Effect of subunit composition and Liddle's syndrome mutations on biosynthesis of ENaC

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Prince, Lawrence S., and Michael J. Welsh. Effect of subunit composition and Liddle's syndrome mutations on biosynthesis of ENaC. Am. J. Physiol. 276 (Cell Physiol. 45): C1346–C1351, 1999.—The epithelial Na⁺ channel (ENaC) is comprised of three homologous subunits: α, β, and γ, all of which are required for formation of the fully functional channel. This channel is responsible for salt reabsorption in the kidney, the airway, and the large bowel. Mutations in ENaC cause human disease by increasing channel function in Liddle's syndrome, a form of hereditary hypertension, or by decreasing channel function in pseudohypoaldosteronism type I, a salt-wasting disease of infancy. We previously showed that ENaC is expressed on the cell surface as a minimally glycosylated, Triton-insoluble protein. In the present study we found that ENaC existed initially as a Triton-insoluble protein that contained high-mannose glycosylation, presumably in the endoplasmic reticulum. This form of the protein disappeared as the Triton-insoluble, minimally glycosylated form became the more prevalent species. In pulse-chase studies of individually expressed subunits, we found that the Triton-soluble form of β-ENaC accumulated initially, whereas the Triton-insoluble form of α-ENaC decreased throughout the time course. However, when all three subunits were coexpressed, the α- and β-subunits showed a similar pattern. The complex became Triton insoluble at some point after the endoplasmic reticulum, as incubation at 15°C blocked the conversion to the insoluble form. Deletion of the carboxy-terminal tail of β-ENaC causes Liddle's syndrome. This mutation increased the amount of newly synthesized Triton-insoluble ENaC heteromultimers but did not affect the half-life of insoluble protein. Therefore, subunit composition and mutations in individual subunits can influence biosynthesis of the ENaC complex.

degenerin/epithelial sodium channel; sodium channel; subunit assembly; Triton solubility

THE RATE OF TRANSEPITHELIAL Na⁺ transport is regulated by apical expression of the epithelial Na⁺ channel (ENaC) (2, 8, 10). ENaC is composed of three homologous subunits, α, β, and γ (3, 4, 13, 15, 16). Each subunit contains two hydrophobic transmembrane domains, intracellular amino and carboxy termini, and a large, cysteine-rich extracellular domain with numerous sites for N-linked glycosylation. Generation of large amiloride-sensitive Na⁺ currents in heterologous cells requires coexpression of α-, β-, and γ-ENaC. Expression of β- and γ-ENaC subunits alone produces no current, whereas expression of α-ENaC alone generates small Na⁺ currents. These findings suggest the formation of a heteromultimeric complex at the plasma membrane. Biochemical evidence of complex formation has come from the findings that ENaC subunits can be communoprecipitated (1, 6, 18) and that they coassemble on sucrose density gradients as large complexes (6, 7).

Our previous study showed that when α-, β-, and γ-ENaC were expressed alone, they could be detected in an intracellular compartment and on the plasma membrane (18). Cell surface ENaC resided in a Triton-insoluble environment and contained only minimal glycosylation. Coexpression of all three ENaC subunits decreased the amount of β-ENaC found intracellularly without dramatically affecting the amount of ENaC at the cell surface. These observations led us to study the kinetics of ENaC biosynthesis and how coexpression of the various ENaC subunits might affect the overall biosynthetic process.

We also examined the trafficking of ENaC proteins containing a disease-associated mutation. Specific mutations in the carboxy terminus of β- and γ-ENaC cause Liddle's syndrome, a genetic form of hypertension resulting from increased Na⁺ absorption in the distal nephron (12, 24). Mutations associated with Liddle's syndrome increase the number of ENaC channels at the apical membrane (25) and may also increase the open probability of channels at the cell surface (9). As a result, Na⁺ absorption increases and hypertension ensues. We asked whether Liddle's mutations increase levels of ENaC at the cell surface at least in part by altering biosynthesis.

MATERIALS AND METHODS

Reagents and cell culture. A monoclonal antibody against the FLAG epitope was obtained from Kodak (Rochester, NY). COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture with DMEM containing 10% FCS. Cells were incubated in a humidified atmosphere containing 5% CO₂. For transfection of COS-7 cells, 1 × 10⁷ cells were electroporated with 30 µg of plasmid DNA. The cells were evenly divided, plated onto five 100-mm dishes, and cultured for 24–48 h in medium containing 10 µM amiloride to prevent cell swelling before study. Cells were 60–80% confluent at the time of experiments.

DNA constructs. Construction of the DNA constructs encoding α-, β-, and γ-subunits of human kidney ENaC (all in the pMT3 expression vector) are described elsewhere (1, 15–17). A form of β-ENaC associated with Liddle's syndrome, βR566X-ENaC, was made by inserting a stop codon after amino acid 565. The FLAG epitope (DYKDDDDK) was introduced into full-length human kidney ENaC with use of the Mutagen-GENE phagemid in vitro mutagenesis kit (Bio-Rad, Hercules, CA). For experiments using βR566X-ENaC, the FLAG epitope was inserted into the extracellular domain of α-ENaC at amino acid 397.

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Epitope-tagged subunits were then cloned into pMT3 for expression.

Immunoprecipitation. For immunoprecipitation, cells were washed three times in ice-cold PBS with 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS c/m) and lysed in Tris-buffered saline (TBS), pH 7.4, with 1% Triton X-100 (Pierce, Rockford, IL) containing the following protease inhibitors: 0.4 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotonin, 20 µg/ml leupeptin, and 10 µg/ml pepstatin A. Lysates were centrifuged at 16,000 g at 4°C, and the supernatant was incubated with 5 µg of anti-FLAG antibody. The pellet was solubilized in 100 µl of 2% SDS, 1% 2-mercaptoethanol, 50 mM Tris (pH 7.4), and 1 mM EDTA by heating to 90°C for 5 min. After solubilization, 1.0 ml of TBS with 1% Triton X-100 was added and ENaC was immunoprecipitated with anti-FLAG antibody. Antigen-antibody complexes were precipitated with immobilized protein A (Pierce), and precipitates were washed three times in TBS with 1% Triton X-100 and eluted with Laemmli sample buffer [4% SDS, 65 mM Tris (pH 6.8), 100 mM dithiothreitol, 20% glycerol, and 0.005% bromphenol blue]. Proteins were separated on 7% polyacrylamide gels by SDS-PAGE.

Pulse-chase experiments and steady-state metabolic labeling. For metabolic pulse-chase experiments, transfected COS-7 cells were starved for 1 h in ethionine-free medium and pulsed for 30 min with 100 µCi/ml [35S]methionine (6,000 Ci/mmol; Amersham, Chicago, IL). After the pulse period, radioactive medium was removed and replaced with complete medium for the chase period. Cells were then lysed, and ENaC was immunoprecipitated as described above. Steady-state labeling of ENaC in COS-7 cells was done by starving the cells in methionine-free medium for 1 h and adding 50 µCi/ml of [35S]methionine (6,000 Ci/ml) to the medium for 4 h. The radiolabeled immunoprecipitates were analyzed by SDS-PAGE on 7% polyacrylamide gels, then the gels were fixed, dried, and developed overnight by autoradiography or phosphorimaging with use of a Storm 600 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands corresponding to ENaC proteins typically contained an integrated pixel intensity of 10⁵–10⁶ units after subtraction of background values with use of Image Quant software (version 1.2, Molecular Dynamics).

RESULTS AND DISCUSSION

Differences in pulse-chase kinetics of α- and β-subunits expressed alone. Our earlier studies found that ENaC subunits at the plasma membrane were Triton insoluble and contained only minimal N-linked glycosylation (18). To investigate the biosynthetic process that generated this protein, we conducted pulse-chase studies in COS-7 cells and followed ENaC through the biosynthetic pathway. As previously shown (18), the Triton-soluble fraction contained glycosylated and
A minimally glycosylated form appeared in the Triton-insoluble fraction. Figure 1A shows that, when expressed alone, the Triton-soluble, unglycosylated form of the α-subunit disappeared very early during the chase. The soluble glycosylated α-subunit disappeared more slowly than the unglycosylated form. During the course of the chase, the Triton-insoluble, minimally glycosylated form accumulated slowly and became the predominant species at later time points.

We observed a similar overall pattern when the β-subunit of ENaC was expressed alone (Fig. 1B). However, interestingly, the glycosylated form of β-ENaC found in the Triton-soluble fraction actually accumulated during the 1st h of the chase and then began to disappear (Fig. 2). This is different from soluble glycosylated α-ENaC, which disappeared as soon as the chase period began. The observed patterns suggested that the glycosylated, Triton-soluble form of ENaC represents a biosynthetic intermediate as ENaC traffics to the cell surface. Because glycosylated, Triton-soluble ENaC possesses high-mannose-type oligosaccharides (1, 18), it probably represents protein resident in the endoplasmic reticulum (ER) (19). Although α- and β-ENaC are capable of trafficking to the cell surface when expressed alone, these data suggest that β-ENaC has a longer residence time in the ER.

Coexpression of subunits changes ENaC biosynthesis. The differences in the time course of Triton-soluble glycosylated α- and β-ENaC expressed alone raised the question of how coexpression of all three ENaC subunits affects biosynthesis. As shown in Fig. 1, C and D, the findings from pulse chase of glycosylated α- and β-ENaC coexpressed with the other two subunits were very similar to each other. More Triton-soluble, glycosylated α-ENaC was present at each time point than when it was expressed alone (Fig. 2). Conversely, there was less Triton-soluble, glycosylated β-ENaC when it was coexpressed with α- and γ-ENaC. These effects were dependent on which subunits were expressed and not which subunit was immunoprecipitated. Therefore, the protein studied represented that present in heteromultimeric complexes. These data suggest that α- and β-ENaC each contribute unique kinetic information to biosynthesis of the heteromultimeric α-β-γ complex.

Fig. 3. Effect of reduced temperature on trafficking of α-ENaC. COS-7 cells were transfected with α-ENaC 48 h before study. Cells in methionine-free medium were labeled with 100 μCi/ml of [35S]methionine for 2 h at 15°C. For chase, cells were warmed to 37°C (n = 3; A) or kept at 15°C for 2 h and then warmed to 37°C (n = 4; B). α-ENaC was immunoprecipitated from Triton-soluble and Triton-insoluble fractions at each time point. A and B: representative autoradiographs. C: quantitation of Triton-insoluble protein. Values are means ± SE.

Fig. 4. Steady-state measurement of wild-type and Liddle’s ENaC. COS-7 cells expressing α-FLAG, βwt, and γ (αβγ) or α-FLAG, βR566X, and γ (αβR566Xγ) were labeled with 50 μCi/ml of [35S]methionine for 4 h. ENaC subunits were immunoprecipitated and analyzed by SDS-PAGE and phosphorimaging. A: autoradiograph of 1 experiment. B: quantitation of 4 independent experiments. Values (means ± SE) are expressed as amount of Triton-insoluble α-ENaC divided by total α-ENaC. *P < 0.05 (paired t-test).
One possibility is that α- and β-ENaC have different inherent rates of trafficking through the biosynthetic pathway. Alternatively, α-ENaC could form multimeric complexes more efficiently than β-ENaC, with the formation of the complete complex limiting trafficking out of the ER. This second hypothesis is supported by sucrose gradient sedimentation data (6).

Effect of a 15°C block on solubility. In earlier work we found that ENaC subunits became Triton insoluble at some intracellular site before they reach the plasma membrane (18). To further delineate the subcellular site at which ENaC becomes Triton insoluble, we conducted pulse-chase experiments with incubation at 15°C during a prolonged (2-h) labeling period. Lowering the temperature to 15°C blocks vesicular transport from the ER to the cis-Golgi network (20). As shown in Fig. 3, very little insoluble α-ENaC was formed at 15°C. However, on warming of the cells to 37°C for the chase, ENaC appeared in the Triton-insoluble fraction. Likewise, maintaining cells at 15°C during the chase period continued to inhibit formation of Triton-insoluble α-ENaC (Fig. 3B). ENaC therefore becomes Triton insoluble at a site between the ER and the cell surface. These data also support the hypothesis that ENaC in the ER is Triton soluble. At this time, we do not know the basis for the insolubility; however, Triton insolubility has been observed with caveolar proteins, cytoskeletal proteins, and some high-molecular-weight oligomers (5, 14, 21, 22). The previous observation that some
Triton-insoluble ENaC (8–37%) may be intracellular (18) is consistent with insolubility occurring in a biosynthetic compartment such as the Golgi apparatus or trans-Golgi network.

Effect of a Liddle’s syndrome-associated mutation on ENaC biosynthesis. Mutations in β- and γ-subunits associated with Liddle’s syndrome increase the number of ENaC channels in the plasma membrane (9, 12, 24, 25). Increased channel number at the plasma membrane could result from increased biosynthesis, slowed degradation, and/or altered cellular localization. Earlier work measuring amiloride-sensitive current in brefeldin A-treated Xenopus oocytes estimated the halflife of wild-type cell surface ENaC as ~3–4 h (23, 27). Additional studies have shown that, in Liddle’s syndrome, reduced endocytosis contributes to the increase in cell-surface protein (23, 25). To study the biosynthesis of ENaC with Liddle’s mutations, we coexpressed wild-type α- and γ-ENaC with βR566X-ENaC. The FLAG epitope was placed in the extracellular domain of α-ENaC, rather than at an intracellular site, to eliminate the possibility that the sequence might interfere with interactions between ENaC and intracellular proteins. By introducing the Liddle’s mutation in β-ENaC and assaying the biosynthesis of α-ENaC, we could be reassured that any differences observed with βR566X were due to biosynthesis of heteromultimeric complexes and not β-ENaC homomultimers.

Figure 4 shows that coexpression of α- and γ-ENaC with a βR566X-subunit increased the amount of α-ENaC found in the Triton-insoluble fraction compared with coexpression with a wild-type β-subunit. Earlier work showed that the Triton-insoluble fraction of ENaC was present at the cell surface (18). To determine whether expression of βR566X slowed degradation, we did pulse-chase experiments comparing the metabolic half-lives of α-ENaC expressed with the βwt-subunit (Fig. 5A) and the βR566X-subunit (Fig. 5B). Quantitation of four independent experiments is shown in Fig. 5C. The observed half-life for the Triton-insoluble form of α-ENaC was ~10 h when it was expressed with βwt or βR566X-ENaC. This result suggests that degradation of the Triton-insoluble α-γ complex is not affected by a Liddle’s mutation.

Even though the half-life of Triton-insoluble α-ENaC was similar when coexpressed with βwt or βR566X-ENaC, there was a visible difference in the relative amount of insoluble α-ENaC at the end of the 30-min pulse in Fig. 5A and B. The difference was similar to that observed at steady state (Fig. 4). These observations suggested the possibility of differences between βwt and βR566X-ENaC early in the biosynthetic pathway. To examine early biosynthetic events, we conducted pulse-chase experiments using a short (5-min) pulse and chase period up to 1 h. We measured the fraction of total α-ENaC that was insoluble at each time point. Figure 6 shows that more Triton-insoluble α-ENaC was present at early time points when coexpressed with the βR566X-subunit than with a wild-type β-subunit. These findings suggest that Liddle’s mutations in β-ENaC may increase channel number in the plasma membrane at least in part by increasing trafficking along the biosynthetic pathway.

How might a Liddle’s mutation in ENaC affect biosynthesis? Earlier work has shown that a PPXY motif in the carboxy terminus of ENaC subunits binds to Nedd4 (26). Liddle’s mutations delete or mutate this motif and subsequent Nedd4 binding. The interaction of Nedd4 with ENaC may decrease the amount of protein at the cell surface, may inhibit the function of ENaC channels, and may increase the rate of ENaC degradation (11, 26, 27). It is possible that Nedd4 first interacts with ENaC during biosynthesis, while the subunits are still in the ER or in the Golgi complex. We speculate that the loss of an interaction with Nedd4 or some other protein may be responsible for the increased relative amount of Liddle’s ENaC that leaves the ER and becomes Triton insoluble.

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