPK in renal physiology and disease, such as podocyte function, renal hypertrophy, ischemia, inflammation, diabetes, and polycystic kidney disease, have rapidly escalated (20–25). We and others have also demonstrated that AMPK regulates a variety of ion transport proteins (16), including inhibition of ENaC by promoting Nedd4-2 interaction with β-ENDaC and enhancing Nedd4-2−dependent ubiquitination, internalization, and degradation of the channel (26–28).

Several small G proteins, including K-Ras, Rho A, and Rab11, have been shown to regulate ENaC activity (29–31). Given that G protein activation relies on the coordinated action of guanine nucleotide exchange factors (GEFs), GEFs could be involved in ENaC modulation. p21-activated kinase (PAK)−interacting exchange factor β (βPix) is a member of the diffuse B cell lymphoma family of Rho-GEFs, existing in two major isoforms, βPix and βPix. In kidneys, βPix has emerged as a main PIP isoform (32, 33) and contains a Dbl homology domain, a pleckstrin homology domain, and an Src homology 3 domain for binding PAK through a proline-rich region. βPix also has a GIT1 (G protein−coupled receptor kinase interactor 1) binding domain and a leucine zipper domain responsible for βPix dimerization (34, 35). By using tagged 14-3-3 proteins (36) and tandem affinity purification and LC-MS methods (37), dimeric βPix was found to bind to dimeric 14-3-3 proteins. Of note, we previously discovered βPix to be a critical regulatory co-factor in the endothelin-1 (ET−1)-dependent regulation of ENaC via Nedd4-2 and 14-3-3 proteins (38).

In this study, we show that AMPK inhibits ENaC by direct phosphorylation of xNedd4-2 at Ser-444 (equivalent to Ser-328 of mNedd4-2), a site we previously showed enhances Nedd4-2 stability (14). Through ENaC current recordings in Chinese hamster ovary (CHO) cells and in mouse polarized CCD cells, we also demonstrate that βPix expression and function are required for ENaC inhibition by AMPK. Treatment with AMPK activators increases the binding of βPix to 14-3-3 proteins in two distinct CCD cell lines. Moreover, βPix knockdown inhibits mNedd4-2 phosphorylation at Ser-328, reducing Nedd4-2 protein expression. Overall, our results suggest that functional βPix is critical for Nedd4-2 stability and AMPK may enhance Nedd4-2-dependent ENaC degradation by promoting the formation of a βPix−Nedd4-2−14-3-3 protein complex. These findings shed new light on the molecular mechanisms by which AMPK regulates ENaC.

**Results**

**AMPK phosphorylates Nedd4-2 at Ser-444**

Our previous work has shown that AMPK could phosphorylate Nedd4-2 in vitro and in intact cells (27). To identify the AMPK phosphorylation site(s) on Nedd4-2, purified GST-xNedd4-2 was expressed in *Escherichia coli* and then subjected to in vitro phosphorylation in the presence of purified active AMPK holoenzyme and [γ-³²P]ATP. Phosphorylation site mapping of tryptic fragments was performed by MALDI-TOF MS and solid-phase sequencing as described previously (39). We found that AMPK phosphorylated xNedd4-2 at Ser-444 (Fig. 1, A and B). AMPK phosphorylation at this site was confirmed by comparing [γ-³²P]ATP incorporation into the WT with a Ser-to-Ala mutant (S444A) Nedd4-2 in vitro, where significantly reduced ³²P incorporation was observed in the S444A mutant compared with WT Nedd4-2 (Fig. 1C).

**Functional βPix is required for ENaC inhibition by AMPK**

It has been reported that phosphorylated Ser-444 on xNedd4-2 could serve as a binding site for 14-3-3 proteins, which may act to sequester Nedd4-2 and thereby prevent its interaction with ENaC (11, 40). Our previous study also showed that a Nedd4-2 mutant (S444A) has a dramatically shorter cellular half-life than WT Nedd4-2, but this property is not dependent on binding to 14-3-3s (14). Because Ser-444 is also phosphorylated by other kinases besides AMPK, including SGK1, PKA, and IkB kinase-β (IKKβ), which have opposite effects on ENaC expression (9−11, 41, 42), we reasoned that AMPK phosphorylation of Nedd4-2 alone could not account for the ENaC regulation. As it has been demonstrated that βPix is involved in long-term ENaC inhibition by ET-1 through impairing 14-3-3β binding to Nedd4-2 (38), we hypothesized that βPix may also play an important role in AMPK-dependent ENaC inhibition. To examine this hypothesis, ENaC was co-expressed with either WT or a dimerization-deficient deletion tract (∆602−611) mutant of βPix in CHO cells. AICAR treatment (1 mM) was used to activate AMPK (43). Representative current sweeps evoked by a voltage ramp from +60 to −100 mV (holding potential at 40 mV) recorded in whole-cell patch-clamp mode are shown before and after treatment with amiloride (10 µM), an inhibitor of ENaC (Fig. 2A). As summarized in Fig. 2B, either AICAR treatment or co-expression of WT βPix significantly decreased ENaC-dependent currents. However, there was no apparent additive effect between WT βPix overexpression and AICAR treatment. These effects were prevented with co-expression of the βPix-Δ602−611 mutant. Besides using the AMPK activator, we also co-expressed ENaC with either pTracer vector alone (control), a dominant-negative (DN) AMPK-α1−K45R mutant, or a constitutively active (CA) AMPK-γ1−R70Q mutant in CHO cells to modulate AMPK activity. As shown in Fig. 2C, CA-AMPK inhibited the ENaC current relative to DN-AMPK and control, but this inhibition was prevented in cells overexpressing the dimerization-deficient ∆602−611 βPix mutant. Together, these findings suggest that AMPK inhibits ENaC activity via βPix. This mechanism requires functional dimeric βPix, which is known to be required for βPix binding to 14-3-3 proteins (44).
Overexpression of WT versus mutant \(\beta_1\text{Pix}\) modulates ENaC currents in polarized mpkCCDc14 cells

To examine whether the inhibitory effect of \(\beta_1\text{Pix}\) on ENaC currents can also be observed in more physiologically relevant mouse kidney cortical collecting duct epithelial cells, V5-tagged WT \(\beta_1\text{Pix}\) or \(\beta_1\text{Pix-}\Delta 602–611\) was stably transduced into mpkCCDc14 cells (45) for inducible overexpression with a Tet-On system. After cells polarized on Transwells were exposed to doxycycline (Dox, 2 \(\mu\text{g/ml}\)) for 3 days, amiloride-sensitive ENaC currents were measured using an epithelial volt-ohm–meter (EVOM). Overexpression of \(\beta_1\text{Pix-}\Delta 602–611\), which is unable to bind 14-3-3 proteins, slightly increased ENaC equivalent short-circuit currents in mpkCCDc14 cells, whereas overexpression of WT \(\beta_1\text{Pix}\) had the opposite effect (Fig. 3A), consistent with the results found in CHO cells. After EVOM measurements, cells were harvested to confirm the expression of V5-tagged WT \(\beta_1\text{Pix}\) or \(\beta_1\text{Pix-}\Delta 602–611\) by immunoblotting (Fig. 3B). WT \(\beta_1\text{Pix}\) and \(\beta_1\text{Pix}\) mutant expression levels were \(\sim 63\) and \(12\%\) of the endogenous \(\beta_1\text{Pix}\) levels, respectively, as determined by comparing total cellular \(\beta_1\text{Pix}\) levels with or without Dox treatment by immunoblotting (data not shown). The lower overexpression level of the \(\beta_1\text{Pix}\) mutant, which we observed in several different mutant clones generated, may result from its instability because the mutant is unable to dimerize and bind to 14-3-3 proteins.

AMPK-dependent interplay of \(\beta_1\text{Pix}\), Nedd4-2, and 14-3-3 proteins in polarized mpkCCDc14 cells with overexpression of WT versus mutant \(\beta_1\text{Pix}\)

To examine how \(\beta_1\text{Pix}\) is involved in AMPK-regulated ENaC inhibition, we also tested whether AMPK modulation alters the associations between \(\beta_1\text{Pix}\), Nedd4-2, and 14-3-3 proteins. Inducible \(\beta_1\text{Pix}\) construct–expressing mpkCCDc14 cells were polarized on Transwell plates, followed by exposure to doxycycline for 3 days and then combined treatment with the AMPK activators AICAR (1 mM) and A769662 (100 \(\mu\text{M}\)) (AA) versus vehicle for 1 day. Cells were lysed, and immunoblotting for various proteins was performed on a small sample of the whole-cell lysates (Fig. 4A, top panel, Input), whereas the remaining cell lysate was subjected to immunoprecipitation (IP) with a pan-14-3-3 protein antibody. Immunoblotting was then performed to detect co-immunoprecipitated V5 (\(\beta_1\text{Pix}\)) and Nedd4-2 (Fig. 4A, bottom panel). Neither overexpression of WT \(\beta_1\text{Pix}\) nor the \(\beta_1\text{Pix-}\Delta 602–611\) mutant affected AMPK
activation (Fig. 4B). In cells overexpressing WT β₁Pix, AMPK activators significantly enhanced the binding of β₁Pix to 14-3-3 proteins (Fig. 4, A and C). As expected, the β₁Pix-Δ602–611 mutant was unable to bind to 14-3-3 proteins. However, the association between Nedd4-2 and 14-3-3 proteins was not significantly altered by AMPK activation and/or overexpression of the WT or the β₁Pix-Δ602–611 mutant in these cells (Fig. 4, D and E).

**Inhibition of ENaC currents by AMPK is blunted with β₁Pix knockdown in mCCDcl1 cells**

To further confirm the importance of β₁Pix in ENaC inhibition by AMPK, β₁Pix was stably knocked down in mCCDcl1 cells (46), which have a higher Nedd4-2 abundance relative to total Nedd4 than mpkCCDc14 cells. Using lentiviral constructs, either scrambled control shRNA or shRNA directed against β₁Pix, stable cell lines were achieved with 45% knockdown of β₁Pix compared with control cells (Fig. 5D). ENaC currents were measured in control and β₁Pix knockdown cells before and during combined treatment with AA versus vehicle (control) for 4 and 24 h (Fig. 5, A and B). Although ENaC current inhibition caused by AA treatment was observed in both control and β₁Pix knockdown cells, in β₁Pix knockdown cells, the decrease of ENaC current was significantly reduced by 55% and 45% at 4 and 24 h, respectively, relative to control cells (Fig. 5C), without any difference in AA treatment–induced AMPK activation between the two cell lines (see below). Together, these results suggest that β₁Pix acts as a negative regulator of ENaC downstream of AMPK.

**Involvement of β₁Pix in Nedd4-2 regulation with AMPK modulation in mCCDcl1 cells**

Compared with mpkCCDc14 cells, mCCDcl1 cells have a higher Nedd4-2 abundance relative to total Nedd4, so we reasoned that performing immunoprecipitation and immunoblotting assays with mCCDcl1 cells could provide a better opportunity to detect differences in the interaction between β₁Pix, Nedd4-2 and 14-3-3 proteins as a function of AMPK activation

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**Figure 2.** β₁Pix is necessary for the inhibition of ENaC by AMPK. A, overlays of typical macroscopic current traces before and after 10-µM amiloride treatment from whole-cell patch-clamped CHO cells expressing mouse ENaC (mENaC) with or without WT β₁Pix or β₁Pix-Δ602–611 either in the presence or absence of pretreatment with AMPK activator (1 mM AICAR, 12 h). Currents were evoked with a voltage ramp (+60 to −100 mV from a holding potential of 40 mV). B, summary graph of the mean ± S.E. for amiloride-sensitive current density at −80 mV for CHO cells expressing mENaC alone or with β₁Pix or β₁Pix-Δ602–611 mutant with or without 1 mM AICAR as indicated. pF, picofarad. C, summary graph of the mean ± S.E. for amiloride-sensitive current density for CHO cells expressing ENaC and either pTracer (control), DN AMPK-α1-K45R, or CA AMPK-γ1-R70Q with or without β₁Pix-Δ602–611. p values are shown for the indicated comparisons.
AMPK regulation of ENaC via β₁Pix

and β₁Pix knockdown (Fig. 6A). As summarized in Fig. 6C, AA treatment significantly enhanced the binding of both β₁Pix and Nedd4-2 to 14-3-3 proteins (p = 0.002 and 0.02, respectively), which was abrogated by β₁Pix knockdown, without disturbing AMPK activation (Fig. 6B). Of note, β₁Pix knockdown caused a significant down-regulation of cellular Nedd4-2 protein expression without reducing Nedd4-2 mRNA levels, as assessed by qPCR (Fig. 6, D and E). Indeed, there was a trend toward increased Nedd4-2 mRNA levels with β₁Pix knockdown, suggesting that these changes in Nedd4-2 gene expression represent a compensatory response to decreased Nedd4-2 protein expression rather than a causal explanation for it. Using a validated phospho-specific antibody (47), we also found that phosphorylation of mNedd4-2 at Ser-328 (equivalent to Ser-444 in xNedd4-2) was concomitantly inhibited with β₁Pix knockdown (Fig. 6F). Cycloheximide chase assays were performed to examine the rates of cellular Nedd4-2 protein degradation and demonstrated decreased Nedd4-2 protein stability in the setting of β₁Pix knockdown (Fig. 6G). This effect is consistent with the known key importance of phosphorylation at this residue (mNedd4-2–Ser-328 or xNedd4-2–Ser-444) in Nedd4-2 protein stability (14). Together, these results demonstrate that β₁Pix plays an important role in Ser-328 phosphorylation and, thus, cellular stability of Nedd4-2 in mCCD₁ cells. Overall, our findings suggest that AMPK activation, through facilitating the association of β₁Pix, 14-3-3 proteins, and Nedd4-2 into a complex, stabilizes and enhances Nedd4-2 binding to ENaC, thereby promoting ENaC ubiquitination and degradation, as described previously (27, 28).

Discussion

We and others showed previously that AMPK is a negative regulator of the ENaC activity in oocytes and polarized epithelial cells (26–28). ENaC inhibition is mediated by decreasing its expression level at the plasma membrane rather than through a change of single channel properties (open probability or conductance) (26). AMPK typically controls its downstream effectors through phosphorylation of target proteins. However, there is no evidence that ENaC subunits are directly phosphorylated by or interact with AMPK. With the presence of a Liddle’s-type mutation (β-mENaC–Y618A), a role of the C-terminal tail of β-ENaC in AMPK-related inhibition was revealed, implying the involvement of the E3 ubiquitin ligase Nedd4-2 (26).

We previously demonstrated that AMPK phosphorylates Nedd4-2 both in vitro and in an intact cellular milieu (27). The association of Nedd4-2 with β-ENaC was enhanced with AMPK activation. A ubiquitin ligase–deficient Nedd4-2 mutant blocked the AMPK inhibitory effect on ENaC, as did addition of the deubiquitinating enzyme Usp2–45, suggesting that Nedd4-2-mediated ubiquitination is necessary for ENaC inhibition (27, 28). In this study, we found that Ser-444 on xNedd4-2 is an AMPK phosphorylation site (Fig. 1). Phosphorylation at Ser-444 has been revealed previously to be critical for Nedd4-2 cellular stability and its association with 14-3-3 (14). This phosphorylation event by AMPK is the first key step in our current simplified working model for how ENaC regulation by AMPK occurs via Nedd4-2 (Fig. 7). However, Ser-444 is a shared phosphorylation site targeted by several other kinases,
such as SGK1, PKA, and IKKβ (10, 42), leading to the hypothesis that, besides Nedd4-2, other molecules may be critical for ENaC regulation by AMPK. Our earlier work studying the mechanism of ENaC regulation by ET-1 demonstrated that the guanine nucleotide exchange factor 1Pix inhibits ENaC expression through the 14-3-3/Nedd4-2 pathway (38). The results from this study show that treatment with an AMPK activator inhibits ENaC-dependent current, an effect that is also observed with overexpression of WT 1Pix as shown in Fig. 2. We also found that there was no synergistic or additive inhibitory effect observed with combined WT-βPix overexpression and AICAR treatment, suggesting that 1Pix and AMPK are components of the same regulatory pathway for ENaC. Moreover, ENaC inhibition caused by either AICAR treatment or overexpression of a constitutively active form of AMPK could be abolished with coexpression of the βPix-A602–611 mutant. The importance of βPix in AMPK-dependent ENaC regulation was also demonstrated in two distinct polarized mouse kidney principal cell lines, mpkCCD<sub>c14</sub> and mCCD<sub>c11</sub>, with either overexpression of WT βPix or βPix-A602–611 or with βPix knockdown in this study. Our data further revealed that βPix knockdown blunts ENaC inhibition caused by combined AICAR and A769662 treatment without affecting AMPK activation. Taken together, these results indicate that βPix is a downstream mediator of AMPK and suggest that its dimerization and binding to 14-3-3 proteins are necessary for ENaC regulation.

As phospho-Ser/phospho-Thr binding proteins, 14-3-3s regulate proliferative, survival, apoptotic, and stress signaling by interacting with a diverse array of binding partners (48). Of note, 14-3-3 proteins are critical for suppressing mTOR complex 1 (mTORC1) activity under conditions of cell stress. AMPK has been shown to inhibit mTORC1 through stimulating the Rheb-GAP (GTPase-activating protein) activity of TSC2 and by phosphorylating Raptor, an mTORC1 scaffolding protein that recruits downstream substrates of mTOR (49, 50). Phosphorylation of Raptor at Ser-722 and Ser-792 by AMPK mediates 14-3-3 binding and is required for mTORC1 inactiva-
tion in vivo (50). Recent work has established a new role of 14-3-3 proteins in ENaC regulation. Phosphorylation of Nedd4-2 by SGK1 has been reported to enhance the association between Nedd4-2 and 14-3-3 proteins and maintain Nedd4-2 in an inactive (sequestered) state, thereby causing a phosphorylation-dependent inhibition of the interaction between Nedd4-2 and ENaC (10, 51). In our previous study of ENaC regulation by ET-1, recruitment of 14-3-3 by 1Pix appeared to prevent the association between Nedd4-2 and 14-3-3, promoting the ubiquitination and degradation of ENaC (38). Although ET-1 has been reported to induce 1Pix translocation and Cdc42 activation via a PKA-dependent pathway in primary human mesangial cells (52), Rac1 and Cdc42 were not involved in 1Pix-dependent ENaC inhibition in CHO cells, suggesting that the GEF activity of 1Pix is not required for ENaC suppression (38). 1Pix is up-regulated in primary human mesangial cells with ET-1 treatment for 24 h (53). However, in this study, there was no significant change in 1Pix expression in polarized renal epithelial cells with AMPK activation for 24 h (Fig. 6B). Importantly, we found that AMPK activation increases the association between 1Pix and 14-3-3 proteins in both CCD cell lines (Fig. 7, step 2) but does not inhibit the binding of Nedd4-2 to 14-3-3 proteins in either cell line (Figs. 4 and 6) under conditions when AMPK inhibits ENaC (Fig. 5). Specifically, in mpkCCDc14 cells, the association between Nedd4-2 and 14-3-3 proteins was not significantly affected either by AMPK activators or by overexpression of WT or mutant 1Pix (Fig. 4, D and E). In contrast, AMPK activation in mCCDcl1 cells promoted Nedd4-2 binding to 14-3-3 proteins, and 1Pix knockdown blunted this increased Nedd4-2 association with 14-3-3s (Fig. 6C). Moreover, as shown in Fig. 6, D–F, 1Pix is required for the phosphorylation and cellular stability of Nedd4-2 (Fig. 7, step 3). Taken together, our findings indicate that AMPK decreases ENaC activity through a 1Pix/Nedd4-2–14-3-3–dependent mechanism that differs from the proposed mechanism of ET-1–dependent ENaC inhibition, although the final regulatory step featuring enhanced Nedd4-2–ENaC interaction with subsequent ENaC ubiquitination and degradation is the same (Fig. 7, step 4).

Pix is well-known as a regulator of cell motility, functioning as a GEF for Cdc42 and Rac1 and a binding partner to the PAK family of Cdc42/Rac1-activated kinases (54). 1Pix has related roles in promoting membrane ruffling (55), focal complex disassembly facilitating migration (56), and maintenance of epi-
and survival (59). Besides cell migration and survival, several studies reported that βPix is critical for cellular transformation and in vivo tumorigenesis, as it can sequester the c-Cbl ubiquitin ligase and prevent the ubiquitination and degradation of various growth factor receptors, including epidermal growth factor receptor, VEGFR2, and IGF1R (60, 61). Here we further demonstrate a novel role of βPix in AMPK-dependent ENaC regulation, suggesting the involvement of βPix in the regulation of the E3 ubiquitin ligase Nedd4-2 during metabolic stress conditions.

The effects of βPix on 14-3-3 proteins and Nedd4-2 downstream of AMPK likely have broader implications for the regulation of additional membrane transport proteins besides ENaC. Specifically, Nedd4-2 is known to regulate a growing list of transport proteins through a direct binding and ubiquitination mechanism similar to that originally characterized for ENaC, including voltage-gated Na⁺, K⁺, and Ca²⁺ channels; Cl⁻ channels; human organic anion transporters; and glutamate transporters in the brain (62). Moreover, it has already been shown that many of these transport proteins are also regulated by AMPK, so it is reasonable to propose that the mechanisms uncovered in this study may be generalizable to the numerous transport proteins that are regulated by AMPK and Nedd4-2. Additional studies to test whether βPix is a critical component in the regulation of these transport proteins in the kidney and other important organs like the heart, lung, and brain are thus warranted.

In summary, this study describes a novel function of βPix as a positive regulator of Nedd4-2 stability and a βPix/Nedd4-2–14-3-3 protein–dependent mechanism of AMPK-regulated ENaC inhibition. Our findings support a model whereby AMPK activation enhances Nedd4-2 cellular stability via βPix and induces ENaC degradation by enhancing the association of βPix, 14-3-3 proteins, and Nedd4-2 into a complex and further strengthening the phosphorylation of Nedd4-2 (Fig. 7). Future work will focus on investigating the mechanisms of how AMPK

Figure 6. βPix regulates AMPK-dependent Nedd4-2–14-3-3 association and Nedd4-2 cellular stability in mCCDcl1 cells. A, representative immunoblot (Input) and co-IP results of βPix, Nedd4-2, and 14-3-3 proteins in total lysates from polarized mCCDcl1 cells with or without βPix knockdown after 24-h treatment with or without AMPK activators (AA). Ab, antibody. B, summary graph of the ratio of pAMPK-α (Thr-172) to total AMPK-α and βPix protein expression levels. IB, immunoblot. C, summary graph of the co-immunoprecipitated βPix (left panel) or Nedd4-2 (right panel) with 14-3-3 proteins under the indicated conditions. D, immunoblot analysis of Nedd4-2 protein expression in cells with or without βPix knockdown and with or without AA treatment. E, RT-PCR analysis of Nedd4-2 mRNA in cells with or without βPix knockdown and with or without AA treatment. F, phosphorylation of Nedd4-2 (Ser-328) in cells with or without βPix knockdown and with or without AA treatment. G, Nedd4-2 cellular stability in control versus βPix knockdown cells. Cells were treated with cycloheximide (100 μg/ml) and harvested at different time points (*, p < 0.008 for comparison of control and βPix knockdown at the 2-h time point). Immunoblot assays were performed to detect changes in cellular Nedd4-2 protein abundance. Data are mean ± S.E. (p values for the indicated comparisons with significant differences are shown).
AMPK regulation of ENaC via βiPix

Figure 7. Proposed model for the roles of Nedd4-2 and βiPix in the regulation of ENaC by AMPK. Activation of AMPK promotes AMPK-mediated phosphorylation of Nedd4-2 at Ser-328 (1) and also promotes the binding of βiPix to the Nedd4-2–14-3-3 complex (2). βiPix association with Nedd4-2 enhances Nedd4-2 stability by helping to maintain Nedd4-2 phosphorylation at Ser-328 (3), which ultimately enhances the association of Nedd4-2 with ENaC (4) and thereby induces ENaC ubiquitination and degradation.

promotes the binding of βiPix to 14-3-3, leading to an increase in ENaC ubiquitination by Nedd4-2.

Experimental procedures
Reagents and chemicals
All chemicals used were purchased from Sigma or Thermo Fisher Scientific unless otherwise noted. [γ-32P]ATP was obtained from MP Biomedicals (Santa Ana, CA). Recombinant active human AMPK holoenzyme (α1-T172D, β1, γ1) was synthesized and purified as described previously (63). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and A769662 were obtained from Toronto Research Chemicals, Inc. and Selleck Chemicals LLC (Houston, TX), respectively.

Cell culture
CHO, human embryonic kidney (HEK-293), mpkCCDc14 and mCCDc11 cells were maintained and cultured as described previously (27, 38, 64). mCCDc11 cells were a kind gift from Dr. Bernard Rossier (University of Lausanne, Lausanne, Switzerland) (46). Immortalized CCD principal cells were grown in defined medium on permeable supports (Costar Transwells, 0.4-μm pore, 6.5- or 24-mm diameter), allowing them to polarize and form monolayers with high resistance and avid Na+ reabsorption.

Plasmids and transfection
For whole-cell patch clamp experiments, CHO cells were seeded on sterile 4 × 4 mm coverglasses in 35-mm Petri dishes, and transfection was performed with Polyfect reagent (Qiagen, Valencia, CA) as described previously (38). Mouse ENaC was reconstituted by co-expressing α-, β-, and γ-channel subunits (38). The expression vectors encoding GSH S-transferase (GST)–tagged WT Xenopus Nedd4-2 (xNedd4-2), rat WT βiPix, βiPix–Δ602–611, AMPK-α1-K45R, and AMPK-γ1-R70Q were also described previously (12, 44, 65). WT and mutant βiPix plasmids were a gift from Dr. Andrey Sorokin (Medical College of Wisconsin).

βiPix shRNA lentiviral particles (Santa Cruz Biotechnology) were used to knock down βiPix expression in mCCDc11 cells. Control shRNA lentiviral particles (Santa Cruz Biotechnology) were used to confirm the transduction efficiency. mCCDc11 cells were seeded on 60-mm Petri dishes and transduced by replacing growth medium with medium containing lentiviral particles and Polybrene (5 μg/ml) according to the manufacturer’s recommendations. The infection medium was removed and replaced with fresh growth medium after overnight incubation. The stable cell line expressing the shRNA was isolated via selection with puromycin (2 μg/ml).

To generate mpkCCDc14 cells with stable conditional over-expression of WT βiPix or βiPix–Δ602–611, V5-tagged WT βiPix and βiPix–Δ602–611 cDNAs were obtained from FLAG pCMV plasmids followed by tag switching from FLAG to the V5 epitope. The cDNAs were then cloned into the pDONR221 entry vector and integrated into pCW57.1 vector containing a tetracycline response element (Addgene) to generate a conditional pCW57.1 V5-tagged WT βiPix or βiPix–Δ602–611 plasmid (66). All constructs were confirmed by DNA sequencing. Lentiviral vector particles were generated by co-transfecting the pCW57.1 V5-tagged βiPix plasmid and a mixture of packaging vectors (pCMVΔR8.2 and pCMV-VSV-G) into HEK293T packaging cells. Vector particles were collected by filtering and concentrating the conditioned medium at 72 h post-transfection. mpkCCDc14 cells were seeded and infected in 6-well plates with lentiviral vector particles and Polybrene. Stable clones were isolated via selection with puromycin for 2 weeks, and expression of recombinant proteins was verified by immunoblotting with a V5 tag antibody.

Mass spectrometry and Edman sequencing
WT GST-xNedd4-2 was expressed in E. coli Rosetta 2 cells (EMD Millipore). The bacteria were harvested after 24 h for elution of GST-xNedd4-2. Purified GST-xNedd4-2 was phosphorylated in vitro with active AMPK holoenzyme, separated by SDS-PAGE, and stained with colloidal Coomassie Blue, and radioactive gel bands were excised. Tryptic digestion, peptide extraction, purification by reverse-phase chromatography, and MALDI-TOF MS analysis were performed by following the reported procedures (67). Selected radioactive peptides were sequenced by solid-phase Edman degradation (68).

In vitro phosphorylation
HEK-293 cells were transiently transfected according to the manufacturer’s protocol using Lipofectamine 2000 (Invitrogen) to express FLAG-tagged WT xNedd4-2 or xNedd4-2 S444A. Cells were lysed 2 days after transfection, and the FLAG-tagged xNedd4-2 (WT and S444A) were immunoprecipitated from cell lysates using the M2 anti-FLAG mAb (Sigma) coupled to protein A/G agarose. In vitro phosphorylation was performed using purified active AMPK holoenzyme with [γ-32P]ATP labeling, as described previously (27). After SDS-PAGE and transfer to nitrocellulose membranes, immunoblotting for expression of the FLAG-tagged xNedd4-2 was first performed and quantified using...
a Versadoc Imager with Quantity One software (Bio-Rad). After the chemiluminescent signal decayed, phosphorylated bands on the membrane were identified by exposure of the same membrane to a phosphoscreen, and the detected bands were quantitated using the same image analysis software. The intensity of each phosphoscreen band was corrected by subtracting the local background in the same lane.

Electrophysiology

A portable epithelial volt-ohm–meter (World Precision Instruments, Sarasota, FL) was used to measure equivalent short-circuit currents across polarized cell monolayers. The electrode was calibrated by placing it into growth medium for 90 min prior to measurement of the potential difference and resistance across the filter. The current was calculated by using the potential difference across the filter divided by the resistance normalized to the surface area to obtain readings measured in microamperes per square centimeter (69). Whole-cell macroscopic patch clamp current recordings of mENaC expressed in CHO cells were made under voltage clamp conditions using our previously described methods (38). Cells were seeded on glass coverslips and then transiently transfected with the bicistronic pTracer plasmid to express GFP and AMPK-α1–K45R, AMPK-γ1–R70Q, or empty vector (65). Whole-cell patch clamping was performed on GFP-positive cells 1–3 days after transfection.

Co-immunoprecipitation assays

To examine the association between β1Pix, Nedd4-2, and 14–3–3 proteins with AMPK modulation, cells were harvested and lysed in ice-cold IP lysis buffer (1% Triton X-100, 2 mM EDTA (pH 8.0), in Dulbecco’s PBS with Ca2+ and Mg2+) after AICAR and A769662 treatment versus vehicle, as indicated, for 24 h. Precleared lysates were incubated with the pan-14-3-3 antibody (1:100) coupled to protein A/G beads (Thermo Fisher Scientific) overnight at 4 °C. Immunoprecipitation in the absence of the pan-14-3-3 antibody was also performed as a no-antibody control. After three washes with lysis buffer, the immunoprecipitation samples were eluted in sample buffer and, along with the cell lysate samples, subjected to immunoblotting to detect β1Pix, Nedd4-2, and 14–3–3 proteins. Relative binding was quantified by dividing the co-IP protein signal by the signal for that protein in the cell lysate, which was then corrected for the amount of immunoprecipitated protein for that condition.

Immunoblotting

Lysis and immunoblotting of cell lysates for Nedd4-2 (EMD Millipore), pNedd4-2 (S328) (Abcam), V5 tag (Cell Signaling Technology), β1Pix (EMD Millipore), phospho-AMPK α (Thr-172, Cell Signaling Technology), AMPK pan α (Cell Signaling Technology), 14–3–3 proteins (Santa Cruz Biotechnology), and β-actin (Sigma) were performed as described previously (25, 38). Gradient gels (4–12%) were used for SDS-PAGE. IRDye® goat anti-rabbit and anti-mouse IgG Dylight 800 and 680 were obtained from LI-COR Biotechnology (Lincoln, NE). Membranes were scanned with an Odyssey Fc imaging system (LI-COR). Bands were quantified by using Image Studio Lite software (LI-COR) on unprocessed raw data files. To compare immunoblotting and co-IP results for specific conditions across multiple replicate experiments, we analyzed the data through the “normalization by sum of the replicate” technique, as described previously (70). Briefly, each data point from an individual group on a blot was divided by the sum of all raw data on the same blot. The data were then further normalized to the average calculated with all of the control group values from all experiments.

RNA extraction and real-time quantitative reverse transcriptase PCR

Total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen), and 0.5–μg aliquots were reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instructions. First-strand cDNA, 1 μl of the 40-μl reverse transcriptase reaction product, was amplified using PowerUp SYBR Green Master Mix (Applied Biosystems) with primers of Nedd4-2 (forward, 5’-AATGACTGGGCTCTCTT-3’; reverse, 5’-GTAAAAGTGGCCCATC-3’) or glyceraldehyde-3-phosphate dehydrogenase (forward, 5’-TCAGCTCTATT- CCGTAGA-3’; reverse, 5’-TAGGCTCCTTTG- CTCAGT-3’). Quantitative real-time RT-PCR was conducted with the ViiATM 7 real-time PCR system (Applied Biosystems) at 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All samples were run in triplicate. The expression of Nedd4-2 was defined from the threshold cycle, and relative expression levels were calculated using the 2–ΔΔCT method. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene for normalization.

Cycloheximide chase assay

mCCDcl1 cells with or without β1Pix knockdown were treated with 100 μg/ml cycloheximide and then chased at 37 °C for 0 to 4 h. At the appropriate chase time, cells were rinsed twice with cold PBS and lysed in radioimmune precipitation assay buffer supplemented with phosphatase and protease inhibitors. Proteins were resolved by SDS-PAGE and immunoblotted with anti-Nedd4 WW2 antibody. The detected bands were then quantitated by using Image Studio Lite software (LI-COR).

Statistical analysis

All summarized data are reported as mean ± S.E. Statistical analyses were performed using corresponding t tests and one-way analysis of variance with post hoc Bonferroni corrections for multiple comparisons. In all cases, p < 0.05 was considered significant.

AMPK regulation of ENaC via β1Pix

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References


β₁Pix exchange factor stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in kidney epithelial cells

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