Nedd4–2 does not regulate wt-CFTR in human airway epithelial cells

Katja Koeppen,1 Chris Chapline,2 J. Denny Sato,2 and Bruce A. Stanton1,2

1Department of Microbiology and Immunology, The Geisel School of Medicine at Dartmouth, Hanover, New Hampshire; 2Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

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Koeppen K, Chapline C, Sato JD, Stanton BA. Nedd4–2 does not regulate wt-CFTR in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 303: L720–L727, 2012. First published August 17, 2012; doi:10.1152/ajplung.00409.2011.—The cystic fibrosis transmembrane conductance regulator (CFTR), a Cl− channel in airway epithelial cells, plays an important role in maintaining the volume of the airway surface liquid and therefore mucociliary clearance of respiratory pathogens. A recent study has shown that the E3 ubiquitin ligase Neural precursor cells expressed developmentally downregulated (Nedd4–2) ubiquitinates ΔF508-CFTR in pancreatic epithelial cells and that siRNA-mediated silencing of Nedd4–2 increases plasma membrane ΔF508-CFTR. Because the role of Nedd4–2 in regulating wild-type (wt)-CFTR in airway epithelial cells is unknown, studies were conducted to test the hypothesis that Nedd4–2 also ubiquitinates wt-CFTR and regulates its plasma membrane abundance. We found that Nedd4–2 did not affect wt-CFTR Cl− currents in Xenopus oocytes. Likewise, overexpression of Nedd4–2 in human airway epithelial cells did not alter the amount of ubiquitinated wt-CFTR. siRNA knockdown of Nedd4–2 in human airway epithelial cells had no effect on ubiquitination or apical plasma membrane abundance of wt-CFTR. Thus Nedd4–2 does not ubiquitinate and thereby regulate wt-CFTR in human airway epithelial cells.

Address for reprint requests and other correspondence: K. Koeppen, Dept. of Microbiology and Immunology, The Geisel School of Medicine at Dartmouth, 604 Remsen, Hanover, NH 03755 (e-mail: Katja.Koeppen@Dartmouth.edu).

Nedd4–2 belongs to the family of homologous to E6-AP COOH-terminus (HECT) E3 ligases that catalyze the final step in the ubiquitination cascade, the conjugation of ubiquitin to lysine residues of their target proteins, thus marking them for degradation (9). Nedd4–2 ubiquitiniates the epithelial sodium channel ENaC (17), the chloride channel CIC-5 (14), voltage-gated potassium and sodium channels (10, 26), the intestinal apical calcium entry channel TRPV6 (28), and the cardiac potassium channel hERG1 (1), but nothing is known about the effect of Nedd4–2 on wt-CFTR in airway epithelial cells.

Here we report that Nedd4–2 did not affect wt-CFTR Cl− currents in Xenopus oocytes and that overexpression did not alter the amount of ubiquitinated wt-CFTR in human airway epithelial cells. siRNA-mediated silencing of Nedd4–2 in human airway epithelial cells had no effect on the amount of ubiquitinated wt-CFTR or the amount of wt-CFTR in the apical membrane. Together, these results suggest that Nedd4–2 does not ubiquitinate and thereby regulate wt-CFTR abundance in human airway epithelial cells.

MATERIALS AND METHODS

Cell culture. CFBE-wt (CFBE41o- cells homozygous for the ΔF508 mutation and stably transduced with wt-CFTR; Ref. 3) cells were maintained in culture and grown as polarized monolayers on collagen coated Transwell permeable supports (Costar) as described in detail previously (4).

Antibodies. CFTR was detected in Western blots using either the COOH-terminus-specific clone 24–1 (R&D Systems, Minneapolis, MN) or clone 596 (Cystic Fibrosis Foundation Therapeutics, Chapel Hill, NC). CFTR was immunoprecipitated using CFTR antibody clone M3A7 (Millipore, Billerica, MA) or COOH-terminus-specific clone 24–1 (R&D Systems, Minneapolis, MN) and polyubiquitinated CFTR were detected with the mouse anti-mono/poly-ubiquitin antibody FK2 (Enzo Life Sciences, Plymouth Meeting, PA). Nedd4–2 was detected with a polyclonal rabbit anti-Nedd4–2 antibody (ab46521; Abcam, Cambridge, MA), which reacts with human and mouse Nedd4–2. Ezrin was a loading control in Western blot studies and was detected with a mouse anti-ezrin antibody (1:1,000; BD Biosciences, San Jose, CA). EBP50 was detected using a mouse monoclonal antibody (no. 611160, BD Biosciences), and SLC26A9 was probed with a mouse polyclonal antibody (H00115019-A01; Abnova, Taipei, Taiwan). USP10 antibody (Bethyl Laboratories, Montgomery, TX) was used as a positive control in communoprecipitation experiments. Mouse IgG1 antibody (Millipore Australia, Boronia, Australia) was used as a negative control in immunoprecipitation (IP) and ubiquitination experiments. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad, Hercules, CA) were used at a dilution of 1:3,000 for Western blots. All of the antibodies used have been shown in previous studies to be specific for their target protein (4, 5, 8).

Synthesis and expression of cRNAs in Xenopus oocytes and voltage-clamp recordings. cRNAs were synthesized and expressed in Xenopus oocytes, and CFTR Cl− currents were analyzed as described in detail previously (22). Briefly, oocytes were injected with either 500 pg Nedd4–2 cRNA, 50 pg CFTR cRNA, or 50 pg CFTR cRNA.
Nedd4–2 does not decrease wt-CFTR currents in Xenopus oocytes. Xenopus oocytes were used as a model to assess whether Nedd4–2 regulates wt-CFTR Cl− currents. If Nedd4–2 ubiquitination of wt-CFTR alone in oocytes increased IBMX-stimulated currents, compared with oocytes expressing only Nedd4–2 (Fig. 1A). Inspection of the current voltage (I/V) plots revealed that wt-CFTR currents had a linear I/V relationship with a reversal potential of −33 mV, characteristics of CFTR Cl− currents, as reported previously (22). Coinjection of Nedd4–2 with wt-CFTR had no effect on the IBMX-stimulated CFTR Cl− currents (Fig. 1A). The I/V plot for oocytes expressing wt-CFTR alone was similar to the I/V plot of oocytes expressing CFTR and Nedd4–2. For example, the IBMX-stimulated Cl− current at −80 mV was increased by 10.2 ± 0.3 mV by Nedd4–2 coinjection (Fig. 1A).

**RESULTS**

**Nedd4–2 construct and overexpression.** Mouse Nedd4–2 cloned into a pCMV-SPORT6 vector was a generous gift from Dr. Ray Frizzell (University of Pittsburgh). A COOH-terminal HA-tag was added onto mouse Nedd4–2 in a three-step process using Taq polymerase (Invitrogen, Carlsbad, CA) and the following sets of primers: set 1 - Forward 5'–CACAGAAGACTTCATGACCAGCTGCTT-3', Reverse 5'–AGGCACCGTCTATGGTACATGAAAG-CTGCTCTGTG-3'; set 2 - Forward 5'–CGTGCTGACAGTCTACCGACAGAAGATCCAAC-3', Reverse 5'–GGTGGATCTTCTGTCGGTAAAGCATAGTCACG-3'; set 3 - Forward 5'–CGTGCCCTGACAGTCAGTTATCCACCGCAGACAGATCCAC-3', Reverse 5'–GTGGATCTGTCTGTCGGTAAAGCATAGTCACG-3'. The resulting clone was verified by sequencing with T7 and SP6 primers (Invitrogen). For all Nedd4–2 overexpression experiments, CFBE-wt cells were seeded at 3 × 10⁶ cells/75-cm² cell-culture flask (T75, no. 3276; Corningware, Corning, NY) the day before transfection. One day after transfection, cells were seeded at 6 × 10⁵ cells/filter on collagen-coated 12-mm Transwell permeable supports (Costar no. 3407) and grown at an air-liquid interface for 4 more days. The COOH-terminal antibody as indicated and the Nedd4–2 antibody.

**CFTR ubiquitination.** Ubiquitination assays were performed as described in detail previously (4). Briefly, cells were treated with the lysosomal inhibitor chloroquine (200 μM) for 4 h. CFTR was precipitated from whole cell lysates (WCL) with the CFTR antibody M3A7 or mouse IgG1 as a negative control using 5 μg antibody/25 μg protein A (Thermo Scientific) was performed as described in detail previously (19, 20).

**CFTR short-circuit current measurements.** Transepithelial Cl− current measurements in Ussing chambers were performed as described in detail previously (25). Amiloride (50 μM, A7410; Sigma-Aldrich, St. Louis, MO) was added to the apical side of the polarized monolayer to inhibit epithelial sodium channel currents. CFTR Cl− currents were stimulated with forskolin (20 μM, Sigma-Aldrich F6886) added to the apical and basolateral sides of the monolayer. CFTR Inhibitor 172 (Thiazolidinone; 5 μM, Sigma-Aldrich C2992) was added to the apical side of the polarized monolayer to inhibit CFTR currents. Data were collected using the Acquire and Analyze software version 2.3 (Physiologic Instruments, San Diego, CA), and data analysis was performed with GraphPad Prism version 5.0c for Mac OS X (GraphPad Software, San Diego, CA). Differences between means were assessed by unpaired t-tests.

**Coimmunoprecipitation.** Coimmunoprecipitation experiments were conducted as described in detail previously (7). Briefly, CFBE-wt cells were seeded at 3 × 10⁶ cells/75-cm² cell-culture flask (T75, no. 3276) 2 days before the experiment. CFTR was immunoprecipitated from WCL with CFTR COOH-terminal antibody or mouse IgG1 as a negative control using 10 μg antibody for 1 mg total protein and 100 μl protein A agarose (Thermo Scientific). WCL and immunoprecipitated proteins were separated by Western blot and probed for CFTR (596 antibody), Nedd4–2, or USP10 (positive control).
mV was 6.2 ± 0.6 μA in oocytes expressing wt-CFTR (Fig. 1B; N = 66) and 6.6 ± 0.7 μA in oocytes expressing wt-CFTR and Nedd4–2 (Fig. 1B; N = 77).

siRNA silencing of Nedd4–2 in human airway epithelial cells. Although Nedd4–2 did not affect CFTR Cl− currents in oocytes, it is possible that Nedd4–2 ubiquitinates wt-CFTR in human airway epithelial cells. To test the hypothesis that Nedd4–2 ubiquitinates wt-CFTR in human airway epithelial cells, we first knocked down Nedd4–2 in CFBE-wt cells using siRNA and then measured ubiquitinated wt-CFTR, as well as plasma membrane wt-CFTR and wt-CFTR Cl− currents.

siRNA against Nedd4–2 reduced Nedd4–2 protein abundance in CFBE-wt cells transfected with siNeg or siNedd4–2. A–C: immunoprecipitation (IP) was conducted with either an anti-CFTR antibody (M3A7) or a control antibody (IgG), and Western blots (WB) were probed for Nedd4–2 (A), ubiquitin (FK2 antibody, B) or wt-CFTR (596 antibody, C). siNeg and siNedd4–2 samples were run on the same gel but are separated for presentation. D: summary of Nedd4–2 levels in siNeg and siNedd4–2 experiments (N = 3). E: summary of the effects of siNedd4–2 on ubiquitinated CFTR, which is reported as the amount of ubiquitinated CFTR (FK2) divided by the amount of total CFTR determined by Western blot (N = 3). ***P < 0.001.

Fig. 2. Ubiquitination assay in CFBE-wild-type (wt) cells transfected with siNeg or siNedd4–2. A–C: immunoprecipitation (IP) was conducted with either an anti-CFTR antibody (M3A7) or a control antibody (IgG), and Western blots (WB) were probed for Nedd4–2 (A), ubiquitin (FK2 antibody, B) or wt-CFTR (596 antibody, C). siNeg and siNedd4–2 samples were run on the same gel but are separated for presentation. D: summary of Nedd4–2 levels in siNeg and siNedd4–2 experiments (N = 3). E: summary of the effects of siNedd4–2 on ubiquitinated CFTR, which is reported as the amount of ubiquitinated CFTR (FK2) divided by the amount of total CFTR determined by Western blot (N = 3). ***P < 0.001.

Fig. 3. Biotinylation experiments in CFBE-wt cells transfected with siNeg or siNedd4–2. Representative Western blots of Nedd4–2 in whole cell lysates (WCL) (A), wt-CFTR and Ezrin in WCL (B), and biotinylated (BT) membrane CFTR (C). D: summary of Nedd4–2 levels in siNeg and siNedd4–2 experiments (N = 3). E: summary of WCL and membrane wt-CFTR for siNeg and siNedd4–2 (N = 3). ***P < 0.001.
siRNA knockdown of Nedd4–2 does not affect the amount of ubiquitinated wt-CFTR in human airway epithelial cells. If Nedd4–2 ubiquitinates wt-CFTR, knockdown of Nedd4–2 with siRNA should reduce the amount of ubiquitinated wt-CFTR (ubi-CFTR). However, knockdown of Nedd4–2 did not decrease the amount of ubi-CFTR in CFBE-wt cells (Fig. 2).

siRNA knockdown of Nedd4–2 does not affect the amount of wt-CFTR in cell lysates or the plasma membrane of human airway epithelial cells. Although siNedd4–2 did not alter the amount of ubi-CFTR, it is possible that Nedd4–2 may affect the amount of wt-CFTR in the plasma membrane or CFTR Cl⁻ currents by other, unknown mechanisms. To test the hypothesis that Nedd4–2 modulates plasma membrane wt-CFTR abundance, cell surface biotinylation experiments were performed to assess the amount of apical membrane wt-CFTR in CFBE-wt cells treated with siNedd4–2 or siNeg. siNedd4–2 did not alter the amount of wt-CFTR in WCLs or at the plasma membrane (Fig. 3).

siNedd4–2 increases wt-CFTR Cl⁻ currents in human airway epithelial cells. To determine whether Nedd4–2 regulates wt-CFTR function, short-circuit currents (Isc) were measured in CFBE-wt cells treated with siNeg or siNedd4–2. siNedd4–2 significantly augmented forskolin-stimulated and I-172-inhibited wt-CFTR Cl⁻ currents in CFBE-wt cells (Fig. 4). Mean forskolin-stimulated Cl⁻ currents were 58.8 ± 7.5 μA/cm² for siNeg control and 100.6 ± 13.8 μA/cm² for siNedd4–2, and mean I-172-inhibited Cl⁻ currents were 66.3 ± 9.3 μA/cm² for siNeg control and 105.8 ± 13.3 μA/cm² for siNedd4–2 (P < 0.05, N = 9). There was no significant difference between Fsk-stimulated and I-172-inhibited currents in either the siNeg or siNedd4–2 experiments.

siNedd4–2 does not alter the abundance of EBP50 or SLC26A9. To explain the increase in wt-CFTR currents that we observed with siNedd4–2, we speculated that siNedd4–2 may reduce the ubiquitination and degradation of positive regulators of CFTR activity such as NHERF1/EBP50 or SLC26A9. EBP50 has been reported to increase CFTR open probability by 2.8-fold (18), and SLC26A9 has been shown to stimulate wt-CFTR currents (2). However, siNedd4–2 did not change the abundance of EBP50 or SLC26A9 (Fig. 5), an observation incon-
consistent with our hypothesis that a decrease in Nedd4–2 may lead to a reduction in the ubiquitination of EBP50 and SLC26A9, which would increase their abundance and increase the activity of wt-CFTR.

**Nedd4–2 overexpression does not increase ubiquitinated CFTR.** Additional studies were conducted to test the hypothesis that Nedd4–2 ubiquitinates and thereby downregulates wt-CFTR abundance. To this end, Nedd4–2 was overexpressed in CFBE-wt cells, and experiments were performed to examine ubi-CFTR as well as wt-CFTR protein levels and CFTR Cl− currents. Overexpression of Nedd4–2 had no effect on the amount of ubi-CFTR (Fig. 6). This observation confirms the finding with siRNA for Nedd4–2, above, that Nedd4–2 does not ubiquitinate wt-CFTR.

**Nedd4–2 overexpression increases whole cell and plasma membrane wt-CFTR.** Paradoxically, overexpression of Nedd4–2 by 195% (Fig. 7, A and D) increased wt-CFTR protein levels in cell lysates (Fig. 7, B and E) and in the plasma membrane (Fig. 7, E)
C and E) by 24 and 21%, respectively. These results were unexpected because Nedd4–2 overexpression did not affect the amount of ubiquitinated wt-CFTR and suggest that overexpression of Nedd4–2 alters wt-CFTR abundance by a mechanism that does not involve ubiquitination of CFTR.

Nedd4–2 overexpression does not decrease CFTR short-circuit currents. To analyze the effect of Nedd4–2 on CFTR function, short-circuit currents were measured in CFBE-wt cells overexpressing Nedd4–2. Nedd4–2 overexpression did not alter forskolin-stimulated or I-172-inhibited CFTR Cl⁻ currents (Fig. 8). Mean forskolin-stimulated Cl⁻ currents were 19.8 ± 1.6 μA/cm² for cells transfected with control vector and 18.7 ± 1.4 μA/cm² for cells transfected with Nedd4–2. Mean I-172-inhibited Cl⁻ currents were 24.3 ± 1.2 μA/cm² for control vector and 23.4 ± 0.9 μA/cm² for Nedd4–2 overexpression (N = 6).

Nedd4–2 does not coimmunoprecipitate with CFTR in CFBE-wt cells. Because Nedd4–2 coimmunoprecipitates with ΔF508-CFTR in CFPAC cells, studies were performed to determine whether Nedd4–2 coimmunoprecipitates with wt-CFTR in CFBE-wt cells. Western blots of wt-CFTR, which had been immunoprecipitated from CFBE-wt cells, revealed that endogenous Nedd4–2 did not coimmunoprecipitate with wt-CFTR in CFBE-wt cells (Fig. 9A, lane 2). As a positive control, USP10 did coimmunoprecipitate with CFTR, as shown previously (Fig. 9A, bottom) (4). Likewise, in cells overexpressing Nedd4–2, wt-CFTR did not coimmunoprecipitate with Nedd4–2 (Fig. 9B, lane 3). The lack of coimmunoprecipitation suggests that there is either no direct interaction between wt-CFTR and Nedd4–2 in this model system, or, if there is any binding, it is of such low affinity that it is below the detection limit.

DISCUSSION

The goal of this study was to test the hypothesis that Nedd4–2, which ubiquitinates ΔF508-CFTR (7), also ubiquitinates wt-CFTR and regulates its expression. Several lines of evidence in this report demonstrate that Nedd4–2 does not ubiquitinate wt-CFTR. First, Nedd4–2 did not alter wt-CFTR Cl⁻ currents in Xenopus oocytes. Second, overexpression of Nedd4–2 in human airway epithelial cells had no effect on the amount of ubiquitinated wt-CFTR or on CFTR Cl⁻ currents. Third, siRNA knockdown of Nedd4–2 in human airway epithelial cells had no effect on the amount of ubiquitinated wt-CFTR. Taken together, the data in this report are consistent with the conclusion that Nedd4–2 does not ubiquitinate and

Fig. 8. Iₘₛₕ recordings of CFBE-wt cells transfected with control vector or Nedd4–2. A: representative current traces. B: summary of Fsk-stimulated and I-172-inhibited wt-CFTR ΔIₘₛₕ (N = 6).
Nedd4–2 does not regulate wt-CFTR abundance in human airway epithelial cells. However, our data suggest that Nedd4–2 may regulate CFTR indirectly. Because E3 ligases are highly promiscuous, Nedd4–2 is likely to ubiquitinate many targets, even though it does not ubiquitinate CFTR in our model system. Thus knockdown or overexpression of Nedd4–2 may have indirect effects on CFTR membrane density and activity by ubiquitinating positive and negative regulators of CFTR. These indirect effects would explain the small but significant increase in whole cell and plasma membrane wt-CFTR without a concomitant increase in CFTR Cl\(^{-}\) currents upon Nedd4–2 overexpression, as well as the increase in wt-CFTR currents without elevation in wt-CFTR membrane density with siNedd4–2. Overexpression of Nedd4–2 may lead to increased ubiquitination and degradation of negative regulators of CFTR abundance as well as positive regulators of CFTR activity, resulting in no net increase in CFTR current. Likewise, siNedd4–2 may reduce the ubiquitination and degradation of positive regulators of CFTR activity, such as NHERF1/EBP50 and SLC26A9, which have been shown to increase wt-CFTR currents (2, 18). However, siNedd4–2 did not change the abundance of EBP50 or SLC26A9, an observation inconsistent with the hypothesis that a reduction in Nedd4–2 led to a reduction in the ubiquitination of EBP50 and SLC26A9, which would increase their abundance. Because the goal of this study was to test whether Nedd4–2 ubiquitates wt-CFTR in airway epithelial cells, the identification of additional Nedd4–2 target proteins that regulate CFTR abundance and function is beyond the scope of this study.

In contrast to the present study, Caohuy and coworkers demonstrated that decreasing endogenous Nedd4–2 with siRNA led to a fivefold reduction in the amount of ubiquitinated ΔF508-CFTR and a fivefold increase in Cl\(^{-}\) transport in CFPAC-1 cells. Moreover, siNedd4–2 increased ΔF508-CFTR membrane density by 2.5-fold in both CFPAC-1 cells and IB3–1 cells and reduced the interaction between Nedd4–2 and ΔF508-CFTR in CFPAC-1 cells, as determined by communoprecipitation studies. These findings led the authors to conclude that ΔF508-CFTR is a target for Nedd4–2–mediated ubiquitination and degradation (7). By contrast, as noted above, we found that, in CFEB-wt cells, siNedd4–2 did not alter the amount of ubiquitinated wt-CFTR or wt-CFTR in the plasma membrane. Furthermore, wt-CFTR did not communoprecipitate with Nedd4–2 in CFEB-wt cells. The most likely explanation for this difference is that the ability of Nedd4–2 to ubiquitinate CFTR requires interaction between wt-CFTR and Nedd4–2 and that interaction is mediated by cell type-specific adaptor protein(s). For example, whereas the WW domain in Nedd4–2 binds directly to PY motifs on ENaC and thereby enhances its ubiquitination (24), CFTR does not have a conventional PY motif that could account for a direct interaction between Nedd4–2 and wt-CFTR (7). However, the NEDD4 family-interacting proteins, Ndfip1 and Ndfip2, assist Nedd4–2 family proteins in binding to target proteins (13, 16, 23, 27). For example, the binding of the divalent metal-ion transporter DMT1 to the NEDD4-like E3 ligase WWP2 and its subsequent ubiquitination and degradation require Ndfips (11). Thus we propose that an unknown adaptor protein may be present in CFPAC cells but not in CFEB cells. In summary, the present study demonstrates that Nedd4–2 does not regulate the ubiquitination and degradation of wt-CFTR in CFBE-wt airway epithelial cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.K. and B.A.S. conception and design of research; K.K., C.C., and J.D.S. performed experiments; K.K., C.C., and B.A.S. analyzed data; K.K. and B.A.S. interpreted results of experiments; K.K. prepared figures; K.K. and B.A.S. drafted manuscript; K.K., J.D.S., and B.A.S. interpreted results of experiments; K.K., C.C., J.D.S., and B.A.S. approved final version of manuscript.

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