Trafficking and Cell Surface Stability of the Epithelial Na\(^+\) Channel Expressed in Epithelial Madin-Darby Canine Kidney Cells*

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The apically located epithelial Na\(^+\) channel (αβγ-ENaC) plays a key role in the regulation of salt and fluid transport in the kidney and other epithelia, yet its mode of trafficking to the plasma membrane and its cell surface stability in mammalian cells are poorly understood. Because the expression of ENaC in native tissues/cells is very low, we generated epithelial Madin-Darby canine kidney (MDCK) cells stably expressing αβγ-ENaC, where each subunit is tagged differentially at the intracellular C terminus and the subunit is also Myc-tagged at the ectodomain (εMycβMyc\(_{-}\)γLam). ENaC expression in these cells was verified by immunoblotting with antibodies to the tags, and patch clamp analysis has confirmed that the tagged channel is functional. Moreover, using electron microscopy, we demonstrated apical, but not basal, membrane localization of ENaC in these cells. The glycosylation pattern of the intracellular pool of ENaC revealed peptide N-glycosidase F and endoglycosidase H sensitivity. Surprisingly, the cell surface pool of ENaC, analyzed by surface biotinylation, was also core glycosylated and lacked detectable endoglycosidase H-resistant channels. Extraction of the channel from cells in Triton X-100 demonstrated that both intracellular and cell surface pools of ENaC are largely soluble. Moreover, flotation assays to analyze the presence of ENaC in lipid rafts showed that both intracellular and cell surface pools of this channel are not associated with rafts. We have shown previously that the total cellular pool of ENaC is turned over rapidly (t1/2 ~ 1–2 h). Using cycloheximide treatment and surface biotinylation we now show that the cell surface pool of ENaC has a similarly short half-life (t1/2 ~ 1 h), unlike the long half-life reported recently for the Xenopus A6 cells. Collectively, these results help elucidate key aspects of ENaC trafficking and turnover rates in mammalian kidney epithelial cells.

The amiloride-sensitive epithelial Na\(^+\) channel (ENaC)\(^{1}\) is an apically located channel expressed primarily in salt-transporting epithelia of the kidney (distal nephron), distal colon, lung, ducts of exocrine glands, and other organs (for review, see Ref. 1). Its critical role in regulating salt and fluid transport is underscored by the findings that inactivating mutations in ENaC cause the salt-wasting nephropathy pseudohypoaldosteronism type I, and gain-of-function mutations cause Liddle syndrome, a hereditary form of hypertension (for review, see Ref. 2). Liddle syndrome is caused by mutations in one of the PY motifs (PPxFY) of ENaC, leading to increased channel numbers and opening at the plasma membrane (3); the increase in channel numbers is believed to be caused by impaired binding to and suppression by the ubiquitin ligase Nedd4 (4–9) and by impaired endocytosis of the channel (10).

ENaC consists of three subunits, αβγ (11, 12), arranged in a stoichiometry of 2α:1β:1γ (13, 14; for another view, see Ref. 15). Each ENaC subunit is comprised of two transmembrane domains, a large ectodomain flanking them and containing numerous N-linked glycosylation sites, and short intracellular N and C termini (16–18). The N termini of α- and γ-ENaC contain conserved Lys residues that serve as ubiquitin acceptor sites (19), and the C termini of all three subunits contain the above mentioned PY motifs (4, 5, 20). Although all three ENaC chains are glycosylated in cells, the role of this glycosylation is not clear, and indeed mutation of all N-linked glycosylation sites in α-ENaC does not seem to affect channel function (16, 18).

In native tissues, ENaC is a rare protein with very low expression levels (21, 22), permitting electrophysiological and limited immunofluorescence analyses to be performed but precluding biochemical studies on such low abundance proteins. Thus, several groups have investigated aspects of ENaC trafficking and protein stability in cell lines and heterologous expression systems, primarily in Xenopus oocytes, Xenopus A6 cells, and mammalian fibroblasts transiently expressing the ENaC subunits (23–26; for review, see Ref. 27). ENaC trafficking in mammalian kidney (polarized) epithelial cells stably expressing αβγ-ENaC has not been reported, hence this was the focus of our studies.

The ENaC chains appear to assemble together early on in the endoplasmic reticulum (28), but details of the route of trafficking to the cell surface are lacking. A major obstacle in following ENaC maturation biochemically has been the lack of an apparent endoglycosidase H (Endo H)-resistant pool, which typically marks mature transmembrane proteins that have acquired complex glycosylation at the medial Golgi compartment. This is unlike the ENaC relative Phe-Met-Arg-Phe-amide-activated Na channel (FaNaC), in which an Endo H-resistant pool is easily detectable during its maturation process (29). Thus, an important issue to be resolved is

* The abbreviations used are: ENaC, epithelial Na\(^+\) channel; Endo H, endoglycosidase H; PNGaseF, peptide N-glycosidase F; MDCK cells, Madin-Darby canine kidney cells; HA, hemagglutinin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline; TfR, transferrin receptor.

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whether ENaC does not acquire complex glycosylation, or whether it does so but the Endo H-resistant pool is below our detection limits. In addition, it has been suggested that in COS cells, the mature ENaC is stripped of its core glycosylation and becomes insoluble in non-ionic detergents upon arrival at the plasma membrane (23, 24).

Our previous work (19) has demonstrated that the total cellular pool of ENaC expressed heterologously in mammalian MDCK or NIH-3T3 cells has a short half-life (τ1/2 ~ 1–2 h), as also shown for A6 cells, which express ENaC endogenously (25, 30). In Xenopus oocytes expressing ectopic ENaC, which are grown at a much lower temperature, this half-life is ~10 h (31). Despite the relatively short half-life of the intracellular pool of ENaC in cultured cells, it has been recently suggested that the half-life of the surface pool of ENaC in A6 cells is quite long (>24 h for α, γ, and ~6 h for the β subunit) (25, 26). This contrasts previous work which suggested, based on functional studies in Xenopus oocytes before brefeldin A treatment, that the active cell surface pool of ENaC is short lived (10, 19), although this was not tested biochemically. The stability of ENaC at the cell surface of mammalian epithelial cells, most relevant for ENaC function, has not been investigated.

In this study, we describe the generation of kidney epithelial MDCK cells stably expressing epitope-tagged αγ-ENaC under an inducible promoter. Each subunit was tagged with a different tag at the intracellular C terminus, and an additional tag was added to the extracellular domain of β-ENaC. These cells express functional ENaC at the apical membrane. Using this cell line, we show here that the cell surface, mature ENaC is Endo H-sensitive (similar to intracellular ENaC), suggesting that the channel does not acquire complex glycosylation during trafficking to the cell surface. We also show that ENaC is not associated with lipid rafts, and its intracellular and cell surface pools are primarily detergent soluble. Moreover, we demonstrate that unlike A6 cells, the cell surface ENaC has a very short half-life, about 1 h. These studies are important for our understanding of the regulation of channel numbers at the plasma membrane, which play a key role in regulating ENaC function under physiological and pathophysiological conditions, such as Liddle syndrome.

EXPERIMENTAL PROCEDURES

Generation of Stable MDCK Cell Lines Expressing Tagged ENaC—MDCK clones expressing rat ENaC chains bearing different epitope tags (see Fig. 1A) were generated from high resistance MDCK cells as follows. For α-ENaC, a triple HA tag (YPYDVPDY) was introduced intracellularly just upstream of the stop codon, and the cDNA was subcloned into pLkneo, which possesses a dexamethasone-inducible promoter and neomycin resistance gene (32). After selection in G418, the stably expressing α-ENaC-MDCK cells were used as a template for the introduction of FLAG-tagged γ-ENaC. FLAG-γENaC was generated by introducing an intracellular FLAG tag (DYKDDDDK) upstream of the stop codon and subcloning into pCEP4 (Invitrogen), which possessed a hygromycin resistance gene. After selection in hygromycin (in the presence of G418), the α-ENaC-MDCK cells were used to introduce a double-tagged β-ENaC into them. For β-ENaC, a Myc tag (AEEQKLISEEDL) was inserted in the ectodomain (between amino acids 138 and 139, in a position previously described to have little effect on channel activity (3)), and an intracellular T7 (MASMTGGQQMG) tag was added just upstream of the stop codon. The tagged β-ENaC was subcloned into pBabe-puro, which expresses the puromycin resistance gene, and the cDNA was introduced into the above cells. Puromycin selection (in the presence of G418 and hygromycin) yielded the α-ENaC-MDCK cells (see Fig. 1A). The α-ENaC-MDCK cells were maintained as described previously (19). MDCK cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2-containing humidified air. The α-ENaC-MDCK cells were maintained in the above medium supplemented with 300 μg/ml G418, 5 μg/ml puromycin, 100 μg/ml hygromycin, and 10 μg/ml amiloride.

To verify protein expression, confluent monolayers grown on 10-cm plates were induced overnight (with 2 mM sodium butyrate, 1 μM dexamethasone, and 10 μM amiloride), washed, and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Triton X-100 (w/v), 10% glycerol (w/v), 1.5 mM MgCl2, 1.0 mM EDTA plus protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride)). Lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C; a 250-μl aliquot (for α or β-ENaC) was boiled in sample buffer, and proteins separated on an 8% SDS-PAGE. Because the expression of the γ7FLAG was very low, a larger amount of protein (6 mg) was used, and proteins were concentrated by methanol/chloroform precipitation prior to separation on a 7% Tricine SDS-PAGE. For comparison, 600 μg of protein from the α-ENaC-MDCK-expressing cells was also run on the same gel. After SDS-PAGE separation, proteins were immobiloblotted with antibodies to the tags (from Sigma (anti-FLAG), BABCO (anti-HA), Novagen (anti-T7), PharMingen (anti-Myc)) and detected by chemiluminescence (ECL from Amersham Biosciences, Inc. or SuperSignal from Pierce). In all experiments, MDCK cells were grown either on permeable filters (2.4-cm diameter, 0.4-μm pore size, Costar) or in 3.25-cm wells or 10-cm plates (plastic, Falcon) to confluence, and assays were performed 4 days later to ensure complete polarization and formation of domes (on plates) in the monolayers of cells. Several assays were performed using both systems, with similar results (see “Results”).

Electroporation—Whole cell current recordings were made from α-ENaC-MDCK cells grown on cover glass using the standard whole cell configuration of the patch clamp technique as reported previously (34). An Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA) was used to measure whole cell currents. The amplifier was driven by PCLAMP 6 software to allow the delivery of voltage step protocols with concomitant digitization of the whole cell current. The patch clamp pipettes, which were pulled from glass capillaries (LG 16, Dagan, Minneapolis) using a vertical puller (model PP-830, Narishige, Tokyo), had resistances of ∼2–3 megohms when filled with a standard cesium-glutamate-rich solution described below. The reference was an Ag/AgCl electrode, which was connected to the bath via an agar bridge filled with a standard NaCl-rich bathing solution.

Current-voltage (I-V) relations were studied using voltage ramps. The command voltage was varied from −124 mV to +16 mV over a duration of 800 ms every 30 s. 10 μM amiloride-sensitive currents were estimated by subtraction of currents measured under identical conditions except for the addition of 10 μM amiloride.

The pipette solution contained (in mM) 100 or 120 cesium-glutamate, 10 CaCl2, 10 NaH2PO4, 0 or 10 Na3-ATP, and 10 EDTA. The pH of the solution was adjusted with CaOH to 7.4. The cells were initially immersed in a bath solution (pH 7.4) containing (in mM) 140 NaCl, 4.3 KCl, 1MgCl2, and 10 HEPES. Before the establishment of whole cell configuration, the bath solution was changed to the one containing (in mM) 145 lithium-glutamate, 1 M NaCl, and 19 HEPES. The pH of the solution was adjusted with LiOH.

Cell Surface Biotinylation—Confluent monolayers grown on either a permeable filter or a solid support were induced overnight and then kept on ice throughout the experiment. Cells were washed three times with ice-cold PBS-CM (PBS with 1 mM MgCl2 and 0.1 mM CaCl2) and incubated 15 min with 1.0 mg/ml EZ-link™ Sulfo-NHS-S-S-biotin (Pierce) in cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, 150 mM NaCl, pH 9.0) with gentle agitation. Cells were then washed once with quenching buffer (192 mM glycine, 29 mM Tris in PBS-CM) and incubated for 10 min with quenching buffer with light agitation. Cells were then rinsed twice with PBS-CM, scraped in cold PBS, and pelleted at 2,000 rpm at 4 °C. They were lysed in lysis buffer and incubated on ice 30 min before centrifugation (10 min at 14,000 × g, 4 °C). Supernatants were transferred to new tubes and after the addition of 50 μl of 50% slurry of streptavidin-agarose beads (Sigma), they were rotated for 2 h at 4 °C. Beads were pelleted by brief centrifugation and aliquots of the supernatant were taken to represent the cell surface. Cells were then washed with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Biotinylated proteins were eluted by boiling in sample buffer supplemented with 5% β-mercaptoethanol, and proteins were separated on 8% SDS-PAGE and immobiloblotted as above. In some experiments, cells were surface biotinylated by treating with prechilled 10 mM sodium periodate on ice for 30 min in the dark. Cells were then incubated with...
1.0 mg/ml EZ-link™ biotin-LC-hydrazide (Pierce) in prechilled 100 mM sodium acetate, pH 5.5, on ice with slight agitation for 30 min.

For all experiments analyzing cell surface expression using the Sulfo-NHS-S-S-biotin for surface biotinylation, to ensure the absence of leakage of biotin into the cell (which could label the intracellular pool of ENaC, an effect reported above), and to verify the results with the quadruple tagged ENaC, we washed three times with HNTG. Biotinylated proteins were eluted by boiling in sample buffer supplemented with 5% β-mercaptoethanol.

**Immunoelectron Microscopy—Confluent, filter-grown αENaC—γyLAC-ENaC-expressing MDCK cells were induced overnight and rinsed three times with cold PBS-CM. Immunogold surface labeling was performed as described previously (37). For experiments in which cells were first surface biotinylated, 50 μl of 50% streptavidin-agarose bead slurry was added to each sample after resuspension of the pellets and rotated for 2 h at room temperature. After brief centrifugation, supernatants were washed three times with cold PBS-CM, surface biotinylated, and quenched as described above, and aliquote supernatants were washed three times with PBS-CM, surface biotinylated, and incubated with goat anti-mouse 15 nm NHS-S-S-biotin for surface biotinylation, to ensure the absence of leakage of biotin into the cell (which could label the intracellular pool of proteins, as described above). Aiquots of supernatant from the streptavidin-agarose bead incubations, representing the intracellular pool of proteins, were boiled. Proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

**Cell Surface Stability—Cells were grown on plates or filters and induced overnight. The following day, the indicated plates/filters were incubated with fresh induction medium supplemented with 20 μg/ml cycloheximide (ICN) for 1–6 h at 37 °C. We used one 10-cm plate or six filters per time point. At the end of the treatment period, the appropriate plates/filters were placed on ice, surface biotinylated, and lysed in lysis buffer as described above. Lysates for each time point were quantitated using the Bio-Rad assay, and total protein was normalized before addition of streptavidin-agarose beads. Lysates were then rotated for 2 h at 4 °C. After a brief centrifugation to pellet the beads, aliquots were taken to represent the unbound, intracellular pool. Beads were washed three times with HNTG, and proteins were eluted by boiling in sample buffer containing 5% β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to the tags followed by chemiluminescence detection. Band quantification was then performed using Fluorchem equipment and software (Alpha Innotech Corp., San Leandro, CA). Bands were quantified using spot densitometry, where only density values within the linear range were used.

**ENaC Distribution in Non-ionic Detergent—Polarized αHA-Myc,γT7-γyLAC-ENaC-expressing MDCK cells grown on filters or plates were induced overnight and placed on ice. Cells were rinsed three times with PBS-CM, surface biotinylated, and quenched as described above, then scraped in PBS. Cells were pelleted and lysed in lysis buffer for 30 min on ice. Samples were centrifuged for 10 min at 14,000 rpm, and pellets (insoluble fraction) were resuspended in a volume of lysis buffer equal to the supernatants (detergent-soluble fractions). Resuspended pellets were sonicated three times for 15 s each on ice to suspend the pellets. Samples were then centrifuged for 10 min at 14,000 rpm, and the supernatants (representing the insoluble fraction) were collected. For ENaC at the cell surface, biotinylated proteins were collected with 50 μl of 50% streptavidin-agarose bead slurry by rotating for 2 h at 4 °C. After brief centrifugation, aliquots of supernatants were then used to represent the intracellular pool and were boiled in sample buffer. Beads were washed, and biotinylated proteins were eluted from the detergent-soluble and -insoluble fractions as described above. Proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting and quantitation of chemiluminescence, as described above. The quadruple tagged ENaC was verified after induction with αENaC in the parental MDCK cells, not sufficient to confer channel activity (34), but suggesting that such MDCK cells could provide a suitable host for heterologous ENaC. We thus epitope tagged each rat ENaC chain with a different tag at the C terminus just upstream of the stop codon. We also introduced a Myc tag at the ectodomain of β-ENaC (Fig. 1A), in the same position shown previously not to affect channel activity when containing a short epitope tag (3). To protect cells from excessive Na+ loading, the α-subunit was expressed under the control of a dexamethasone-inducible promoter, and the other two subunits were expressed constitutively (see “Experimental Procedures”). The tagged ENaC subunits were then stably transfected into the parental MDCK cells, and protein expression of the quadruple tagged ENaC was verified after induction with dexamethasone (in the presence of amiloride). As seen in Fig. 1B, antibodies against the tags recognized the expected size of each ENaC subunit.

To verify that the tagged ENaC is localized properly to the apical membrane, we grew the αHA-Myc,γT7-γyLAC-ENaC-expressing MDCK cells on permeable filters and used immunogold labeling with anti-Myc antibodies to stain the apical or basal compartments (4). Cells were washed three times (5 min each) with cold PBS-CM and incubated with goat anti-mouse 15 nm gold in PBS-CM (1:12, British Biolabs) at 4 °C for 1 h on ice. Cells were rinsed three times (5 min each) with ice-cold PBS and incubated in Karnovsky’s fixative (2.5% glutaraldehyde, 3.2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. Samples were rinsed three times with PBS and incubated with 1% OsO4 for 15 min in the dark. Following PBS washes, filters were incubated with 2% uranyl acetate for 15 min in the dark. Samples were then washed x3 with distilled water and dehydrated using ethanol incubations in 50, 70, 85, and 100% ethanol (2 times 5 min each). Filters were infiltrated with 1:1 ethanol:methanol, 1:1 ethanol:xylene, and then 100% xylene overnight, 4 °C. Fresh Epon was applied to filters and incubated at room temperature for 5 h. Epon was again replaced and polymerized for 48 h at 65 °C. Blocks containing filter were sectioned (90-nm thickness) and placed on slotted grids. Sections were viewed using a Hitachi H600 transmission electron microscope.

**RESULTS**

**Characterization of Epitope-tagged αβγENaC Stably Expressed in MDCK Cells—**The expression of the ENaC protein in native kidney epithelia is very low, thus precluding biochemical analyses of the channel in these tissues. To analyze ENaC function in cultured kidney epithelial cells, we utilized high resistance MDCK cells, which form polarized monolayers and which are well characterized with respect to cellular trafficking (37). In addition, our unpublished work has identified traces of αENaC in the parental MDCK cells, not sufficient to confer channel activity (34), but suggesting that such MDCK cells could provide a suitable host for heterologous ENaC. We thus epitope tagged each rat ENaC chain with a different tag at the C terminus just upstream of the stop codon. We also introduced a Myc tag at the ectodomain of β-ENaC (Fig. 1A), in the same position shown previously not to affect channel activity when containing a short epitope tag (3). To protect cells from excessive Na+ loading, the α-subunit was expressed under the control of a dexamethasone-inducible promoter, and the other two subunits were expressed constitutively (see “Experimental Procedures”). The tagged ENaC subunits were then stably transfected into the parental MDCK cells, and protein expression of the quadruple tagged ENaC was verified after induction with dexamethasone (in the presence of amiloride). As seen in Fig. 1B, antibodies against the tags recognized the expected size of each ENaC subunit.

To verify that the tagged ENaC is localized properly to the apical membrane, we grew the αHA-Myc,γT7-γyLAC-ENaC-expressing MDCK cells on permeable filters and used immunogold labeling with anti-Myc antibodies to stain the apical or basal surface of unpermeabilized cells. Fig. 2A shows that the tagged ENaC is indeed localized to the apical membrane of polarized MDCK cells. Basal labeling was not detected, as expected from the apical localization seen in the apical apertures of the parental untransfected MDCK cells (Fig. 2A). Interestingly, although the tagged ENaC chains are overexpressed in these cells, the density of ENaC channels actually expressed at the apical cell surface appears very low (probably <1% of total ENaC pool, based on our crude estimate).

The parental MDCK cells do not possess detectable ENaC.
has shown that the pool of ENaC at the cell surface is very small relative to the total cellular pool, we had to ensure that none of the biotin enters the cells (and thus labels intracellular ENaC) during surface biotinylation. Thus, for each treatment, we analyzed both surface biotinylation of ENaC and biotinylation of an abundant intracellular protein such as actin, annexin II, or enolase, used as a marker for biotin entry into the cell. Fig. 3A shows the validity of the assays, demonstrating surface biotinylation of α-ENaC but not of actin, despite high intracellular expression of both proteins. Such cell surface biotinylation reflecting cell surface expression of ENaC was seen both when the ENaC-expressing MDCK cells were grown (and became polarized) on plates (solid support) or on permeable filters. Using this cell surface biotinylation approach, we then analyzed the susceptibility of the cell surface, mature ENaC to PNGaseF and Endo H. As seen in Fig. 3B the surface pool of α-ENaC was PNGaseF- and Endo H-sensitive, similar to the intracellular pool. We were not able to detect cell surface biotinylation of β-ENaC (Fig. 3C) even though this subunit is expressed at the apical surface (Fig. 2A). The intracellular pool of β-ENaC was sensitive to both PNGaseF and Endo H (Fig. 3C), as expected. Cell surface expression of γ-ENaC in the αβγFLAG-ENaC-expressing cells was too low to allow quantitative biochemical studies. However, we were able to perform cell surface biotinylation of γ-ENaC in the αβγFLAG-ENaC cells (Fig. 3D, boxed), which demonstrated lack of Endo H-resistant cell surface or intracellular pools of γ-ENaC, similar to α-ENaC. Our confocal immunofluorescence analysis of the steady-state pool of ENaC revealed extensive colocalization with the Golgi marker galactosyltransferase (green fluorescent protein-tagged), suggesting, as expected, that ENaC transits through the Golgi en route to the plasma membrane (data not shown). Thus, these results demonstrate that ENaC does not acquire complex sugar modifications during its traffic from the endoplasmic reticulum through the Golgi to the plasma membrane.

**Detergent Solubility and Lack of Raft Association of ENaC**—ENaC has been proposed previously to become insoluble in non-ionic detergent (such as Triton X-100) during its maturation (23, 24). To analyze whether the mature, cell surface pool of ENaC is detergent-soluble/insoluble, we compared solubility of surface biotinylated and intracellular pools of ENaC. Fig. 4 (top panel) depicts solubility of ENaC in increasing concentrations of Triton X-100 and shows that with concentrations as low as 0.2%, the majority of the cellular and all of the surface pool of ENaC was soluble. At 0.5 and 1% Triton X-100, virtually all of the intracellular and cell surface pools of the channel were found in the soluble fraction. This was unlike annexin II, which is associated with rafts (see below) and was used here as a control that is known to possess a substantial insoluble fraction (Fig. 4, bottom panel).

To investigate further the detergent solubility of ENaC, we tested its possible association with lipid rafts. This was particularly important because the ENaC-interacting protein Nedd4 is associated with lipid rafts, and this association helps recruit Nedd4 to the apical plasma membrane in MDCK cells (38). We thus performed flotation assays (36, 38, 39) to test for the presence of ENaC in the light fractions (20–25%) of an Optiprep gradient that was overlaid on top of detergent (1% Triton X-100, 4 °C)-extracted ENaC followed by ultracentrifugation. As controls, we used caveolin and annexin II, two proteins well established to become raft-associated (40, 41), and TfnR, which is not associated with rafts (42). As seen in Fig. 5A, both caveolin and annexin II were present in lipid rafts (in the 20 and 25% fractions), as expected, and the association of annexin II with the rafts, which is calcium-dependent, was disrupted by
the addition of the chelator EGTA. As also expected, the TfnR was not associated with rafts. As evident from Fig. 5A (bottom panel), the total cellular pool of ENaC did not float in the light raft fractions, and its distribution matched that of the TfnR and not of caveolin or annexin II. The same results were obtained when the ENaC-expressing MDCK cells were grown on permeable filters (Fig. 5B). Moreover, Fig. 5B (second from bottom panel) demonstrates that the biotinylated, cell surface pool of ENaC is also not associated with lipid rafts. Therefore, these results suggest that ENaC does not associate with rafts during its intracellular trafficking or at the plasma membrane.

Half-life of ENaC at the Cell Surface—Our previous work (19) and that of others (25, 30) has demonstrated a relatively short half-life of the total (largely intracellular) pool of ENaC (1–2 h) in cultured cells. The stability of ENaC at the cell surface where it functions, however, is a more important parameter. To determine the half-life of ENaC at the plasma membrane, we surface biotinylated ENaC after cycloheximide treatment (for 0–6 h), which blocks protein synthesis and thus prevents the synthesis and arrival of new channels to the plasma membrane. We opted not to use brefeldin A because of earlier reports questioning its effectiveness in disrupting the Golgi apparatus in MDCK and other kidney cells and demonstrating its inhibitory effect on transcytosis instead (43, 44).

Half-life of ENaC at the cell surface pool of α-ENaC (in the context of αβγ-MDCK-ENaC) is short lived, with half-life of $1.04 \pm 0.19$ (n = 5). As expected, the intracellular pool is also turned over relatively quickly ($t_{1/2} = 1.7 \pm 0.16$ (n = 5)), as also shown for the other two ENaC subunits (Fig. 6A) and as documented previously (19). There was no difference in the half-life of ENaC when cells were grown to postconfluence on either solid support or permeable filters ($t_{1/2}$ for cell surface-ENaC grown on filters $= 0.9$ h) (Fig. 6, A and B). A similar short half-life of cell surface and intracellular γ-ENaC (analyzed in the αβγ-MDCK-ENaC cells) was also observed (Fig. 6C, boxed).

**DISCUSSION**

The regulation of ENaC trafficking and cell surface stability is of fundamental importance to its function. Studying the factors that control these parameters has been difficult, however, because biochemical analyses are hard to perform on such a low abundance protein in native tissues. Thus, several groups have used surrogate systems, including Xenopus A6 cells, which express γ-ENaC endogenously, and Xenopus oocytes or mammalian fibroblasts ectopically (usually transiently) expressing ENaC to study ENaC trafficking and stability. In this report, we describe the generation of a MDCK cell line stably expressing epitope-tagged ENaC, which allowed us to perform...
biochemical analyses and study trafficking of ENaC in these polarized kidney epithelial cells.

Although ENaC contains multiple Asn-linked glycosylation sites (18), which are utilized in cells, the role of glycosylation of this channel is obscure. Moreover, our present work indicates that the glycosylation pattern commonly seen in transmembrane proteins, i.e. the acquisition of complex glycosylation during protein maturation, is not seen in ENaC. This was demonstrated by analyzing the glycosylation pattern of the cell surface, mature pool of ENaC (Fig. 3), which we found is still core-glycosylated and Endo H-sensitive. Other groups have described the lack of a detectable Endo H-resistant pool of total cellular ENaC (e.g. 17, 23, 31), but because ENaC efficiency of maturation is so low it was not possible to conclude whether this reflects the complete absence of such a pool or its presence in amounts below detection limits. Thus, our approach of studying the cell surface pool of ENaC (mature by definition) helps resolve this issue. Although uncommon, there have been reports on transmembrane proteins trafficking to the plasma membrane without acquiring complex sugar modifications. Examples include the anion exchanger AE1 (45), the Torpedo acetylcholine receptor (46), and the Shaker potassium channel (47), although in the latter two, a lack of complex glycosylation was seen upon expression in Xenopus oocytes (which still resulted in the presence of functional channels at the cell surface). Our results showing retention of core glycosylation of the mature ENaC are not in agreement with those of Prince and Welsh (23, 24) who proposed that ENaC is stripped of its
carbohydrates before arriving at the plasma membrane. We do not know the reason for the difference, although their study focused mainly on individual subunits expressed in nonpolarized (COS-7 or 293) cells, which renders comparison with our work difficult. What is clear from several studies, at least in cells heterologously expressing ENaC (e.g. (31)) is that ENaC maturation is very inefficient, with only a fraction of synthesized channels making it to the cell surface. Indeed, our crude estimate suggests that less than 1% of total α-ENaC expressed in our αENaC-MDCK cells is present at the cell surface under steady-state conditions.

Our results show that both intracellular and cell surface pools of ENaC are largely soluble in the non-ionic detergent Triton X-100. This differs from recent reports (23, 24), which suggested that the mature ENaC is insoluble, although the cause of such proposed insolubility was not identified. In accord with our observation of solubility of ENaC we found, using floatation assays, that ENaC is not associated with rafts after extraction in 1% Triton X-100 in the cold. We cannot preclude the possibility, however, that insolubility in other non-ionic detergents would allow sequestration of the channel in some rafts. Lipid rafts are cholesterol- and sphingolipid-containing microdomains in membranes that play an important role in trafficking, sequestration, or exclusion of proteins and in signal transduction (48). We have demonstrated previously that Nedd4, a binding partner of ENaC, employs apical rafts to localize to the apical membrane of polarized MDCK cells; it does so by associating, via its C domain, with annexin XIIIb, an apical raft-enriched protein (38, 49). Our present work thus suggests that the interaction between ENaC and Nedd4 likely occurs late, at the plasma membrane, and that Nedd4 is not involved in mediating trafficking of ENaC to the apical membrane. What does control apical plasma membrane localization of ENaC is not known. Our earlier work had suggested that the interaction of α-ENaC C terminus with α-spectrin helps stabilize ENaC at the apical membrane of polarized alveolar epithelial cells (50). However, such an interaction is clearly not necessary for membrane targeting because removal of the C termini of all ENaC chains does not interfere with plasma membrane localization of the channel. Recent reports have implicated the SNARE protein syntaxin 1A in regulating ENaC transport to the cell surface (51, 52), but the regulation of this process is not known.

The stability of ENaC at the cell surface has critical implications for its regulation, and indeed Liddle syndrome mutations in the PY motifs of β- or γ-ENaC are associated with increased channel retention at the plasma membrane (6–8, 10). The cell surface stability of ENaC is regulated by several factors, including ubiquitination (19) and the ubiquitin ligase Nedd4 (4, 6–8). Thus, investigating the cell surface stability of the channel in polarized epithelial cells is very important. Our results presented here suggest that the cell surface pool of ENaC is turned over rapidly, with a half-life of ~1 h. This value is in agreement with functional data in Xenopus oocytes, dem-

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**Fig. 6. Half-life of cell surface and intracellular pools of αENaC.** Postconfluent MDCK cells expressing αENaC grown on plates (A) or permeable filter support (B) were treated with cycloheximide (CHX) for the indicated times. The half-lives of the cell surface biotinylated pool and intracellular pool were then determined as described under “Experimental Procedures.” Only the half-lives of intracellular β- and γ-ENaC pools expressed in these cells are depicted in A and B because their cell surface pool was too small for biochemical analyses using either surface biotinylation or labeling with anti-Myc antibodies to detect β-ENaC. C, boxed area, shows the t1/2 of γ-ENaC expressed in the context of αENaC-ENaC cells. Half-life values: for cell surface α-ENaC, 1.04 ± 0.19 h (mean ± S.E., n = 5) on plates and ~0.9 h on filters (n = 2); for intracellular α-ENaC, 1.66 ± 0.16 h (mean ± S.E., n = 5) on plates and ~1.3 h (n = 2) on filters; for intracellular β-ENaC, 0.94 ± 0.16 h on plates and 1.03 ± 0.16 h on filters; for γ-ENaC, 0.75 ± 0.16 h at the cell surface (n = 2) and ~2.5 h intracellularly (n = 4). For all experiments, cells from a 10-cm plate or six filters (pooled) were used for each time point. The faint upper band in A (top panel) and the lower bands (asterisk) in A (middle panel) or B (middle panel) represent cross-contaminating bands recognized by the anti-HA antibodies (see also Fig. 1B).
onstrating that functional channels disappear rapidly from the plasma membrane after brefeldin A treatment, which blocks anterograde transport of transmembrane proteins from the Golgi apparatus (10, 19), although channel numbers were not analyzed directly. In contrast to our observations of a short half-life of the cell surface pool of ENaC, recent reports in A6 cells suggested that endogenous x-ENaC is very stable at the plasma membrane (t1/2 > 24 h, but 6 h for β2-ENaC) (25, 26). Although A6 cells grow at a lower temperature (27°C) than mammalian MDCK cells (37°C), this proposed half-life is still quite long relative to what we observe in MDCK cells (Fig. 6) or in Xenopus oocytes (19), which grow at 18°C. It was assessed by using antibodies raised against x-ENaC (26) to immunoprecipitate surface-biotinylated x-ENaC from A6 cells. The antibody to α-ENaC recognizes three bands: at 75 (the expected size), 150, and 180 kDa. The 180 kDa band was proposed to be a dimer of the mature, glycosylated α-ENaC, and its half-life at the cell surface was shown to be ~30 h (26). This long half-life (relative to the ~1 h reported here) thus suggests that the mode of regulation of the channel in A6 cells may be different from that seen in mammalian epithelial cells, although the nature of such putative differences in regulation is unknown.

In summary, our work described here provides important new insights into the mode of trafficking and cell surface stability of ENaC expressed in mammalian kidney epithelial cells.

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