Nedd4-1 and β-Arrestin-1 Are Key Regulators of Na⁺/H⁺ Exchanger 1 Ubiquitylation, Endocytosis, and Function*§

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Alexandre Simonin*§ and Daniel Fuster†§

From the ‡Department of Nephrology and Hypertension, University Hospital of Bern, Freiburgstrasse 15, 3010 Bern, Switzerland and the †Institute of Biochemistry and Molecular Medicine, Bühlstrasse 28, 3012 Bern, Switzerland

The ubiquitously expressed mammalian Na⁺/H⁺ exchanger 1 (NHE1) controls cell volume and pH but is also critically involved in complex biological processes like cell adhesion, cell migration, cell proliferation, and mechanosensation. Pathways controlling NHE1 turnover at the plasma membrane, however, are currently unclear. Here, we demonstrate that NHE1 undergoes ubiquitylation at the plasma membrane by a process that is unprecedented for a mammalian ion transport protein. This process requires the adapter protein β-arrestin-1 that interacts with both the E3 ubiquitin ligase Nedd4-1 and the NHE1 C terminus. Truncation of NHE1 C terminus to amino acid 550 abolishes binding to β-arrestin-1 and NHE1 ubiquitylation. Overexpression of β-arrestin-1 or of wild type but not ligase-dead Nedd4-1 increases NHE1 ubiquitylation. siRNA-mediated knockdown of Nedd4-1 or β-arrestin-1 reduces NHE1 ubiquitylation and endocytosis leading to increased NHE1 surface levels. Fibroblasts derived from β-arrestin-1 and Nedd4-1 knock-out mice show loss of NHE1 ubiquitylation, increased plasmalemmal NHE1 levels and greatly enhanced NHE1 transport compared with wild-type fibroblasts. These findings reveal Nedd4-1 and β-arrestin-1 as key regulators of NHE1 ubiquitylation, endocytosis, and function. Our data suggest a broader role for β-arrestins in the regulation of membrane ion transport proteins than currently known.

Na⁺/H⁺ exchangers (NHEs) are ion transporters catalyzing the exchange of sodium with protons in prokaryotes and eukaryotes (1). The ubiquitous mammalian NHE isoform 1 (NHE1) controls cellular volume and pH and is therefore often referred to as the “housekeeping” NHE (2). In recent years, however, it has become clear that NHE1 is a highly dynamic protein at the plasma membrane with pivotal importance for mammalian biology that extends beyond ion translocation (3, 4). NHE1 regulates cell shape, adhesion, proliferation and migration (5). Moreover, NHE1 senses mechanical stress directly, thereby serving as a cellular mechanosensor (6). In resting cells, NHE1 is found at sites of focal adhesions in nonpolarized cells and at basolateral membranes in polarized epithelial cells, where it is required for assembly of stress fibers and focal adhesions. In migrating cells, NHE1 is endocytosed at the rear end of the cell and enriched at the leading edge of the cell, promoting cell migration by the development of pseudopodial protrusions and retraction at the rear end (7, 8). NHE1-deficient cells display reduced adhesion, loss of polarity and greatly diminished motility and chemotaxis (7). Conversely, up-regulation of NHE1 is associated with increased tumor growth and tumor cell invasion (9, 10). Regulation of these complex biological processes is thought to require both NHE transport directly as well as anchoring of cytoskeletal elements and scaffolding of signaling molecules by the large, intracellular C terminus of NHE1 (4). Clearly, the dynamics of NHE1 at the plasma membrane mandate rapid and tight regulation of NHE1 turnover. The molecular mechanisms underlying plasmalemmal NHE1 turnover are not known.

Turnover of eukaryotic ion transport proteins often involves the posttranslational conjugation of one or several ubiquitin residues to target proteins by E3 ubiquitin ligases (11). This process, called ubiquitylation, typically targets plasmalemmal proteins for internalization by endocytosis, sorting into multivesicular bodies and delivery to lysosomes. So far, involvement of the ubiquitin pathway in the regulation of NHEs has not been explored.

In some instances, downregulated proteins interact directly with their cognate E3 ubiquitin ligases through PY motifs (PPXY or LPXY). However, many proteins (including NHE1) lack PY motifs and are not capable of directly binding their E3 ubiquitin ligases (11). In the case of G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors, β-arrestins function as adaptors linking receptors with their cognate E3 ubiquitin ligases (12–15).

Here we find that NHE1 turnover at the plasma membrane is regulated by ubiquitylation through a process that is unprecedented for mammalian ion transport proteins, requiring the concerted action of both β-arrestin-1 and Nedd4-1. Our data suggest that Nedd4-1 and β-arrestin-1 are key regulators of NHE1 ubiquitylation, endocytosis, and function.

EXPERIMENTAL PROCEDURES

Unless specified otherwise, all chemicals and reagents were obtained from Sigma. Densitometric quantifications were performed using National Institutes of Health Scion Image software. Statistical analysis was done using Student’s t test or one-way analysis of variance (ANOVA) to correct for multiple comparisons, as appropriated. All statistical tests were...
two-sided and a $p$ value $<0.05$ was considered statistically significant.

Cell Culture, DNA Transfection, and siRNA Transfection Experiments—HEK293 cells were obtained from ATCC. β-arrestin-1 single knock-out mouse embryonic fibroblasts (MEFs) and corresponding wild-type MEFs derived from littermate mice were obtained from R. Lefkowitz. Generation and characterization of β-arrestin-1 knock-out and wild-type MEFs was described previously (16). Nedd4-1 wild-type and knock-out MEFs were a gift of H. Kawabe and N. Brose and were described in two recent publications (17, 18). Generation and characterization of immunized skin fibroblasts derived from NHE1 wild-type and knock-out mice was described (19). Transient cDNA and siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Octameric, N-terminally tagged HA-ubiquitin and His$_6$-ubiquitin constructs were gifts of M. Treier and described previously (15, 22, 23). The siRNA sequences (5′-3′) used were: Nedd4-1: UAGAGCCUGGCUGGGUUGUUU; Nedd4-2: AACCACAACACAAAGUCACAG; β-arrestin-1: AGCCUCUGCGCGGAAAU. The control non-targeting sequence used was: UCAUCUAAGCGGCUCUUUGCTT.

Cell Surface Biotinylation, Endo-, and Exocytosis Assays—Cell surface biotinylation, endo-, and exocytosis assays were essentially conducted as described (24). For surface biotinylation, cells were rinsed with PBS and surface proteins were biotinylated by incubating cells with 1.5 mg/ml sulfo-NHS-LC-biotin in 10 mM triethanolamine (pH 7.4), 1 mM MgCl$_2$, 2 mM CaCl$_2$, and 150 mM NaCl for 90 min with horizontal motion at 4 °C. After labeling, plates were washed with quenching buffer (PBS containing 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, and 100 mM glycine) for 20 min at 4 °C, then rinsed once with PBS. Cells were then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS), and lysates were cleared by centrifugation. Cell lysates of equivalent amounts of protein were equilibrated overnight with streptavidin-agarose beads at 4 °C. Beads were washed sequentially with solutions A (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM EDTA) three times, B (50 mM Tris-HCl (pH 7.4) and 500 mM NaCl) two times, and C (50 mM Tris-HCl, pH 7.4) once. Biotinylated proteins were then released by heating to 95 °C with 2.5× Lämmli buffer.

For sequential cell surface biotinylation/immunoprecipitation, monomeric avidin agarose beads were used which allow, in contrast to streptavidin-agarose beads, elution of biotinylated proteins by excess of free biotin. For this purpose, monomeric avidin beads were pre-washed according to the manufacturer’s instructions and subsequently used to precipitate surface membrane proteins that had been biotinylated before as described above. Beads were washed with solutions A–C as described above with additional two washes with a buffer containing PBS/1% Triton X-100 at the end. Biotinylated proteins were then eluted at 4 °C for 1 h in a buffer containing PBS/1% Triton X-100, 20 mM biotin, fresh protease inhibitors (Roche), 2.5 mM N-ethylmaleimide and 10 μM MG132. The resulting bead supernatant was then used for immunoprecipitation experiments.

To measure NHE1 endocytosis, cells were surface-labeled with sulfo-NHS-SS-biotin instead of sulfo-NHS-LC-biotin and quenched as described above. Cells were then warmed to 37 °C for 3 h to allow protein trafficking to occur, control cells were kept at 4 °C. After twice washing with TBS, surface biotin was cleaved with the small cell-impermeant reducing agent sodium 2-mercaptoethane sulfonate (10 mM MesNa, 1 mM EDTA, 0.2% BSA in 50 mM Tris, pH 8.6). The biotin bound to endocytosed proteins is protected from MesNa cleavage. After MesNa quenching with iodoacetamide and one wash with TBS, cells were then solubilized in RIPA buffer and biotinylated proteins were retrieved with streptavidin-agarose affinity precipitation as described above.
For measurement of NHE1 exocytosis, cells were rinsed with PBS thrice and the apical surface was then exposed to 1.5 mg/ml sulfo-NHS-acetate in 0.1 M sodium phosphate (pH 7.5) and 0.15 M NaCl (3 times 40 min at 4 °C) to saturate NHS-reactive sites on the cell surface. After quenching for 20 min (see above for quench conditions), cells were warmed to 37 °C for 3 h to permit protein trafficking, control cells were kept at 4 °C. Cells were then surface-labeled with 1.5 mg/ml sulfo-NHS-LC-biotin and lysed with RIPA buffer. The biotinylated fraction, which represents newly inserted surface proteins, was then affinity-precipitated with streptavidin-agarose, and the precipitate was subjected to SDS-PAGE and immunoblotting. MesNa, sulfo-NHS-acetate, sulfo-NHS-SS-biotin, sulfo-NHS-LC-biotin, monomeric avidin agarose beads, streptavidin-agarose beads and biotin were obtained from Pierce.

Isolation of His6-ubiquitylated Proteins—Purification of His6-ubiquitylated proteins was essentially performed as described by Treier et al. (20). Briefly, 24 h after transfection, cells grown on 100-mm dishes were lysed in 2 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 8.0), 1% Triton X-100 and sonicated to reduce viscosity. Lysate was mixed on a rotor with 50 μl (settled volume) of Ni-NTA-agarose (Qiagen) for 4 h at room temperature. The slurry was applied to Bio-Rad chromatography columns and successively washed with the following: 2 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 8.0); 4 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 5.8); 4 ml of (6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 pH 8.0, 1% Triton X-100:protein buffer) 1:1; 4 ml of (6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 pH 8.0, 1% Triton X-100:protein buffer) 1:3; 4 ml of protein buffer; 2 ml of protein buffer plus 10 mM imidazole. Elution was 1 ml of protein buffer plus 200 mM imidazole. Protein buffer was 50 mM Na2HPO4/NaH2PO4 (pH 8.0), 10 mM

![FIGURE 2. NHE1 is both polyubiquitylated and multi-monoubiquitylated at the plasma membrane.](image)

A, HEK293 cells were co-transfected with NHE1–3xFLAG and wild-type or indicated mutant HA-ubiquitin K/R constructs. After isolation of surface proteins as described in Fig. 1A, immunoprecipitation was performed using a polyclonal α-FLAG antibody. Immunoblotting was then performed with either a monomodal α-FLAG antibody (upper panel) or α-HA (lower panel). Note that cellular expression levels of HA-ubiquitin K/R constructs in HEK293 cells were not identical. Data are representative of three individual experiments. B, immunoblotting of a Lys63-linked (1–7) polyubiquitin ladder by monoclonal P4D1 and FK1-ubiquitin antibodies and corresponding Coomassie (CM)-stained gel. C, detection of ubiquitylated proteins in a native HEK293 cell lysate (20 μg of lysate protein loaded per lane) by P4D1 and FK1-α-ubiquitin antibodies. D, ubiquitylation of endogenous NHE1 in skin fibroblasts derived from NHE1 wild-type (+/+) and knock-out (−/−) mice. Endogenous NHE1 was immunoprecipitated with a polyclonal α-NHE1 antibody after denaturing samples in 1% SDS for 15 min at 95 °C. Immunoblotting was performed with monomodal α-NHE1 and monoclonal P4D1 or FK1 α-ubiquitin antibodies. Data are representative of three individual experiments.

![FIGURE 3. Intracellular NHE1 C terminus is required for NHE1 ubiquitylation.](image)

A, scheme of C-terminally truncated NHE1 constructs used in experiments. NHE1 transmembrane domain is shown in gray and intracellular NHE1 C terminus in white color. B, HEK293 cells were transiently co-transfected with HA-ubiquitin and either wild-type (NHE1WT) or C-terminally truncated (NHE1Δ747, NHE1Δ675, and NHE1Δ550, respectively) NHE1–3xFLAG constructs. Plasma membrane protein isolation and subsequent immunoprecipitation were performed as described in the legend to Fig. 1A. Asterisks mark mature, fully N- and O-glycosylated NHE1 forms. Data are representative of three independent experiments.

**Isolation of His6-ubiquitylated Proteins—**Purification of His6-ubiquitylated proteins was essentially performed as described by Treier et al. (20). Briefly, 24 h after transfection, cells grown on 100-mm dishes were lysed in 2 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 8.0), 1% Triton X-100, and 5 mM imidazole and sonicated to reduce viscosity. Lysate was mixed on a rotor with 50 μl (settled volume) of Ni-NTA-agarose (Qiagen) for 4 h at room temperature. The slurry was applied to Bio-Rad chromatography columns and successively washed with the following: 2 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 8.0), 1% Triton X-100; 4 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 (pH 5.8), 1% Triton X-100; 2 ml of 6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 pH 8.0, 1% Triton X-100:protein buffer) 1:1; 4 ml of (6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4 pH 8.0, 1% Triton X-100:protein buffer) 1:3; 4 ml of protein buffer; 2 ml of protein buffer plus 10 mM imidazole. Elution was 1 ml of protein buffer plus 200 mM imidazole. Protein buffer was 50 mM Na2HPO4/NaH2PO4 (pH 8.0), 10 mM

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**FIGURE 2. NHE1 is both polyubiquitylated and multi-monoubiquitylated at the plasma membrane.** A, HEK293 cells were co-transfected with NHE1–3xFLAG and wild-type or indicated mutant HA-ubiquitin K/R constructs. After isolation of surface proteins as described in Fig. 1A, immunoprecipitation was performed using a polyclonal α-FLAG antibody. Immunoblotting was then performed with either a monomodal α-FLAG antibody (upper panel) or α-HA (lower panel). Note that cellular expression levels of HA-ubiquitin K/R constructs in HEK293 cells were not identical. Data are representative of three individual experiments. B, immunoblotting of a Lys63-linked (1–7) polyubiquitin ladder by monoclonal P4D1 and FK1-ubiquitin antibodies and corresponding Coomassie (CM)-stained gel. C, detection of ubiquitylated proteins in a native HEK293 cell lysate (20 μg of lysate protein loaded per lane) by P4D1 and FK1 α-ubiquitin antibodies. D, ubiquitylation of endogenous NHE1 in skin fibroblasts derived from NHE1 wild-type (+/+) and knock-out (−/−) mice. Endogenous NHE1 was immunoprecipitated with a polyclonal α-NHE1 antibody after denaturing samples in 1% SDS for 15 min at 95 °C. Immunoblotting was performed with monomodal α-NHE1 and monoclonal P4D1 or FK1 α-ubiquitin antibodies. Data are representative of three individual experiments.
KCl, 20% glycerol, and 0.2% Nonidet P-40. The eluate was then trichloroacetic acid/acetone precipitated for further analysis.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation and immunoblotting have been described (25). For chemical cross-linking, cells grown on 6-cm dishes were washed 3x with PBS and incubated with 1 mM DTME (Pierce) with PBS to remove unreacted DTME, lysed with RIPA buffer, and then used for immunoprecipitation experiments. Antibodies were obtained from the following sources: α-β-arrestin-1, α-Nedd4-1 and α-NHE1 polyclonal (Santa Cruz Biotechnology), α-Nedd4-2 (Abcam), α-ubiquitin P4D1 (Cell Signaling, recognizes monoubiquitin and polyubiquitin chains (26, 27)), α-ubiquitin FK1 (BioMol, recognizes polyubiquitin chains only (26, 27)), secondary HRP-coupled antibodies and avidin-HRP (Bio-Rad). Cellular lysis buffers always contained protease inhibitors (Roche), 2.5 mM chlormerodan (Sigma-Aldrich), 50 μg of lyase protein were loaded per lane. D, HEK293 cells were co-transfected with NHE1–3xFLAG, HA-ubiquitin, and WT or ligase-dead (ΔNedd4-1) WT or knock-out (KO) MEFs (n = 3). To detect endogenous and transfected Nedd4-1, 20 μg of lyase protein were loaded and probed with a polyclonal α-Nedd4-1 antibody. Data are representative for three individual experiments. E, NHE1 ubiquitylation in Nedd4-1 WT or knock-out (KO) MEFs (n = 2). Immunoblotting was performed with monoclonal α-NHE1 or α-ubiquitin (P4D1) antibodies.

**RESULTS**

**NHE1 Is Ubiquitylated at the Plasma Membrane**—As it is currently unknown if NHE1 or any other NHE are ubiquitylated at the plasma membrane, we first tested if plasmalemmal NHE1 is ubiquitylated. NHE1–3xFLAG and HA-ubiquitin constructs or corresponding empty plasmids (pCMV and pMH, respectively) were transiently transfected in HEK293 cells. Then, after labeling of plasma membrane proteins by sulfo-
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Together, these findings clearly indicate that NHE1 itself and not an NHE1-associated protein is subject to ubiquitylation.

The 76 amino acid protein ubiquitin contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) all capable of conjugating ubiquitin (33, 34). It is the current belief that Lys48-linked chains primarily serve as recognition signal for proteasomal degradation whereas monoubiquitylation and Lys63-linked polyubiquitylation seem to be involved in cellular trafficking (34, 35). Recent evidence, however, suggests that all non-Lys63-linked chains can target proteins for degradation in eukaryotes (33). We next generated several HA-ubiquitin constructs with arginine replacement of key lysine residues and assessed plasmalemmal NHE1 ubiquitylation. As shown in Fig. 2A, cellular expression levels of the different K/R HA-ubiquitin constructs in HEK293 cells varied greatly. Clearly, however, NHE1 ubiquitylation seemed to be unaffected by arginine replacement of key ubiquitin lysine residues, including Lys31, Lys48, or Lys63 (Fig. 2A, left panel). Although poorly expressed when compared with the wild-type ubiquitin construct, even a ubiquitin construct lacking all seven lysine residues led to a NHE1 ubiquitylation signal (Fig. 2A, right panel). These results suggested that NHE1 is either only multi-mono-ubiquitylated or both multi-mono- and polyubiquitylated. To further differentiate between multi-mono- and polyubiquitylation, we employed two monoclonal α-ubiquitin antibodies with different affinities to monoubiquitin and tested native NHE1 ubiquitylation in untransfected mouse skin fibroblasts derived from NHE1 wild-type and knock-out animals, respectively (19, 26, 27). As shown in Fig. 2C, both α-ubiquitin antibodies recognize ubiquitylated proteins in a Western blot of cell lysates. However, whereas the α-ubiquitin antibody P4D1 recognizes both monoubiquitin and polyubiquitin chains of a ubiquitin ladder, the α-ubiquitin antibody FK1 only recognizes the polyubiquitin chains (Fig. 2B). As shown in Fig. 2D, both the P4D1 and FK1 α-ubiquitin antibody detected endogenous ubiquitylated NHE1 in NHE1 wild-type fibroblasts compared with NHE1 knock-out fibroblasts. Together with results obtained with mutant ubiquitin constructs, these findings suggest that NHE1 is subject to both multi-mono- and polyubiquitylation.

NHE1 C Terminus Is Required for NHE1 Ubiquitylation—

The large, ~320 amino acid spanning intracellular C terminus of the 820 amino acid long NHE1 protein contains 27 conserved lysine residues making it a likely target for the attachment of...
ubiquitin moieties. Whereas the short intracellular NHE1 N terminus does not contain any lysine residues, several short intracellular loops between transmembrane domains also contain conserved lysine residues that would theoretically be accessible to the ubiquitin machinery. To map regions important for NHE1 ubiquitylation, we expressed wild-type (NHE1 WT) or C-terminally truncated (Δ550) NHE1–3xFLAG were transfected in HEK293 cells, followed by immunoprecipitation 2 days after transfection. β-arrestin-1 and Nedd4-1 co-immunoprecipitate with native NHE1. HEK293 cells were grown to confluence, subjected to chemical cross-linking (DTME) as described under “Experimental Procedures” and then solubilized by RIPA buffer. Immunoprecipitation was performed with 1 mg of cell lysate protein by either a polyclonal α-Nedd4-1 or a monoclonal α-β-arrestin-1 antibody or equal amounts of rabbit or mouse IgG, respectively. For immunoblotting, a monoclonal α-arrestin-1 antibody was used.

Silencing of endogenous Nedd4-1 was achieved by the respective siRNA treatments compared with control siRNA-treated cells (Fig. 4C). Residual NHE1 ubiquitylation despite knockdown of Nedd4-1 suggests that small amounts of E3 ligase activity are sufficient for partial NHE1 ubiquitylation, an observation that was also made in the case of Nedd4-1-mediated ubiquitylation of the β2-adrenergic receptor (15). To confirm results obtained with siRNA targeting Nedd4-1, wild-type, and ligase-dead human Nedd4-1 were transiently overexpressed and NHE1 ubiquitylation was assessed. Despite significant amounts of endogenous Nedd4-1 in HEK293 cells, overexpressed wild-type but not ligase-dead Nedd4-1 further enhanced NHE1 ubiquitylation compared with cells transfected with the empty vector (Fig. 4D). In a next step we aimed to validate these findings in the recently generated Nedd4-1 wild-type and knock-out mouse embryonic fibroblasts (MEFs) (17, 36). As shown in Fig. 4E, whereas NHE1 is ubiquitylated in Nedd4-1 wild-type MEFs, Nedd4-1 knock-out MEFs show loss of plasmalemmal NHE1 ubiquitylation. Taken together, our data suggest that the ubiquitous E3 ligase Nedd4-1 is responsible for NHE1 ubiquitylation at the plasma membrane.

β-Arrestin-1 Is Essential for NHE1 Ubiquitylation—Nedd4 ligases typically interact directly with targets via PY motifs, but many membrane proteins, including NHE1, lack PY motifs (11). β-Arrestins are a family of versatile adapter proteins that were shown to bind to several E3 ligases, including Nedd4-1 (14, 15, 37). Moreover, β-arrestins were shown to interact with the NHE5 isoform, and their overexpression reduced NHE5 surface levels (38).

We thus hypothesized that β-arrestins could play a role in the ubiquitylation process of NHE1. To test this, we used siRNA targeting endogenous β-arrestin-1 in HEK293 cells and assessed plasmalemmal NHE1 ubiquitylation (Fig. 5A). siRNA targeting β-arrestin-1 led to a ~50% inhibition of NHE1 ubiquitylation (Fig. 5B). Typically, > 80% knock-down of β-arrestin-1 was achieved compared with control siRNA treated cells.
We next tested if NHE1 associates physically with β-arrestin-1 and Nedd4-1. In HEK293 cells, as depicted in Fig. 6, overexpression of endogenous Nedd4-1 and endogenous β-arrestin-1 partially co-localized with NHE1 in the cell (Fig. 6, C–E), supporting the idea of a physical interaction of these three proteins as suggested by functional ubiquitylation studies and co-immunoprecipitation experiments.

**Knock-down of Nedd4-1 or β-Arrestin-1 Increases Plasmalemmal NHE1 Levels by Inhibiting NHE1 Endocytosis without Affecting NHE1 Exocytosis**—We next studied the consequences of Nedd4-1 or β-arrestin-1 deficiency on NHE1 abundance at the plasma membrane in HEK293 cells. As shown in Fig. 7, A and B, knock-down of Nedd4-1 or β-arrestin-1 led to an increase of NHE1 levels at the plasma membrane, while total cellular NHE1 levels were unaffected (Fig. 7, C and D). The observed increase of plasmalemmal NHE1 levels could be due to decreased NHE1 internalization from the plasma membrane or increased NHE1 exocytotic insertion into the plasma membrane or a synergistic combination of both.

To measure NHE1 internalization, we prelabeled membrane proteins with cleavable sulfo-NHS-SS-biotin and measured during 3 h the amount of NHE1 that moved to a location inaccessible to cleavage by the membrane impermeant reagent sodium 2-mercaptoethane sulfonate (MesNa, Fig. 8A). As shown in Fig. 8B, both knock-down of Nedd4-1 or β-arrestin-1 led to a reduction of NHE1 internalization compared with cells transfected with control siRNA. This assay, however, measures net internalization. The observed reduction of NHE1 internalization could thus be either due to a reduction of NHE1 endocytosis or due to an increase in NHE1 recycling. To differentiate between the two possibilities, we measured NHE1 internalization in the presence of 25 μM monensin, a well documented inhibitor of endosomal recycling (39, 40). As demonstrated in Fig. 8B, NHE1 internalization was not significantly increased by monensin treatment, indicating that endocytosis itself was inhibited. To confirm that the monensin dose used was indeed effective in inhibiting endosomal recycling in our cells, we assessed Alexa 594-coupled transferrin uptake in HEK293 cells by confocal microscopy in the presence or absence of 25 μM monensin. In the absence of monensin, endocytozed transferrin is found in the typical perinuclear recycling endosomal compartment (supplemental Fig. S3). In the presence of 25 μM monensin, transferrin
cells, we next utilized MEFs derived from Nedd4-1 and β-arrestin-1 knock-out mice to further test our findings obtained in HEK293 cells (16, 17). Plasmalemmal NHE1 levels were increased in β-arrestin-1 (Fig. 9, A and B) and Nedd4-1 (Fig. 9, D and E) knock-out MEFs by ∼50% compared with MEFs derived from wild-type littermates. These findings support our observations made in HEK293 cells with siRNA-mediated knock-down of Nedd4-1 and β-arrestin-1. When tested for NHE1 transport activity under V_{max} conditions employing maximal sodium and proton gradients (19), we observed a robust increase of NHE1-mediated proton flux in β-arrestin-1 (Fig. 9, C and G) and Nedd4-1 (Fig. 9F) knock-out MEFs compared with their wild-type counterparts. Enhanced NHE1-mediated transport under V_{max} conditions is compatible with the observed increase of NHE1 at the cell surface in knock-out MEFs. Thus, collectively, our data indicate that both Nedd4-1 and β-arrestin-1 are key regulators of NHE1 ubiquitylation, endocytosis, and function.

**DISCUSSION**

In this study, we demonstrate the first time that a member of the large NHE family, the ubiquitous NHE1 isoform, is ubiquitylated. Using various ubiquitin K/R mutants and α-ubiquitin antibodies with differential sensitivity to monoubiquitin residues suggest that NHE1 is both poly- and multi-monoubiquitylated. Experiments including: 1) siRNA-mediated knock-down of endogenous Nedd4-1 in HEK293 cells, 2) overexpression of wild-type and ligase-deficient Nedd4-1 constructs in HEK293 cells and 3) analysis of NHE1 ubiquitylation in Nedd4-1 knock-out and wild-type MEFs suggest that the E3 ubiquitin ligase Nedd4-1 is critical for NHE1 ubiquitylation. As shown in Fig. 4A, we occasionally observed diminished plasmalemmal NHE1 expression when Nedd4-1 was knocked down in cells transiently transfected with NHE1–3xFLAG, which was never the case for endogenous NHE1 (Fig. 7, A and B). Possible explanations for this discrepancy include a decrease of protein synthesis/maturation or altered protein trafficking during forced expression of NHE1 with simultaneous Nedd4-1 deficiency. Quantification of NHE1 ubiquitylation thus always included normalization to surface NHE1 lev-
els. Also, effects of Nedd4-1 or β-arrestin-1 deficiency on NHE1 trafficking and surface levels were always performed on endogenously expressed proteins to avoid overexpression artifacts.

In addition to Nedd4-1, the intracellular NHE1 C terminus seems to be required for the ubiquitylation process as truncation of the C terminus to amino acid 550 leads to loss of NHE1 ubiquitylation at the plasma membrane. Whereas the NHE1 C terminus constitutes the most likely site of direct NHE1 ubiquitylation, intracellular loops between transmembrane segments are theoretically also accessible to the ubiquitylation machinery. Thus, it is also conceivable that the NHE1 C terminus plays only an indirect role in NHE1 ubiquitylation e.g. by harboring regulatory sites necessary for NHE1 ubiquitylation. That the NHE1 C terminus is at least modifying the ubiquitylation process is supported by the finding that two partial C-terminal truncations (NHE1/H9004747 and NHE1/H9004675) are more intensively ubiquitylated than wild-type NHE1, as shown in Fig. 3B. Because of the known redundancy of ubiquitylation sites, however, definitive mapping of NHE1 ubiquitylation sites in the C terminus will require site-specific mutations of all conserved 27 lysine residues in future studies (41).

In addition to Nedd4-1, presence of the adapter protein β-arrestin-1 seems to be essential for NHE1 ubiquitylation. The functional importance of β-arrestin-1 for NHE1 ubiquitylation as revealed by silencing and overexpression experiments and β-arrestin-1-deficient MEFs is supported by the finding that β-arrestin-1 co-immunoprecipitates with NHE1 and Nedd4-1. Both Nedd4-1 and β-arrestin-1 are cytosolic proteins, but both proteins were shown previously to also interact with and regulate plasma membrane proteins (13, 36, 42, 43). In support of this, we observed partial co-localization of NHE1 with Nedd4-1 and β-arrestin-1 by confocal imaging at or close to the plasma membrane levels. Also, effects of Nedd4-1 or β-arrestin-1 deficiency on NHE1 trafficking and surface levels were always performed on endogenously expressed proteins to avoid overexpression artifacts.

In addition to Nedd4-1, the intracellular NHE1 C terminus seems to be required for the ubiquitylation process as truncation of the C terminus to amino acid 550 leads to loss of NHE1 ubiquitylation at the plasma membrane. Whereas the NHE1 C terminus constitutes the most likely site of direct NHE1 ubiquitylation, intracellular loops between transmembrane segments are theoretically also accessible to the ubiquitylation machinery. Thus, it is also conceivable that the NHE1 C terminus plays only an indirect role in NHE1 ubiquitylation e.g. by harboring regulatory sites necessary for NHE1 ubiquitylation. That the NHE1 C terminus is at least modifying the ubiquitylation process is supported by the finding that two partial C-terminal truncations (NHE1Δ747 and NHE1Δ675) are more intensively ubiquitylated than wild-type NHE1, as shown in Fig. 3B. Because of the known redundancy of ubiquitylation sites, however, definitive mapping of NHE1 ubiquitylation sites in the C terminus will require site-specific mutations of all conserved 27 lysine residues in future studies (41).

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membrane (Fig. 6, C–E). We also observed intracellular co-localization of the three proteins, the reasons of which are unclear at the moment. This could be due to an overexpression artifact or indicate the possibility that once internalized, NHE1 remains associated with Nedd4-1 and β-arrestin-1 for a certain time period.

Given experimental evidence provided within this study and the documented scaffolding function of β-arrestins for E3 ligases, β-arrestin-1 most likely recruits Nedd4-1 to the NHE1 C terminus for subsequent NHE1 ubiquitylation (14, 15, 44). However, other scenarios are theoretically also possible, we cannot exclude the possibility that β-arrestin-1 affects NHE1 ubiquitylation independently of Nedd4-1.

siRNA-mediated knock-down of either Nedd4-1 or β-arrestin-1 in HEK293 cells increases NHE1 levels at the plasma membrane by attenuating NHE1 endo- but not exocytosis. These findings are in agreement with our observation of increased plasmalemmal NHE1 levels and increased NHE1 transport activity in MEFs deficient in either Nedd4-1 or β-arrestin-1. The magnitude of the increase in transport activity in knock-out MEFs, however, clearly exceeds the observed increase of surface NHE1 levels. Nedd4-1 and β-arrestin-1 may also influence the balance of active and inactive NHE1 transporters at the plasma membrane, e.g. by altering NHE1 distribution between plasma membrane lipid microdomains or interaction with associated proteins (3, 45). Alternatively, Nedd4-1 and β-arrestin-1 may influence sodium and/or proton kinetics of NHE1 (6, 19). These hypotheses will need to be addressed in detail in future studies.

β-Arrestins have been studied extensively in the regulation of GPCRs, RTKs and cytokine receptors, but few reports demonstrating interaction of β-arrestins with plasmalemmal transport proteins exist (38, 46–48). In the cases of NHE5, the Na/K-co-transporters at the plasma membrane, e.g. by altering NHE1 distribution between plasma membrane lipid microdomains or interaction with associated proteins (3, 45). Alternatively, Nedd4-1 and β-arrestin-1 may influence sodium and/or proton kinetics of NHE1 (6, 19). These hypotheses will need to be addressed in detail in future studies.

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