Epithelial sodium channel (ENaC) is multi-ubiquitinated at the cell surface

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The human ENaC (epithelial sodium channel), a complex of three subunits, provides the rate-limiting step for sodium uptake in the distal nephron, and therefore plays a key role in salt homeostasis and in regulating blood pressure. The number of active sodium channel complexes present at the plasma membrane appears to be tightly controlled. In Liddle's syndrome, a form of hypertension caused by an increase in the number of active sodium channels at the cell membrane, the β ENaC or γ ENaC subunit gene contains a mutation that disrupts the binding site for the Nedd4 (neuronal precursor cell expressed developmentally down-regulated gene 4) family of ubiquitin-protein ligases. Therefore ubiquitination of channel subunits may be involved in altering cell surface ENaC. Here, we provide evidence that the ENaC subunits located at the

cell surface are modified with multiple mono-ubiquitins (multi-ubiquitination) and that Nedd4-2 modulates this ubiquitination. We confirm that ENaC is associated with the μ 2 subunit of the AP-2 (adaptor protein 2) clathrin adaptor. Since mono- or multi-ubiquitination of other membrane proteins is a signal for their internalization by clathrin-mediated endocytosis and subsequent trafficking, our results support a model whereby ubiquitin and clathrin adaptor binding sites act in concert to remove ENaC from the cell surface.

Key words: endocytosis, kidney, Liddle's syndrome, neuronal precursor cell expressed developmentally down-regulated gene 4-2 (Nedd4-2), protein trafficking, ubiquitin.

INTRODUCTION

The ENaC (epithelial sodium channel) is a protein complex composed of three subunits: α , β and γ [1,2]. ENaC is expressed in the apical membrane of epithelia found in the lung, colon and distal kidney. In the distal tubule and collecting duct of the kidney the activity of ENaC is controlled by aldosterone, to regulate sodium uptake from the urine to maintain body salt balance [3]. As a result, ENaC is an important player in long-term blood pressure regulation, and mutations in ENaC subunit genes cause Liddle's syndrome, an inherited form of hypertension [4].

Regulation of ENaC is thought to be achieved by adjusting the number of channels present in the plasma membrane [5,6], by changes in channel open probability [5,7], and by cleavage of ENaC subunits [8] to form active channels. To adjust the number of sodium channel complexes, the rates of insertion into and removal from the plasma membrane have to be regulated (reviewed in [9,10]). The rate of insertion is affected by the delivery of newly synthesized protein as well as reinsertion of ENaC from recycling vesicles. Removal of ENaC from the cell surface is also under tight regulation. Rotin and co-workers [10,11] proposed that both clathrin-mediated endocytosis and ubiquitination contribute to internalization of ENaC from the cell surface.

Nedd4 (neuronal precursor cell expressed developmentally down-regulated gene 4), Nedd4-2 and WWP2 (WW domain containing E3 ubiquitin protein ligase 2) are HECT (homologous to E6-associated protein C-terminus) domain E3 ubiquitin-protein ligases implicated in ENaC ubiquitination and down-regulation. Nedd4, Nedd4-2 and WWP2 contain interaction motifs, called WW domains (protein-protein interaction domain containing two conserved tryptophan residues), that bind to PY motifs (PPPXY, P = proline, X = any amino acid and Y = tyrosine) in the ENaC

subunit C-terminal domains [12–17]. The PY motif is deleted or mutated in Liddle's syndrome leading to ENaC overactivity, while on the other hand co-expression of Nedd4 family members with ENaC results in down-regulation of channel activity, which is dependent on the ubiquitin ligase activity of Nedd4 [13,17,18]. ENaC subunits are ubiquitinated [11,19], and Nedd4/Nedd4-2 are strongly implicated in the regulation of ENaC surface expression [16,18,20]. However, the exact ENaC ubiquitination pattern, and the cellular location(s) where ubiquitination occurs have not been reported.

The PY motif in all three ENaC subunits overlaps with a YXX Φ -endocytosis motif (Φ = hydrophobic amino acid), sharing the same tyrosine: PPPXYXXL. In addition, both α ENaC and β ENaC have a YXX Φ motif in their N-terminal cytoplasmic domain. These tyrosine-based endocytosis motifs (YXX Φ) may mediate direct ENaC binding to the medium subunit (μ 2) of the clathrin adaptor protein AP-2 (adaptor protein 2), and facilitate entry into clathrin-coated pits. ENaC activity is increased in the presence of dominant-negative dynamin [21,22], suggesting that internalization can occur via clathrin-mediated endocytosis. Ubiquitinated ENaC could also enter clathrin-coated vesicles indirectly through an interaction of ubiquitin with a ubiquitin-binding protein such as epsin, which binds both ubiquitin and the μ 2 subunit. In strong support of both these pathways being involved in ENaC retrieval from the cell surface, Wang et al. [23] have recently shown that all three ENaC subunits are present in clathrin-coated vesicles isolated from collecting duct epithelial cells, that the ENaC subunits co-precipitate with clathrin-coated vesicle-associated proteins including μ 2, and that epsin binds to ubiquitinated ENaC. Co-expression of epsin with ENaC in CHO cells (Chinese-hamster ovary cells) or in *Xenopus* oocytes resulted in current inhibition dependent on the presence of epsin's ubiquitin-binding domain [22,23].

Abbreviations used: AP-2, adaptor protein 2; ENaC, epithelial sodium channel; ER, endoplasmic reticulum; HA, haemagglutinin; MVB, multivesicular body; Nedd4, neuronal precursor cell expressed developmentally down-regulated gene 4; NHS, *N*-hydroxysuccinimido; ROMK1, renal outer-medullar potassium 1; TRPV4, transient receptor potential (protein) vanilloid; WWP2, WW domain containing E3 ubiquitin protein ligase 2.

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Mono- or multi-ubiquitination (where more than one mono-ubiquitin moiety is attached to a substrate protein) can also act as an internalization signal for membrane proteins in both yeast [24] and mammalian cells [25]. A network of ubiquitin-binding proteins such as epsin, eps15 (epidermal-growth-factor-receptor pathway substrate 15) and Hrs (hepatocyte-growth-factor-regulated tyrosine kinase substrate) has been implicated in the endocytosis and subsequent trafficking of membrane proteins through the endosomal system to the MVB (multivesicular body). Mono-ubiquitin also acts as a signal for protein sorting into the MVB, and from the MVB proteins can be degraded in the lysosome. Polyubiquitinated proteins, on the other hand, are targeted to the proteasome for degradation [26].

In this study, we present evidence that ENaC subunits at the cell surface are modified by multiple mono-ubiquitins, and that Nedd4-2 can alter ubiquitination of surface-localized ENaC.

EXPERIMENTAL

cDNA constructs

Full-length HA (haemagglutinin)- α ENaC, HA- β ENaC_{Y620A}, Nedd4-2-FLAG, μ 2-HA (internal tag) and FLAG-ubiquitin containing a HA or FLAG tag were cloned into pMT3. HA/FLAG-tagged α ENaC, β ENaC and γ ENaC are described elsewhere [27]. Amino acids 43–49 of β ENaC, including three lysine residues were changed to alanine using the Genetailor kit (Invitrogen). cDNA was sequenced by the Allan Wilson Centre, Massey University (Palmerston North, New Zealand).

Cell culture and transient transfection

COS7 cells were grown in low bicarbonate Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 10 units/ml penicillin and 10 mg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂. On the day before transfection, COS7 cells were plated at a density of 3×10^5 cells in 35 mm plates. Cells were transfected with 1.5 μ g of each cDNA construct using FuGENE™ 6 (Roche) as described in [13]. In one experiment, FLAG-Nedd4-2 in pMT3 was also added in various amounts, along with empty pMT3 vector to keep the total amount of cDNA added constant.

Biotinylation of surface proteins and fractionation of ENaC

COS7 cells were transfected with HA-ENaC subunits in 35 mm dishes, and six plates were used for one experiment. Medium was replaced 6–8 h after transfection with fresh medium containing 10 μ M amiloride to block the channel, and 36 h after transfection the medium was replaced again with fresh medium containing 10 μ M amiloride and 10 μ M proteasome inhibitor MG-132 (carbobenzoxycarbonyl-L-leucyl-L-leucyl-L-leucinal; Sigma). In some cases cells were treated with 100 μ M of lysosome inhibitor chloroquine (Sigma) instead of MG-132 or received no treatment. Cell surface proteins were labelled with sulfo-NHS (*N*-hydroxysuccinimido)-LC-biotin (Pierce) 48 h after transfection as follows. The medium was removed from plates, and the cells were washed three times with 2 ml of ice-cold PBS (pH 8.0). The cells were incubated for 30 min on ice with 0.4 ml of 1.0 mg/ml sulfo-NHS-LC-biotin in PBS (pH 8.0). The biotinylation step was repeated. The reagent was finally removed by aspiration, and the cells were washed four times with 2 ml of ice-cold PBS (pH 8.0). Cells were then lysed in 125 μ l of boiling 1% SDS in PBS (pH 7.2) to avoid isopeptidase activity. Lysates were sheared by passing them through a 22-gauge needle and boiled for 5–10 min. If required, protein concentration was determined by using the Bio-Rad RC-DC protein assay

kit. Immunoprecipitation buffer (2 $\times$ PBS, 0.4% Triton X-100, 20 μ g/ml PMSF, 4 μ g/ml aprotinin, 4 μ g/ml leupeptin, 2 μ g/ml pepstatin and 80 μ M MG-132) was then added to the lysates along with 2.5 μ g/ml anti-HA antibody (Sigma). After incubation for 2 h at 4°C with constant motion, 50 μ l of Protein G-Sepharose (Sigma) slurry was added, and incubation continued for 1 h. Protein G-Sepharose beads were collected by centrifugation and washed four times with 1% Triton X-100 in PBS (pH 7.2). If required, beads were treated with 3 units of N-glycosidase F (Roche) for 18 h at 4°C. ENaC immune complexes bound to the Protein G-Sepharose beads were eluted for 15 min at 100°C in 100 μ l of 1% SDS in PBS (pH 7.2) and diluted into 900 μ l of PBS (pH 7.2). In order to isolate the biotinylated ENaC surface fraction the solution was incubated for 1 h at 4°C with 50 μ l of Ultralink™ streptavidin (Pierce) slurry. The streptavidin beads were collected by centrifugation, and washed twice with 1% Triton X-100 in PBS (pH 7.2). Proteins were eluted at 100°C for 15 min in 5 $\times$ sample buffer [312.5 mM Tris/HCl, 10% (w/v) SDS, 0.05% Bromophenol Blue, 25% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, pH 6.8]. To isolate the intracellular pool of ENaC the supernatant of the streptavidin precipitation step was incubated again with 2.5 μ g/ml anti-HA antibody and Protein G-Sepharose as described above. Proteins were eluted from the Protein G beads with 5 $\times$ sample buffer. In some cases the surface fraction of ENaC was precipitated directly from the cell lysate with streptavidin without prior immunoprecipitation of total cell ENaC.

Immunoblotting analysis of ubiquitin modifications of ENaC

Protein samples were separated by SDS/8% PAGE and transferred to PVDF membranes. Membranes were blocked in TBS (50 mM Tris/HCl and 150 mM NaCl, pH 7.4) containing 3% (w/v) BSA and 0.05% Tween 20, and then consecutively probed with mouse anti-polyubiquitin antibody FK1 (Biomol) at a 1:1000 dilution, mouse anti-poly- and mono-ubiquitin antibody P4D1 (Cell Signaling Technology) at a 1:1000 dilution and rabbit anti-HA antibody (Sigma) at a 1:2500 dilution, all overnight at 4°C. The secondary HRP (horseradish peroxidase)-coupled antibodies used were goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgG (all from Sigma) respectively at 1:10000 dilutions. Antigen-antibody complexes were detected by chemiluminescence using Lumilight (Roche). The ubiquitin ladder (Affiniti Research Products) was separated on an SDS/16.5% PAGE gel and processed as described above. After each round of antibody probing the membranes were incubated for 1 h at 50°C in stripping buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS and 10 mM 2-mercaptoethanol), followed by two wash steps for 10 min with 0.05% Tween 20 in TBS, and reblocking as described above.

Co-immunoprecipitation of β ENaC with μ 2

COS7 cells were transfected with FLAG-tagged β ENaC or β ENaC_{Y620A} alone or together with HA- μ 2 or Nedd4-2. After 48 h cells were washed and lysed in TBS+1% Triton containing protease inhibitors. HA- μ 2 was immunoprecipitated with anti-HA and Protein G-Sepharose, and β ENaC was detected by Western blotting with anti-FLAG M2 (1:2000; Sigma).

RESULTS

ENaC subunits are present at the cell surface in COS7 cells

A number of mammalian cell surface proteins, such as TRPV4 [transient receptor potential (protein) vanilloid] [28], ROMK1

Figure 1 Surface labelling of ENaC subunits

COS7 cells expressing the indicated HA-epitope-tagged ENaC subunits and FLAG-tagged Nedd4-2 were biotinylated. Cells were then lysed and biotinylated surface proteins precipitated with immobilized streptavidin. (A) Whole cell lysate (5 %) and (B) surface fraction samples were tested for the presence of ENaC subunits and Nedd4-2 respectively. All four ENaC subunits were detectable at the surface of COS7 cells. $n = 3$.

(renal outer-medullar potassium 1; [29]), and the interleukin-1 [30], EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) receptors [25] are mono- or multi-ubiquitinated in mammalian cells. This modification is sufficient to promote internalization from the cell surface [25]. We hypothesized that mono- or multi-ubiquitination of ENaC may occur at the cell surface, and that this modification may contribute to endocytosis followed by lysosomal degradation and/or recycling, whereas unassembled and/or misfolded ENaC subunits in the ER (endoplasmic reticulum) are polyubiquitinated and degraded by the proteasome. Therefore it should be possible to identify different populations of ENaC, one that is mono-/multi-ubiquitinated and one that is polyubiquitinated. As a first step to test this hypothesis we expressed HA-epitope-tagged ENaC subunits in COS7 cells, separated the ENaC proteins into cell surface and intracellular pools, and asked what type of ubiquitin modification these ENaC subunits were tagged with using two ubiquitin antibodies with different specificities.

First, we established a surface protein labelling assay. Individual ENaC subunits were transfected into COS7 cells and after 48 h expression the cell surface proteins were labelled with biotin, the cells were lysed and the surface protein fraction was isolated. Figure 1(A) shows the expression of α ENaC, β ENaC, γ ENaC, β ENaC_{Y620A} and Nedd4-2 in whole cell lysate. Figure 1(B) shows that ENaC subunits are present at the cell surface, whereas the cytosolic protein Nedd4-2 was not detected at the cell surface, indicating that the biotin reagent selectively labels proteins that reside in the plasma membrane. No surface ENaC was detected in the absence of biotin (results not shown). The portion of whole cell ENaC that is located at the cell surface is relatively small (1–5 % of total cell ENaC), which is consistent with other studies {MDCK (Madin–Darby canine kidney): <1 % [31]; oocytes: <1 % [32]}. The total cell expression of the subunits is similar (Figure 1A); however, the two β ENaC subunits are present at the cell surface in higher amounts than α ENaC and γ ENaC.

The surface fraction of ENaC is multi-ubiquitinated in COS7 cells

In order to independently study the ubiquitination of ENaC at the cell surface and in the intracellular pool, we combined surface labelling and immunoprecipitation methods. COS7 cells transiently expressing HA–ENaC subunits were biotinylated 48 h after transfection. Cells were then lysed, and total cell ENaC was isolated from the lysate by immunoprecipitation. ENaC immune complexes were eluted, and the surface fraction of ENaC was captured. The unlabelled intracellular pool of ENaC was re-immunoprecipitated. Cell surface and intracellular pool fractions were then separated by SDS/PAGE along with 2 % of the initial cell lysate. After the Western transfer FK1, P4D1 and anti-HA antibodies were added sequentially to the membranes, in order to detect ubiquitin modifications of ENaC and the subunits themselves. FK1 detects polyubiquitin chains, whereas P4D1 detects polyubiquitin chains as well as ubiquitin monomers attached to substrate proteins. Both antibodies used in combination should therefore allow us to identify the character of the ubiquitin modifications on ENaC, similar to other studies [25,28,30]. To verify the specificity of the two antibodies we tested them on a mixture of ubiquitin monomers and ubiquitin chains of different lengths separated by SDS/PAGE (Figure 2A). As expected [25], only P4D1 detected the ubiquitin monomer, FK1 did not. However, both antibodies detected ubiquitin chains of two or more ubiquitin moieties. In addition, both antibodies labelled proteins located in the cell surface fraction (Figure 2B), and appeared to have similar sensitivities (Figure 2C).

Figure 3 shows the ubiquitination patterns of whole cell, intracellular and surface pools of ENaC. The presence of ENaC in the intracellular pool, cell surface fraction and total cell lysate was confirmed by blotting with anti-HA antibody for the subunits (Figure 3B). Membranes blotted with P4D1 antibody (Figure 3A) show a typical smeary signal for both intracellular and cell surface pools of ENaC subunits ranging from the size of unmodified ENaC (75–85 kDa) to 150–200 kDa. This suggests that several ubiquitins are attached to both intracellular and cell surface pools of ENaC. Whether these ubiquitin attachments involve several single ubiquitin moieties or polyubiquitin chains cannot be concluded, since the anti-ubiquitin antibody P4D1 detects both single ubiquitins as well as polyubiquitin chains. When blotting the same membranes with the anti-ubiquitin antibody FK1 only the ENaC intracellular pools produced a signal, whereas the surface fraction did not (Figure 3C). Combined with the result of the P4D1 antibody this firstly suggests that the ENaC intracellular pool is polyubiquitinated, although a portion of the intracellular pool could be mono-ubiquitinated at one or more lysine residues. We would expect this result as misfolded and unassembled ENaC proteins will be removed from the ER and degraded by the proteasome after polyubiquitination.

Secondly, the result suggests that the surface fraction of ENaC is multi-ubiquitinated, meaning that several single ubiquitins have been attached to ENaC subunits residing in the membrane. The smeary high-molecular-mass signal detected by P4D1 in the surface fraction indicates that multiple lysine residues in ENaC are modified by single ubiquitins. We predict that the multi-ubiquitination of ENaC subunits facilitates their internalization and trafficking to the lysosome for degradation, or to recycling endosomes.

It can be argued that the glycosylation of ENaC proteins contributes to the smeary high-molecular-mass bands detected by P4D1. Therefore this experiment was repeated in the presence of the deglycosylating enzyme N-glycosidase F, which removes all N-linked sugars. The result (Figure 4A) shows that the ubiquitination pattern of β ENaC is not affected by

Figure 2 Specificity of the two anti-ubiquitin antibodies FK1 and P4D1

(A) A ubiquitin ladder was separated by SDS/16.5% PAGE. The number of ubiquitin units is indicated on the left. The ladder was visualized by Coomassie Brilliant Blue staining, or by immunoblotting with the two anti-ubiquitin antibodies FK1 and P4D1. Only antibody P4D1 detects the ubiquitin monomer. (B) Untransfected COS7 cells were biotinylated, lysed and the surface protein fraction was isolated by streptavidin precipitation. The lysate and surface protein fractions were tested for ubiquitin modification with antibodies FK1 and P4D1 as indicated. Both antibodies produced a signal for the lysate and the biotinylated surface fraction. No signal was produced when the cells were not biotinylated prior to lysis. (C) Serial dilutions of whole cell lysate from COS-7 cells treated with MG-132 were used to test the sensitivities of the FK1 and P4D1 antibodies.

deglycosylation. Similar results were obtained for α ENaC and γ ENaC (results not shown).

To provide further evidence for the cell surface ENaC being multi- and not poly-ubiquitinated we reasoned that mutation of a subset of lysine residues in β ENaC should change the pattern of multi-ubiquitination. β ENaC contains ten cytosolic lysine residues and we tested a β ENaC lacking three of the nine N-terminal domain lysine residues (β ENaCK3A). We co-expressed wild-type β ENaC and β ENaCK3A with FLAG-ubiquitin and detected cell surface ENaC. A smeary ladder of bands corresponding to ubiquitinated β ENaC was observed. Loss of three lysine residues resulted in the appearance of two lower molecular mass bands (Figure 4B, arrows,) showing that removal

of a subset of acceptor lysine residues alters the ubiquitination pattern, supporting our hypothesis that multiple lysine residues are modified by single ubiquitins.

Liddle's syndrome β ENaC_{Y620A} is also ubiquitinated

ENaC activity is decreased by the ubiquitin ligases Nedd4 and Nedd4-2 [9,16–18]. They bind to the subunits of the channel via the PY motif present in all three subunits [12]. Although direct evidence is lacking, it is consensus that Nedd4 and Nedd4-2 down-regulate ENaC activity by catalysing its ubiquitination. If the PY motif is mutated, as it is in Liddle's syndrome, Nedd4 and Nedd4-2 cannot bind to the mutated ENaC subunit, and ENaC

Figure 3 Ubiquitination of intracellular and surface fractions of ENaC subunits

The indicated HA-ENaC subunits were expressed in COS7 cells. Prior to cell lysis, surface proteins were labelled with biotin. ENaC was then immunoprecipitated with anti-HA antibody and Protein G-Sepharose beads. ENaC was released from the beads and separated into surface and intracellular pool fractions using streptavidin precipitation to capture surface ENaC, and then reimmunoprecipitation to capture the ENaC intracellular pool. Both fractions, along with 2% of the initial lysate, were analysed by SDS/PAGE and sequential Western blotting with FK1, followed by P4D1 and finally anti-HA antibody with antibody stripping in between. **(A)** Ubiquitinated cellular proteins in the lysate, ubiquitinated ENaC subunits in the intracellular pool and multi-ubiquitinated ENaC subunits in the cell surface fraction. **(B)** Expression of ENaC in all the fractions. **(C)** Polyubiquitinated proteins in the lysate and ENaC intracellular pool. No signal was detectable for the surface fraction of ENaC when blotting with FK1. Exposure times for the FK1 and P4D1 antibodies were similar. An asterisk (*) indicates multi-ubiquitinated surface ENaC. $n = 4$.

Figure 4 The ubiquitination pattern of ENaC is affected by removal of lysine residues but not by deglycosylation

(A) β ENaC-HA was expressed in COS7 cells. Cell surface and intracellular pool fractions were separated as described in the Figure 3 legend, and treated with N-glycosidase F (+ lanes). Fractions were analysed by SDS/PAGE and Western blotting with P4D1. Treatment of β ENaC protein with N-glycosidase F did not affect its smeary ubiquitination pattern, $n = 2$. **(B)** HA- β ENaC or HA- β ENaCK3A were expressed with FLAG-ubiquitin in COS7 cells, and the surface fraction was isolated. Ubiquitinated ENaC was detected with anti-FLAG, $n = 2$. The arrows indicate lower molecular mass forms of ubiquitinated β ENaCK3A.

activity is up-regulated [4,16]. Preventing Nedd4 and Nedd4-2 from binding to ENaC might therefore prevent ubiquitination of the channel. To test this hypothesis we expressed a β ENaC_{Y620A} mutant, which does not bind Nedd4 [13] in COS7 cells, and

studied the ubiquitination pattern of the intracellular and surface pools of ENaC. As shown in Figure 3 (right panel) the result for β ENaC_{Y620A} did not differ from wild-type β ENaC. Both the intracellular pool and surface β ENaC_{Y620A} showed ubiquitination

Figure 5 β ENaC and β_{Y620A} ENaC subunits bind to $\mu 2$

FLAG- β ENaC or FLAG- β_{Y620A} ENaC were transiently expressed in COS7 cells, alone (lanes 3 and 4) or in combination with HA- $\mu 2$ (lanes 1 and 2) or HA-Nedd4-2 (lanes 5 and 6). After immunoprecipitation of $\mu 2$ or Nedd4-2, FLAG- β ENaC was detected (upper panel). Lower panel: 1% of lysates were blotted with anti-HA to detect $\mu 2$ and Nedd4-2. $n = 3$.

signals, suggesting that surface β ENaC $_{Y620A}$ is multi-ubiquitinated and β ENaC $_{Y620A}$ in the intracellular pool is polyubiquitinated. From this result we suggest that, at least in COS7 cells, ENaC can be ubiquitinated by ubiquitin ligases other than Nedd4 and Nedd4-2, that do not need the PY motif to catalyse ENaC ubiquitination, or that Nedd4 enzymes can ubiquitinate ENaC via an adaptor protein.

Binding of ENaC subunits to $\mu 2$

Recently it has been reported that $\alpha\beta\gamma$ ENaC are co-localized in clathrin vesicles, that ubiquitinated ENaC binds to epsin, and that α ENaC, β ENaC and γ ENaC co-immunoprecipitate with the $\mu 2$ clathrin adaptor subunit [23]. To investigate whether lack of Tyr⁶²⁰ in β ENaC would impair the binding of this subunit to $\mu 2$ we carried out co-immunoprecipitation experiments. Figure 5 shows that both β ENaC and β_{Y620A} ENaC interact with $\mu 2$, and there did not appear to be a quantitative difference in the amount of β_{Y620A} ENaC co-immunoprecipitated with $\mu 2$ compared with wild-type β ENaC. As a negative control we show that β_{Y620A} ENaC does not interact with Nedd4-2, as expected. This implies that β ENaC containing a Liddle's mutation can still associate with clathrin adaptors, similar to wild-type β ENaC. Since β_{Y620A} ENaC is also ubiquitinated at the cell surface (Figure 3), this association could be via a ubiquitin-binding protein such as epsin. Alternatively, a direct interaction could occur between adaptor protein subunits and the N-terminal domain YKEL or LL motifs of β ENaC.

Nedd4-2 decreases surface expression of α ENaC by increasing its ubiquitination

Of the Nedd4-like family of ubiquitin ligases, Nedd4-2 is the most potent candidate for mediating ENaC ubiquitination [18]. Therefore we tested whether Nedd4-2 affects the ubiquitination and surface expression of ENaC in transfected cells. COS7 cells were transfected with constant amounts of α ENaC and increasing amounts of Nedd4-2. The surface population of α ENaC was then separated from the intracellular pool, and analysed for expression and ubiquitination. The results, in Figure 6, show that increasing concentrations of Nedd4-2 decrease the overall expression of α ENaC (Figure 6, upper panels) as well as its surface expression (Figure 6, lower panels), similar to a report by Knight et al. [33]. As expected, Nedd4-2 increased the ubiquitination of α ENaC in both the surface and intracellular pools (Figure 6, lower panels). This result suggests that Nedd4-2 decreases ENaC activity by catalysing its ubiquitination, therefore decreasing its surface and total cell expression.

Figure 6 Nedd4-2 alters the expression and ubiquitination of ENaC

COS7 cells were co-transfected with α ENaC-HA cDNA (1.5 μ g) and increasing amounts of Nedd4-2-FLAG cDNA (lanes 1–4: 0, 0.5, 1.0 and 1.5 μ g). Cells were lysed after labelling with biotin. α ENaC was then immunoprecipitated from cell lysates containing equal amounts of protein. The immune complexes were eluted, and the surface fraction separated from the intracellular pool of α ENaC as described in the Experimental section. Samples were separated by SDS/8% PAGE along with 5% of the initial lysates. The upper panel shows the total cell expression of α ENaC and Nedd4-2 as indicated. The lower panel shows the cell surface and the intracellular pool expression of α ENaC (Blot: HA), as well as the ubiquitination of α ENaC in the cell surface and intracellular pools (Blot: P4D1) as indicated. α ENaC expression was decreased, and ubiquitination increased, in the presence of Nedd4-2. $n = 3$.

ENaC surface expression is altered by proteasome and lysosomal inhibitors

Our results suggest that both poly- and multi-ubiquitination modification of ENaC can occur. This suggests an involvement of the proteasome to which polyubiquitinated ENaC would be targeted, and the lysosome to which multi-ubiquitinated ENaC may be targeted. Other groups have reported that just proteasomal [34] or both proteasomal and lysosomal inhibitors [11] affect ENaC stability. We tested this in our system by treating COS7 cells with the proteasomal inhibitor MG-132 or with the lysosomal inhibitor chloroquine, and then analysing the total and cell surface expression of ENaC subunits. Figure 7 shows that both inhibitors increased surface as well as total cell expression of ENaC subunits, supporting the hypothesis that both degradation pathways are involved in the regulation of ENaC.

DISCUSSION

Here we show that the subunits forming the ENaC can undergo differential ubiquitination dependent on their subcellular location. ENaC in the intracellular pool appears to be primarily

Figure 7 ENaC surface expression is increased by proteasomal and lysosomal inhibitors

The indicated HA-epitope-tagged ENaC subunits were expressed in COS7 cells for 24 h. Cells were treated with MG-132, chloroquine or water as indicated. Prior to cell lysis, surface proteins were labelled with biotin. Surface ENaC was precipitated from equal amounts of total cell lysate protein with streptavidin. The surface fractions and 2% of the cell lysates were tested for ENaC expression. The resulting bands were analysed densitometrically. Total ENaC expression and ENaC surface expression was increased when cells were treated with MG-132 or chloroquine. Bars represent means $\pm$ S.E.M. An asterisk (*) indicates a statistical significance of $P < 0.05$ (ANOVA, $n = 3$).

Figure 8 Working model: the internalization of ENaC

Multi-ubiquitin modifications of ENaC are recognized by a ubiquitin-binding protein such as epsin. The adaptor complex AP-2 is then recruited to ENaC via interaction of its μ 2 subunit with the PY motif of ENaC subunits, and an interaction with epsin. The AP-2 complex then triggers the formation of a clathrin-coated vesicle. Once the vesicle is internalized, clathrin and other proteins involved in vesicle formation are released. ENaC can then undergo deubiquitination and recycling, or degradation by the lysosome or the proteasome. ●, Ubiquitin.

polyubiquitinated, whereas cell surface ENaC is multi-ubiquitinated. Our working model (Figure 8, similar to that previously described by Rotin et al. [10]) suggests that multi-ubiquitination assists the removal of ENaC from the plasma membrane via clathrin-mediated endocytosis. Thus, after ENaC is multi-ubiquitinated at the cell surface, it is then bound by both a

ubiquitin-binding adaptor protein, such as epsin, as well as μ 2, and is then internalized via clathrin-coated vesicles. This is consistent with reports from others showing that both epsin- and clathrin-mediated endocytoses are important for ENaC regulation [21–23].

Once internalized, ENaC enters an early endosomal compartment [23] and from here could follow a number of paths: it

could be deubiquitinated and recycled to the cell surface via a recycling pool that has been described by others [35]. Alternatively, the ubiquitin tags could mediate movement through the endosomal system to the MVB and lysosome where ENaC would be degraded. A further possibility is that single ubiquitins on ENaC could be extended to form polyubiquitinated ENaC that is targeted to the proteasome. This pathway would be consistent with our results (Figure 7) and with previous reports studying ENaC in A6 cells, suggesting that ENaC surface expression is regulated by polyubiquitination, although the latter study did not separate the intracellular pool from the surface ENaC to study ENaC ubiquitination [19].

A number of cell surface proteins from both yeast and mammals are regulated by mono- or multi-ubiquitination to stimulate their internalization from the cell surface (reviewed in [26,36]). Mono-ubiquitin tags interact with a variety of endocytic-protein-containing ubiquitin-binding motifs such as UIM (ubiquitin interacting motif), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation), UBA (ubiquitin-associated) or UEV (ubiquitin-conjugating enzyme E2 variant) domains, which localize to different compartments along the endocytosis pathway [26]. The ion channels TRPV4 and ROMK1 are reported to be multi- and mono-ubiquitinated respectively and this regulates surface expression of both channels [28,29].

The size of the multi-ubiquitinated ENaC ranged up to 180–200 kDa (Figure 3). Since the addition of only one ubiquitin would result in one distinct band the smear suggests several ubiquitins are attached. Glycosylation was eliminated as a contributor to the smear, and removal of three potential acceptor lysine residues in β ENaC significantly altered the cell surface ubiquitination pattern. The sizes of multi-ubiquitinated ENaC suggest between 1 and 15 ubiquitins are attached. There are three to nine acceptor lysine residues in the N-terminal domains of ENaC subunits, and a number of these lysine residues in α ENaC and γ ENaC have previously been implicated in ENaC cell surface regulation [11]. It is possible that ENaC C-terminal domain lysine residues are also modified with ubiquitin since a C-terminal domain lysine in δ ENaC is able to be modified by ubiquitin (K. Ly and F.J. McDonald, unpublished work). Inclusion of both N- and C-terminal lysine residues brings the total acceptor lysine residues to 4 for α , 10 for β and 9 for γ ENaC, fitting more closely the molecular mass of the ubiquitinated ENaC patterns reported here. Alternatively, multi-ubiquitinated ENaC migration by SDS/PAGE may be aberrant, similar to that reported for sumoylated BKLF (basic Krüppel-like factor; [37]). We found ENaC in the intracellular pool to be polyubiquitinated, although we cannot exclude a mixture of multi- and poly-ubiquitinated ENaC being present. As others have suggested previously [11], this fraction of polyubiquitinated ENaC is likely to represent unassembled or misfolded ENaC subunits that are tagged by the ER quality control system for degradation in the proteasome.

Nedd4, and perhaps Nedd4-2, are able to both mono- [38] and poly-ubiquitinate [39] substrate proteins. When the catalytic activity of Nedd4 is inhibited, Nedd4 is unable to down-regulate ENaC, suggesting that Nedd4/Nedd4-2 directly ubiquitinate ENaC [16,20]. Here we show that Nedd4-2 decreases expression of α ENaC in the surface and intracellular pools, with a parallel increase in ENaC ubiquitination, supporting the hypothesis that Nedd4-2 has a key role in regulating the amount of cell surface ENaC. In yeast, the Nedd4 orthologue RSP5 (a ubiquitin ligase) is involved at multiple steps in the trafficking pathway: at the *trans*-Golgi network, at the cell surface and at the MVB [26].

We found that when Nedd4/Nedd4-2 binding was inhibited, β ENaC_{Y620A} was still ubiquitinated, suggesting that other ubiquitin ligases, which do not require the PY motif for binding, are

also able to ubiquitinate and target the channel for internalization or degradation. Similar to the chloride channel CFTR (cystic fibrosis transmembrane conductance regulator), ENaC in the intracellular pool might be regulated by the CHIP [C-terminus of the Hsc (heat-shock cognate) 70-interacting protein] [40] or SCF^{Fbx2} [41] ubiquitin ligases; however, it is not known if other ubiquitin ligases might act on ENaC at the cell surface. Alternatively, β ENaC_{Y620A} may be indirectly associated with Nedd4. A recent report [33] linked both ubiquitination and Nedd4-2 to activation of ENaC by proteolytic cleavage. Liddle's mutations appear to increase the fraction of cleaved active ENaC at the cell surface, supporting a model whereby changes in both open probability and trafficking may lead to the Liddle's phenotype. These results suggest that Nedd4-2 may have more than one role in regulating ENaC.

The study presented here is limited by the use of transfected non-epithelial cells to analyse the ubiquitination pattern of ENaC. The ubiquitination of ENaC in native tissue might be different from the scenario described here. However, we have established that multi-ubiquitination of ENaC subunits is possible. The next challenge is to study the ubiquitination of ENaC residing in the plasma membrane and intracellular pools in epithelial cells using epitope-tagged ENaC subunits, and ultimately to study endogenous ENaC in native mammalian tissue. Multi-ubiquitination of ENaC at the cell surface is likely to act in concert with endocytic proteins to facilitate removal of ENaC from the cell surface, and keep ENaC levels in check to prevent excess sodium absorption and hypertension.

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REFERENCES

- Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D. and Rossier, B. C. (1994) Amiloride-sensitive epithelial Na⁺ channel is made of three homologous subunits. *Nature* **367**, 463–467.
- McDonald, F. J., Price, M. P., Snyder, P. M. and Welsh, M. J. (1995) Cloning and expression of the beta- and gamma-subunits of the human epithelial sodium channel. *Am. J. Physiol.* **268**, C1157–C1163.
- Garty, H. and Palmer, L. G. (1997) Epithelial sodium channels: function, structure, and regulation. *Physiol. Rev.* **77**, 359–396.
- Shimkets, R. A., Warnock, D. G., Bositis, C. M., Nelson-Williams, C., Hansson, J. H., Schambelan, M., Gill, Jr, J. R., Ulick, S., Milora, R. V. and Findling, J. W. (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell* **79**, 407–414.
- Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P. and Rossier, B. C. (1995) A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5699–5703.
- Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, B. G., Stokes, J. B. and Welsh, M. J. (1995) Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na⁺ channel. *Cell* **83**, 969–978.
- Firsov, D., Schild, L., Gautschi, I., Merillat, A. M., Schneeberger, E. and Rossier, B. C. (1996) Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15370–15375.
- Hughey, R. P., Bruns, J. B., Kinlough, C. L., Harkleroad, K. L., Tong, Q., Carattino, M. D., Johnson, J. P., Stockand, J. D. and Kleyman, T. R. (2004) Epithelial sodium channels are activated by furin-dependent proteolysis. *J. Biol. Chem.* **279**, 18111–18114.
- Snyder, P. M. (2005) Minireview: regulation of epithelial Na⁺ channel trafficking. *Endocrinology* **146**, 5079–5085.
- Rotin, D., Kanelis, V. and Schild, L. (2001) Trafficking and cell surface stability of ENaC. *Am. J. Physiol. Renal Physiol.* **281**, F391–F399.
- Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L. and Rotin, D. (1997) Regulation of stability and function of the epithelial Na⁺ channel (ENaC) by ubiquitination. *EMBO J.* **16**, 6325–6336.

- 12 Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J. and Rotin, D. (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na⁺ channel deleted in Liddle's syndrome. *EMBO J.* **15**, 2371–2380
- 13 Farr, T. J., Coddington-Lawson, S. J., Snyder, P. M. and McDonald, F. J. (2000) Human Nedd4 interacts with the human epithelial Na⁺ channel: WW3 but not WW1 binds to Na⁺-channel subunits. *Biochem. J.* **345**, 503–509
- 14 McDonald, F. J., Western, A. H., McNeil, J. D., Thomas, B. C., Olson, D. R. and Snyder, P. M. (2002) Ubiquitin-protein ligase WWP2 binds to and downregulates the epithelial Na⁺ channel. *Am. J. Physiol. Renal Physiol.* **283**, F431–F436
- 15 Harvey, K. F., Dinudom, A., Komwatana, P., Jolliffe, C. N., Day, M. L., Parasivam, G., Cook, D. I. and Kumar, S. (1999) All three WW domains of murine Nedd4 are involved in the regulation of epithelial sodium channels by intracellular Na⁺. *J. Biol. Chem.* **274**, 12525–12530
- 16 Goulet, C. C., Volk, K. A., Adams, C. M., Prince, L. S., Stokes, J. B. and Snyder, P. M. (1998) Inhibition of the epithelial Na⁺ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. *J. Biol. Chem.* **273**, 30012–30017
- 17 Kamynina, E., Tauxe, C. and Staub, O. (2001) Distinct characteristics of two human Nedd4 proteins with respect to epithelial Na⁺ channel regulation. *Am. J. Physiol. Renal Physiol.* **281**, F469–F477
- 18 Snyder, P. M., Steines, J. C. and Olson, D. R. (2004) Relative contribution of Nedd4 and Nedd4-2 to ENaC regulation in epithelia determined by RNA interference. *J. Biol. Chem.* **279**, 5042–5046
- 19 Malik, B., Yue, Q., Yue, G., Chen, X. J., Price, S. R., Mitch, W. E. and Eaton, D. C. (2005) Role of Nedd4-2 and polyubiquitination in epithelial sodium channel degradation in untransfected renal A6 cells expressing endogenous ENaC subunits. *Am. J. Physiol. Renal Physiol.* **289**, F107–F116
- 20 Abriel, H., Loffing, J., Rebhun, J. F., Pratt, J. H., Schild, L., Horisberger, J. D., Rotin, D. and Staub, O. (1999) Defective regulation of the epithelial Na⁺ channel by Nedd4 in Liddle's syndrome. *J. Clin. Invest.* **103**, 667–673
- 21 Shimkets, R. A., Lifton, R. P. and Canessa, C. M. (1997) The activity of the epithelial sodium channel is regulated by clathrin-mediated endocytosis. *J. Biol. Chem.* **272**, 25537–25541
- 22 Staruschenko, A., Pochynyuk, O. and Stockand, J. D. (2005) Regulation of epithelial Na⁺ channel activity by conserved serine/threonine switches within sorting signals. *J. Biol. Chem.* **280**, 39161–39167
- 23 Wang, H., Traub, L. M., Weixel, K. M., Hawryluk, M. J., Shah, N., Edinger, R. S., Perry, C. J., Kester, L., Butterworth, M. B., Kleyman, T. R., Frizzell, R. A. and Johnson, J. P. (2006) Clathrin-mediated endocytosis of ENaC: role of epsin. *J. Biol. Chem.* **281**, 14129–14135
- 24 Shih, S. C., Sloper-Mould, K. E. and Hicke, L. (2000) Mono-ubiquitin carries a novel internalization signal that is appended to activated receptors. *EMBO J.* **19**, 187–198
- 25 Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P. and Dikic, I. (2003) Multiple mono-ubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat. Cell Biol.* **5**, 461–466
- 26 Hicke, L. and Dunn, R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* **19**, 141–172
- 27 Adams, C. M., Snyder, P. M. and Welsh, M. J. (1997) Interactions between subunits of the human epithelial sodium channel. *J. Biol. Chem.* **272**, 27295–27300
- 28 Wegierski, T., Hill, K., Schaefer, M. and Walz, G. (2006) The HECT ubiquitin ligase AIP4 regulates the cell surface expression of select TRP channels. *EMBO J.* **25**, 5659–5669
- 29 Lin, D.-H., Sterling, H., Wang, Z., Babilonia, E., Yang, B., Dong, K., Hebert, S. C., Giebisch, G. and Wang, W.-H. (2005) ROMK1 channel activity is regulated by mono-ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 4306–4311
- 30 Brissoni, B., Agostini, L., Kropf, M., Martinon, F., Swoboda, V., Lippens, S., Everett, H., Aebi, N., Janssens, S., Meylan, E. et al. (2006) Intracellular trafficking of interleukin-1 receptor 1 requires tollip. *Curr. Biol.* **16**, 2265–2270
- 31 Hanwell, D., Ishikawa, T., Saleki, R. and Rotin, D. (2002) Trafficking and cell surface stability of the epithelial Na⁺ channel expressed in epithelial Madin–Darby canine kidney cells. *J. Biol. Chem.* **277**, 9772–9779
- 32 Valentijn, J. A., Fyfe, G. K. and Canessa, C. M. (1998) Biosynthesis and processing of epithelial sodium channels in *Xenopus* oocytes. *J. Biol. Chem.* **273**, 30344–30351
- 33 Knight, K. K., Olson, D. R., Zhou, R. and Snyder, P. M. (2006) Liddle's syndrome mutations increase Na⁺ transport through dual effects on epithelial Na⁺ channel surface expression and proteolytic cleavage. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2805–2808
- 34 Malik, B., Schlanger, L., Al-Khalili, O., Bao, H. F., Yue, G., Price, S. R., Mitch, W. E. and Eaton, D. C. (2001) ENaC degradation in A6 cells by the ubiquitin–proteasome proteolytic pathway. *J. Biol. Chem.* **276**, 12903–12910
- 35 Butterworth, M. B., Edinger, R. S., Johnson, J. P. and Frizzell, R. A. (2005) Acute ENaC stimulation by cAMP in a kidney cell line is mediated by exocytic insertion from a recycling channel pool. *J. Gen. Physiol.* **125**, 81–101
- 36 Haglund, K., Di Fiore, P. P. and Dikic, I. (2003) Distinct mono-ubiquitin signals in receptor endocytosis. *Trends Biochem. Sci.* **28**, 598–603
- 37 Perdomo, J., Verger, A., Turner, J. and Crossley, M. (2005) Role for SUMO modification in facilitating transcriptional repression by BKL. *Mol. Cell. Biol.* **25**, 1549–1559
- 38 Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P. P. (2002) A single motif responsible for ubiquitin recognition and mono-ubiquitination in endocytic proteins. *Nature* **416**, 451–455
- 39 Hatakeyama, S., Jensen, J. P. and Weissman, A. M. (1997) Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases. *J. Biol. Chem.* **272**, 15085–15092
- 40 Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M. and Cyr, D. M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol.* **3**, 100–105
- 41 Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K. and Tai, T. (2002) E3 ubiquitin ligase that recognizes sugar chains. *Nature* **418**, 438–442

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