

Original Paper

Post-Transcriptional Modulation of α ENaC mRNA in Alveolar Epithelial Cells: Involvement of its 3' Untranslated Region

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Key Words

Epithelial sodium channel (ENaC) • RNA-binding protein • mRNA stability • 3'UTR

Abstract

Background/Aims: The epithelial sodium channel (ENaC) expressed in alveolar epithelial cells plays a major role in lung liquid clearance at birth and lung edema resorption in adulthood. We showed previously that α ENaC mRNA expression is downregulated in part via posttranscriptional regulation of mRNA stability. In the present work, the role of the α ENaC 3' untranslated region (3'UTR) in the regulation of mRNA stability was studied further. **Methods:** Quantitative reverse transcription PCR (qRT-PCR) was performed to investigate the expression of α ENaC in alveolar epithelial cells. The role of the α ENaC 3'UTR was evaluated through sequential deletions. RNA affinity chromatography and mass spectrometry were achieved to investigate the nature of the proteins that could bind this sequence. The function of these proteins was assessed through knockdown and overexpression *in vitro*. **Results:** First, we found that α ENaC mRNA half-life was much shorter than expected when using a transcriptionally controlled plasmid expression system compared to Actinomycin D treatment. Sequential deletions of the α ENaC 3'UTR revealed that the α ENaC 3'UTR plays an important role in the modulation of α ENaC mRNA stability, and that there is a complex stabilizing and destabilizing interplay between different regions of the 3'UTR that modulate this process. Finally, we identified RNA-binding proteins that interact with the α ENaC 3'UTR and showed that Dhx36 and Tial1 are involved in the decrease in α ENaC mRNA stability via the proximal region of its 3'UTR. **Conclusion:** Taken together, these findings indicate that the α ENaC 3'UTR plays an important role in modulating transcript levels, and Dhx36 and Tial1 seem to be involved in posttranscriptional regulation of α ENaC expression in alveolar epithelial cells.

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Introduction

Active sodium transport has been demonstrated to be important in the lungs for fluid movement across the alveolar epithelium [1, 2]. The main channel involved in this process is the epithelial sodium channel (ENaC), which is expressed by alveolar epithelial cells [2]. Although it is composed of three subunits (α , β , γ) [3, 4], the α subunit has been shown to be the most important since α ENaC KO mice are unable to clear fluid from alveolar spaces, develop respiratory distress and die shortly after birth [5]. In addition to its role in the absorption of lung liquid at birth [6], ENaC is known as a key player in pulmonary edema resolution in adults [7, 8].

A number of pathophysiological insults have been found to modulate ENaC activity and expression. Pulmonary inflammation is an important feature involved in the inhibition of ENaC expression and could alter the resolution of pulmonary edema [9]. We reported previously that α ENaC expression is downregulated in alveolar epithelial cells by TNF- α , in part via posttranscriptional regulation of mRNA stability [10]. We also reported that α ENaC 3' untranslated region (3'UTR) could be involved in this process [11].

While the modulation of α ENaC gene transcription has been thoroughly investigated under various pathophysiological stress conditions [12-15], there have been no studies on either the regulation of α ENaC mRNA stability or the mechanisms involved in this modulation. One reason that could explain the lack of interest in this question is the very long half-life of α ENaC mRNA reported in the literature after inhibiting transcription with Actinomycin D (> 10 h) [10, 16-20]. This finding is in contradiction with some of our results showing that, in a model of canine lung transplantation, the expression of α ENaC mRNA was decreased by 75% 4 h after reperfusion [21]. Although ischemia-reperfusion injury in transplanted lungs could modulate α ENaC mRNA stability, these data also raise the hypothesis that Actinomycin D stabilize α ENaC mRNA.

For this reason, we developed a transcriptionally controlled plasmid expression system to investigate the stability of an α ENaC mRNA transcript in rat alveolar epithelial cells in primary culture. We decided to focus on the 3'UTR portion of α ENaC mRNA since numerous data show that the 3'UTR of a transcript can play an important role in modulating mRNA stability via the interaction of cis elements with trans-acting RNA-binding proteins (RBPs) (reviewed in [22]). The rat α ENaC 3'UTR is 900 nt in length (NM_031548) and represents about one third of the transcript, which is significantly longer than the mean 3'UTR length in rat (500 nt) [23]. In the present work, we investigated the hypothesis that the rat α ENaC 3'UTR could play a role in modulating the stability of the transcript. To study this question, we tested the effect of sequential deletions of the α ENaC 3'UTR on the half-life of the transcript.

The data in the present study show that the half-life of α ENaC mRNA is much shorter than what has been previously reported. Furthermore, we found that the stability of α ENaC mRNA is the result of a complex interplay between 3'UTR sequences that either stabilize or frequently destabilize the transcript. Finally, we show that α ENaC 3'UTR is able to bind several RBPs, and that two of those RBPs, Dhx36 and Tial1, are involved in decreased α ENaC stability.

Materials and Methods

Materials

Minimum essential medium (MEM) was purchased from Life Technologies (Burlington, Ont.). Fetal bovine serum (FBS) was acquired from WISENT Inc (Saint-Bruno, Qc). Porcine pancreatic elastase was obtained from Worthington Biochemical (Lakewood, NJ). Doxycycline hyclate and Actinomycin D were procured from Sigma (St. Louis, MO). Primary antibody against hnRNPK was from Cell Signaling Technology (Beverly, MA) and primary antibodies against Dhx36 and Tial1 were from Abcam (Toronto, ON).

Plasmids

pTet-Off Advanced and pTRE-tight were obtained from Clontech Laboratories Inc. (Mountain View, CA). To construct the pTRE-V5- α ENaC plasmids, the V5- α ENaC ORF + 3'UTR (NM_031548) sequence was generated by PCR-amplification with the forward primer coding for the V5 epitope upstream of the rat α ENaC ORF and a reverse primer at the 3' end of the cDNA. The primers for cloning and generation of the different mutants are shown in Table 1. The amplification product was inserted in the pTRE-tight vector following NheI-ClaI digestion. The different V5- α ENaC 3'UTR deletion mutants were generated using the forward V5- α ENaC primer and reverse primers coding for the polyadenylation site that gradually delete the 3' end of α ENaC 3'UTR (Table 1). For V5- α ENaC 3'UTR deletion mutant 5, the proximal part of α ENaC 3'UTR (379 bp) was deleted by PCR-directed mutagenesis and cloned into the pTRE-tight vector following NheI-ClaI digestion. Cloning of the α ENaC-Luc plasmid, a 2.9-kb BamHI-MscI fragment of the mouse α ENaC promoter cloned upstream of the firefly luciferase (Luc) reporter gene has been described previously [14]. pRL-SV40, a plasmid expressing Renilla reniformis luciferase, was purchased from Promega (Madison, WI), and pcDNA3 was purchased from Life Technologies. The pcDNA3-Dhx36 and pcDNA3-Tial1 expression vectors were generated by PCR amplification from their respective ORFs: nt 38 to 3114 of NM_001107678.1 (Dhx36) and nt 209 to 1387 of NM_001013193.1 (Tial1) and the amplicons cloned at the HindIII and XhoI sites of pcDNA3 vector. For the pcDNA3-hnRNP K expression clone, the hnRNP K ORF (nt 31 to 1419 of NM_057141.1) was PCR-amplified and inserted at the EcoRI and NotI sites of pcDNA3. Each construct was verified by sequencing.

Alveolar epithelial cell isolation and experimental conditions

Alveolar epithelial cells were isolated from male Sprague-Dawley rats (Charles River Canada, St. Constant, Quebec, Canada), as described previously [11, 13], and according to a procedure approved by our institutional animal care committee. Perfused lungs were digested with elastase, and the cells were purified by a differential adherence technique on bacteriological plastic plates coated with rat Immunoglobulin G. The cells were maintained in MEM containing 10% FBS, 0.08 mg/l tobramycin, 0.2% NaHCO₃, 0.01 M HEPES, and 2 mM L-glutamine. They were plated at a 1x10⁶ cells/cm² density in plastic dishes and cultured at 37 °C with 5% CO₂ in a humidified incubator. The medium was supplemented with Septra (3 µg/ml trimethoprim and 17 µg/ml sulfamethoxazole) for the first 3 days. After this time, the medium was replaced, and the cells were cultured without Septra. Cells were seeded in different culture plates according to the experimental protocol.

Quantitative RT-PCR

Total RNA was isolated from a 24 mm well by directly lysing the cells with RiboZol Reagent according to the manufacturer's protocol (Amresco, OH). mRNA expression was estimated by qPCR. One microgram of total RNA was pretreated with RNase free-DNase I (Life Technologies) and reverse-transcribed subsequently to cDNA with iScript Reverse Transcription Supermix (BioRad Life Science, Ont.) according to the manufacturer's protocols. For the qPCR amplification, 5 ng of cDNA were amplified with the forward and reverse primers at 225 nM each with SsoAdvanced Universal SYBR Green Supermix (Biorad Life Science) at a final volume of 10 µL. For α ENaC, forward 5'-CGT CAC TGT CTG CAC CCT TA-3 and reverse 5'-CCT GGC GAG TGT AGG AAG AG-3 primers amplified a 128-bp amplicon between exon 1 and 2 of the rat Scnn1a gene (between nt 830 and 957 of NM_031548). The β -actin signal was used for normalization. Forward 5'-ACC GTG AAA AGA TGA CCC AGA T-3' and reverse 5'-CAC AGC CTG

Table 1. List of primers for cloning and generation of the different deletion mutants

Template	3'UTR	Sens	Sequence
α ENaC	Complete	F	5'-ATCGCAGCTAGCACCATGGGTGGTAAGCCTATCCCTAACCCCTCTC-3'
		R	5'-GCACTAATCGATTTTATTGAGTACCTGCCTACCCGTC-3'
α ENaC	Del 1	R	5'-GCACTAATCGATTTTATTGTTCTGAGGGACAGTGAAAG-3'
α ENaC	Del 2	R	5'-GCACTAATCGATTTTATTAACTAACAAGGGGGCTTTTGGG-3'
α ENaC	Del 3	R	5'-GCACTAATCGATTTTATTGTGTCCTGAAGGCAGTGAGGC-3'
α ENaC	Del 4	R	5'-GCACTAATCGATTTTATTTCAGAGCGCCGCCAGGGCAGAC-3'
α ENaC	Del 5	R	5'-ATCGCAGAATTCTCAGAGCGCCGCCAGGGCAGAC-3'
		F	5'-ATCGCAGAATTCTGATGTCTGCTCCTCTCTCTTG-3'
DHX 36	-	F	5'-ATCGCAAAGCTTACCTGTAGTCTGCTCTCGGACA-3'
		R	5'-GCACTACTCGAGTTTATTGCCAAGTGAGAGGCAGAACT-3'
Tial 1	-	F	5'-ATCGCAAAGCTTAGAGCCGGGTTTCAGTACCTT-3'
		R	5'-GCACTACTCGAGTTTATTCTTCAGAGTGTACAGGAGCC-3'
hnRNP K	-	F	5'-ATCGCAGAATTCTGAGACCGAACAGCCA-3'
		R	5'-GCACTAGCGCGCGCTTATTTTAGAAAACTTCCAGAAT-3'

GAT GGC TAC GT-3 primers amplified a 78-bp amplicon between exon 3 and 4 of *Actb* gene (between nt 421 and 498 of NM_031144.2). For V5- α ENaC, forward 5'- CCT AAC CCT CTC CTC GGT CT-3 and reverse 5'-TTG AAT TGG TTG CCC TTC AT-3 primers amplified a 122-bp amplicon of the V5 epitope to distinguish it from the endogenous α ENaC gene expression. Expression of the tTA-Ad transcript (Forward 5'-GCC TGA CGA CAA GGA AAC TC-3' and reverse 5'-AGT GGG TAT GAT GCC TGT CC-3 primers, 129-bp amplicon) was used for normalization of the transfected pTRE-tight clones. The PCRs were amplified in a StepOne Plus (Life Technologies) for 40 cycles. After a 10 min, 95 °C incubation to activate Taq polymerase, the samples were amplified for 40 cycles with a 10-sec denaturation step at 95 °C, a 15-sec annealing step at 58 °C and a 20-sec elongation step at 72 °C. A high resolution melting curve was generated after the amplification cycles to assess that only amplicon that was amplified. The fold change of mRNA levels was calculated with the comparative Cq method. The expression of a given gene was reported as a percentage compared to untreated cells from the same animal.

Transient transfection of alveolar epithelial cells

Alveolar epithelial cells were transiently transfected as described previously [11]. Briefly, on day 2, the cells were trypsinised, washed with PBS and resuspended in Resuspension Buffer R. For each well, 400 000 cells were mixed with 1 μ g of pTet-Off and 1 μ g of pTRE-V5- α ENaC for half-life assays and with 2 μ g of α ENaC-Luc and 0.4 μ g of pRL-SV40 for luciferase assays. For coexpression experiments with RNA-binding proteins, 1 μ g of pcDNA3-Dhx36, pcDNA3-hnRNPk or pcDNA3-Tial1 was added to the DNA/cells mix. The cells were transfected with the NEON Transfection System (Life Technologies) and cultured without antibiotics in MEM + 10% FBS. On day 4, the cells were rinsed, and fresh medium (MEM + antibiotics) was added. On day 5, the cells were processed according to the respective assay.

Luciferase assay

For the promoter activity, cells were transiently transfected with a 2.9-kb BamHI-MscI fragment of the mouse α ENaC promoter (α ENaC-Luc) cloned upstream of the firefly Luc reporter gene and pRL-SV40 expressing Renilla reniformis luciferase (RL) for normalization of the Luc response. On day 5, firefly and RL assays were undertaken with the Dual-Luciferase Reporter Assay System, as specified by the manufacturer (Promega). Luminometry measurements were undertaken in an EnVision Multilabel reader (PerkinElmer, QC).

mRNA stability

To measure V5- α ENaC mRNA stability, alveolar epithelial cells were transfected with pTRE-V5- α ENaC plasmids and pTet-Off. On day 5, cells were treated with doxycycline (1.0 μ g/mL) for 15 min to 6 h and then washed with ice-cold phosphate-buffered saline (PBS) and harvested with RiboZol reagent according to the manufacturer's protocol. To study the impact of transcription inhibition on V5- α ENaC mRNA stability, cells were pretreated with Actinomycin D (5 μ g/mL) 30 min prior treatment with doxycycline. V5- α ENaC mRNA expression was measured by quantitative RT-PCR and presented as percentage of V5- α ENaC mRNA expression of cells at the starting point (t=0) after normalization with tTA-Ad. The half-lives were measured from the rate constant (K) of the V5- α ENaC mRNA degradation curve using the following equation: $t_{1/2} = \ln 2/K$.

Cell lysis and protein isolation

The cells were washed twice in ice-cold PBS, lysed in buffer A (50 mM Tris-HCl pH 7.4, 150 mM KCl, 0.1 mM EDTA, 1% (w/v) NP-40, 4 mM DTT, 20 mM NaF, 100 nM PMSF) supplemented with protease inhibitor and phosphatase inhibitor cocktails (Sigma) and incubated on ice for 15 min under agitation. The lysates were centrifuged for 10 min at 13 000 g and the supernatants removed and used immediately or frozen in liquid nitrogen and stored at -80 °C. Protein concentration of the supernatants was evaluated with the Bradford method (BioRad Laboratories Inc., Mississauga, ON).

RNA affinity chromatography

To isolate RNA-binding proteins, *in vitro* transcripts corresponding to the 3'UTR of α ENaC mRNA or an irrelevant RNA (negative strand of rat β -actin ORF, NM_031144.3 from nt 760 to nt 413 [348 nt]) for negative control were synthesized using the AmpliScribe T7-Flash Biotin-RNA Transcription kit from Epicenter Technologies Corp. (Chicago, IL) and bound to RNase-free streptavidin-coated magnetic beads (Life technologies) according to the manufacturer's instructions. Similar amounts of biotinylated RNAs were bound to the magnetic beads as measured by absorbance of the nonbound fraction after bead preparation. Lysate (1 mg protein) from alveolar epithelial cells were incubated for 1 h at 4 °C with *in vitro* labeled transcript (10 μ g) in binding solution consisting of equal volumes of buffer A and buffer B (50 mM KCl, 10 mM MgCl₂, 10% glycerol, 2 μ g/ μ L heparin, 2 μ g/mL tRNA, 50 U/mL RNaseOUT (Life Technologies)). The beads were washed five times in buffer C (25 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 5% glycerol), and the RNA-binding proteins were eluted directly in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 0.2% bromophenol blue, 10% glycerol, and 7.7% β -mercaptoethanol) and subjected to SDS-PAGE for silver staining or immunoblotting. After silver staining, bands for the putative RNA-binding proteins were excised for tryptic digestion and analyzed by mass spectrometry.

Protein digestion with trypsin

The in-gel digestion protocol is based on the results obtained by Havlis et al. [24]. Gel pieces were first washed with water for 5 min and destained twice with the destaining buffer (100 mM sodium thiosulfate, 30 mM potassium ferricyanide) for 15 min. An extra wash of 5 min was performed after destaining with a buffer of ammonium bicarbonate (50 mM). Gel pieces were then dehydrated with acetonitrile. Proteins were reduced by adding the reduction buffer (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 40 °C and then alkylated by adding the alkylation buffer (55 mM iodoacetamide, 100 mM ammonium bicarbonate) for 20 min at 40 °C. Gel pieces were dehydrated and washed at 40 °C by adding ACN for 5 min before discarding all reagents. Gel pieces were dried for 5 min at 40 °C and then rehydrated at 4 °C for 40 min with the trypsin solution (6 ng/ μ L of trypsin sequencing grade from Promega, 25 mM ammonium bicarbonate). Protein digestion was performed at 58 °C for 1 h and stopped with 15 μ L of 1% formic acid/2% acetonitrile. The supernatant was transferred into a 96-well plate and peptide extraction was performed with two 30-min extraction steps at room temperature using the extraction buffer (1% formic acid/50% ACN). All peptide extracts were pooled in a 96-well plate and subsequently dried in a vacuum centrifuge. The plate was sealed and stored at 20 °C until LC-MS/MS analysis.

LC-MS/MS analysis

The LC column was a PicoFrit fused silica capillary column (15 cm x 75 μ m i.d; New Objective, Woburn, MA) self-packed with C-18 reverse-phase material (Jupiter 5 μ m particles, 300 Å pore size; Phenomenex, Torrance, CA) using a high-pressure packing cell. This column was installed on the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to a Q Exactive (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray Flex ion source. The buffers used for chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid (buffer B). Peptides were loaded on-column and eluted with a 2-slope gradient at a flowrate of 600 nL/min. Solvent B first increased from 2 to 40% in 15 min and then from 40 to 80% B in 5 min. LC-MS/MS data were acquired using a data-dependent top12 method combined with a dynamic exclusion window of 5 sec. The mass resolution for full MS scan was set to 70,000 (at m/z 400), and lock masses were used to improve mass accuracy. The mass range was from 360 to 2000 m/z for MS scanning with a target value of 1×10^6 and a maximum ion fill time (IT) of 100 ms. The data-dependent MS2 scan events were acquired at a resolution of 17,500 with a maximum ion fill time of 50 ms, a target value of 1×10^5 , an intensity threshold of 1×10^4 and an underfill ratio of 0.5%. The normalized collision energy used was 27, and the capillary temperature was 250 °C. Nanospray and S-lens voltages were set to 1.3-1.7 kV and 50 V, respectively.

Protein identification

Protein database searches were performed using Mascot 2.3 (Matrix Science) against the NCBI rat database (version 20120718). The mass tolerance for precursor ions was set to 10 ppm and to 0.5 Da for fragment ions. The enzyme specified was trypsin, and two missed cleavages were allowed. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation was a variable modification.

Immunoblotting

RNA-binding protein eluates were subjected to SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were blocked with 5% w/v bovine serum albumin (BSA) in Tris-buffered saline at a pH of 7.4 with 0.05% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibody (anti-Dhx36, anti-hnRNP or anti-Tial1) in TBST plus 5% BSA. After washing with TBST, the membranes were incubated with goat anti-rabbit (Santa Cruz Biotechnologies, Santa Cruz, CA) IgG linked to horseradish peroxidase for 1 h. After TBST washes, the membranes were incubated with an Immun-Star WesternC Chemiluminescence Kit (BioRad Laboratories Inc.) before the luminescent signals were recorded with a ChemiDoc XRS+ system (BioRad Laboratories Inc.).

siRNA inhibition of Dhx36 and Tial1

siRNA against Dhx36 (SR512228A; OriGene, Rockville, MD, USA) and Tial1 (SR512213A; OriGene, Rockville, MD, USA) were diluted at a 500 nM concentration in buffer R and the cells transfected by electroporation with the NEON system as described above. After 72 h, total RNA was extracted as described above, and the levels of α ENaC mRNA were estimated by RT-qPCR. After normalization of the signal with HPRT, the expression of α ENaC mRNA was expressed as a ratio to cells transfected with nonspecific siRNA, siCtrl (SR30004, OriGene, Rockville, MD, USA).

Statistical analysis

All data are presented as the mean \pm SEM. Groups were compared by the Mann-Whitney U-test, one-phase decay nonlinear regression, one-sample t-test, or Kruskal-Wallis test with post-hoc Dunn's test with GraphPad Prism 5 software (GraphPad software Inc., San Diego, CA). $P < 0.05$ was considered significant. Two-sided P values and statistical tests are reported for each experiment.

Results

Actinomycin D stabilizes α ENaC mRNA

The half-life of α ENaC mRNA reported when transcription was inhibited with Actinomycin D ranged from 8 to 22 h (Table 2). Since Actinomycin D affects the transcription of many genes [25-27], we suspected that it could inhibit the expression of factors involved in α ENaC mRNA degradation. For this reason, we developed a Tet-Off model to assess α ENaC mRNA half-life without affecting the transcription of the whole genome. α ENaC, with its complete 3'UTR, was cloned in pTRE-tight plasmid bearing a V5 epitope upstream of its

Table 2. Estimation of α ENaC mRNA half-life in different studies

Author	Method	Cell type	Half-life (hrs)
Current study	Tet-Off Dox 1 μ g/mL	Alveolar epithelial cells	~ 1.5
Zentner, MD [16]	Act D 5 μ g/mL	Rat parotid cells	8
Otulakowski, G [17]	Act D 5 μ g/mL	Fetal distal lung epithelial cells	22
Mick, VE [18]	Act D 1 μ M	MDCK-C7 cells(kidney)	14
Itani, OA [19]	Act D 1 μ M	MDCK-C7 cells(kidney)	9
Dagenais, A [10]	Act D 5 μ g/mL	Alveolar epithelial cells	15
Mustafa, SB [20]	Act D 1 μ M	SMG-C6 cells(submandibular gland)	17

Fig. 1. Degradation kinetics of V5- α ENaC mRNA with a Tet-Off system in the presence and absence of Actinomycin D. Alveolar epithelial cells were transiently cotransfected with the pTet-Off plasmid and the pTRE-tight plasmid coding for α ENaC cDNA bearing a V5 epitope upstream of its open reading frame and complete 3'UTR sequences. The cells pretreated or not with Actinomycin D (5.0 μ g/mL) for 30 min were incubated thereafter with doxycycline (1.0 μ g/mL) from 15 min to 6 h. Expression of V5- α ENaC mRNA was measured by quantitative RT-PCR and presented as percentage \pm SEM of V5- α ENaC mRNA expression of untreated cells (t=0) after normalization with tTA-Ad. V5- α ENaC mRNA half-life was estimated by one-phase decay nonlinear regression for each cell preparation. Multiple regression analysis revealed a statistically significant difference in V5- α ENaC mRNA stability in cells treated with Actinomycin D compared to untreated cells ($P < 0.0001$). Cells from at least four different rats ($n \geq 4$) were used for each experimental condition.

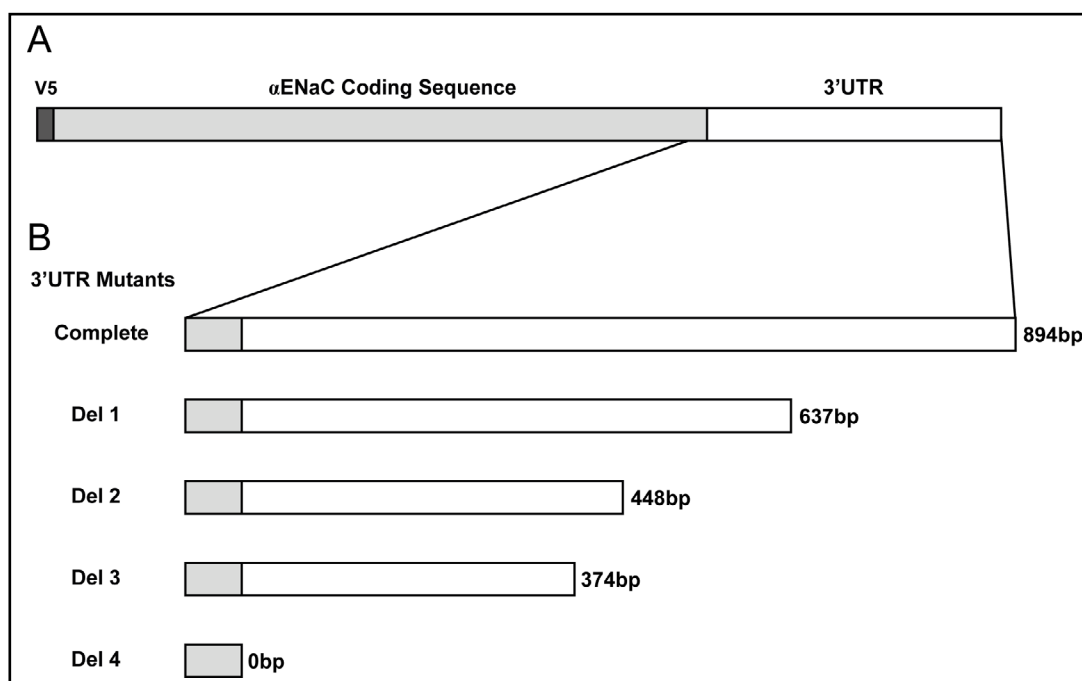
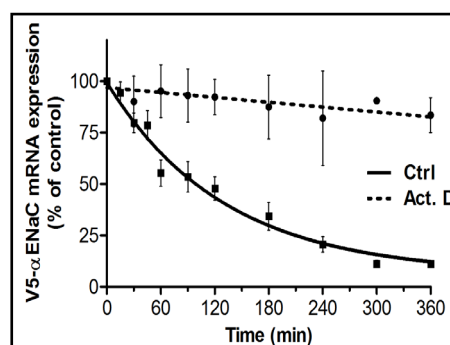


Fig. 2. Map of the α ENaC 3'UTR deletion mutants. (A) Schematic map of the V5- α ENaC transcript with the complete 3'UTR inserted in the pTRE-tight expression vector. The open reading frame is depicted as a gray box while the 3'UTR is shown as a white box. (B) The 3'UTR portion of α ENaC mRNA is depicted for the clone bearing a complete 3'UTR and for the different deletion mutants (Del 1 to Del 4).

open reading frame to allow specific RT-qPCR amplification of the V5- α ENaC transcript. The construct was cotransfected with the pTet-Off plasmid in alveolar epithelial cells that were either pretreated or not with Actinomycin D (5 μ g/mL) for 30 min. The degradation kinetics of the V5- α ENaC mRNA were determined by RT-qPCR of RNA isolated at different time points following inhibition of pTRE-tight clone transcription with doxycycline (1 μ g/mL). The half-life of V5- α ENaC mRNA was estimated to be \sim 99 min with the Tet-Off system. In contrast, Actinomycin D significantly stabilized the α ENaC transcript compared to controls (Fig. 1), increasing its half-life to an estimated 21 h. These results are consistent with an effect of Actinomycin D on the expression of additional mRNA modulating factors that modulate α ENaC mRNA stability.

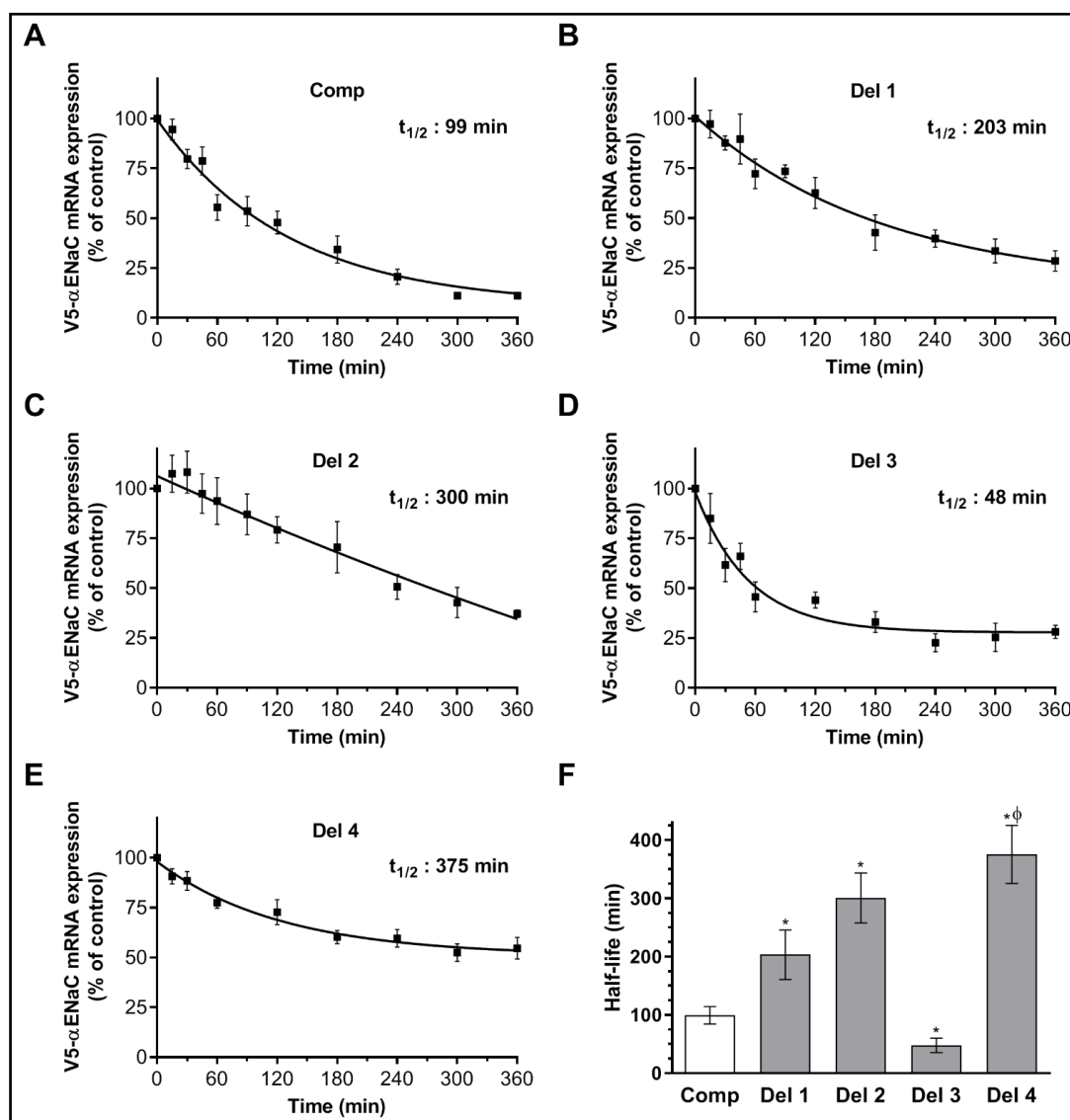


Fig. 3. Role of different regions of the α ENaC 3'UTR in the modulation of mRNA stability. Alveolar epithelial cells were transiently cotransfected with the pTRE-tight plasmid coding for V5- α ENaC mRNA with the different α ENaC 3'UTR deletion mutants along with pTet-Off plasmid that express tTA-Ad that allows the specific expression of the construct and its inhibition by doxycycline. Seventy-two h after transfection, cells were treated with doxycycline (1.0 μ g/mL) from 15 min to 6 h. Expression of V5- α ENaC mRNA was measured by quantitative RT-PCR and presented as percentage \pm SEM of V5- α ENaC mRNA expression of untreated cells ($t=0$) after normalization with tTA-Ad. V5- α ENaC mRNA half-life for each construct was estimated by one-phase decay nonlinear regression. The V5- α ENaC mRNA decay is shown for the clone with (A) complete 3'UTR (Comp) and (B-E) for the Del 1 to Del 4 3'UTR deletion mutants. (F) The half-life ($t_{1/2}$) of mRNA decay is given for each clone. In the lower right quadrant, the graph shows a comparison of the different $t_{1/2} \pm$ SEM estimated for each clone. $P < 0.05$ by Kruskal-Wallis tests between the different groups. * $P < 0.05$ by Mann-Whitney U-tests compared to complete 3'UTR. $\Phi P < 0.05$ by Mann-Whitney U-tests compared to Del 1. Cells from at least five different animals ($n \geq 5$) were used for each experimental condition.

Sequences present in the 3'UTR of α ENaC mRNA modulate transcript stability

The rat α ENaC 3'UTR is almost 30% of the total transcript length, suggesting that cis-elements important to the modulation of α ENaC mRNA stability could be present. To study the role of α ENaC 3'UTR on transcript stability, 3'UTR deletion mutants were designed to remove progressively different fragments of the 3'UTR (Fig. 2). The mRNA degradation kinetics of the different 3'UTR V5- α ENaC mutants were tested as described above. All the deletion mutants led to significant changes in V5- α ENaC mRNA stability compared to the control. Deletion mutants 1, 2 and 4 showed a significant stabilization of V5- α ENaC mRNA, with half-lives of 203, 300 and 375 min, respectively. In contrast, the half-life for mutant 3 decreased significantly to 47 min (Fig. 3).

The α ENaC 3'UTR binds Dhx36, hnRNPK and Tial1

RNA-binding proteins (RBPs) from alveolar epithelial cell extracts were purified by RNA chromatography using the α ENaC 3'UTR as an affinity matrix. The affinity-purified proteins were subsequently separated by SDS-PAGE, silver-stained and extracted for protein identification by LC-MS/MS analysis. RNA affinity chromatography using the α ENaC 3'UTR specifically enriched four bands with approximate molecular weights of 100, 68, 59 and 40 kDa corresponding to Dhx36, hnRNPK, Pabp, and Tial1, respectively (Fig. 4A). Since Pabp

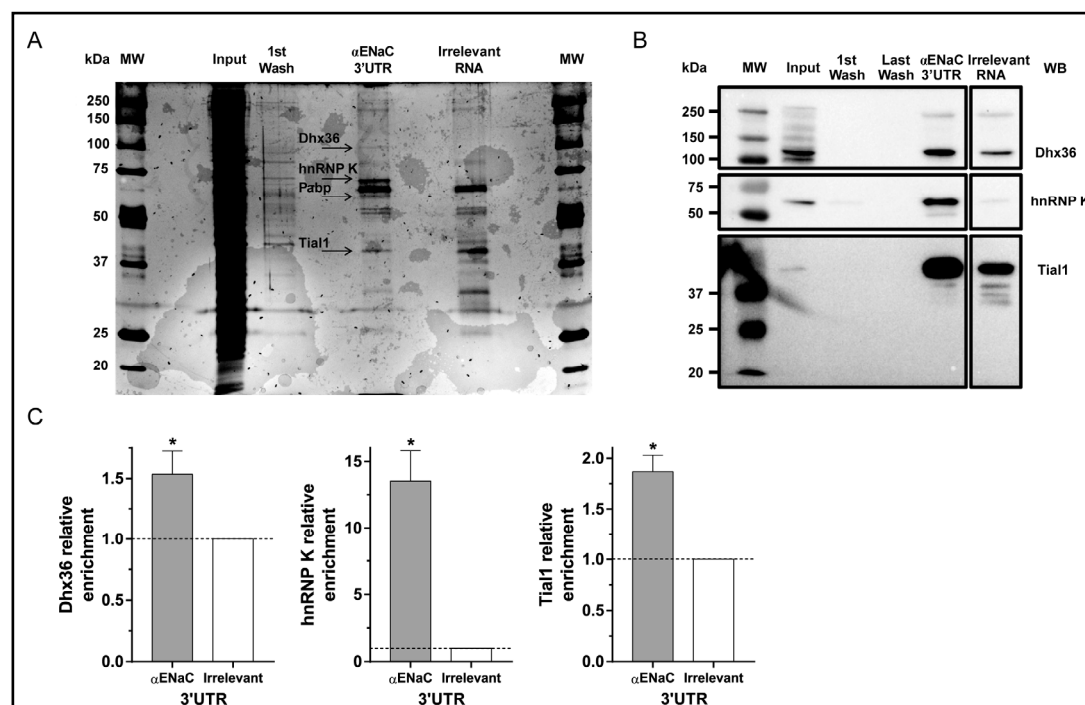
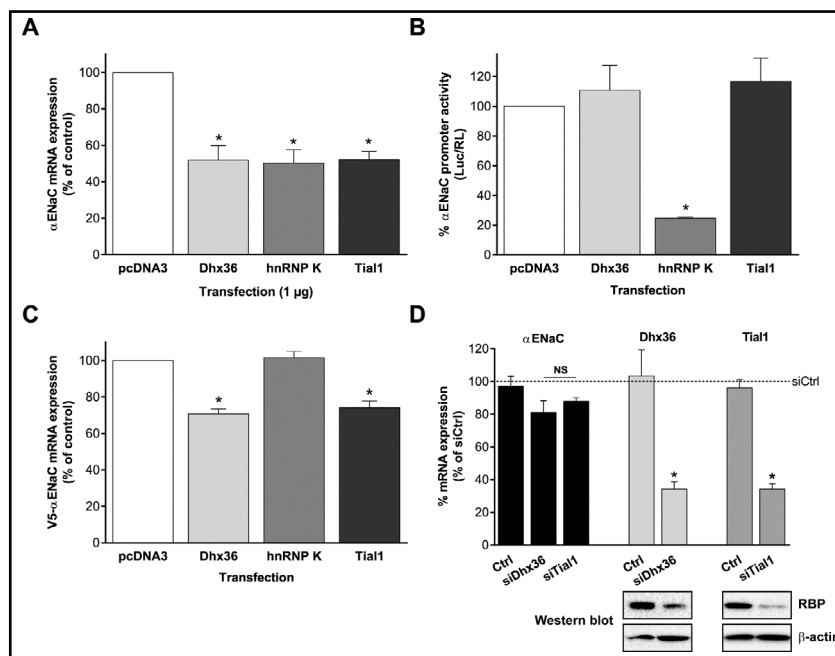


Fig. 4. Identification of proteins bound to the α ENaC 3'UTR by RNA affinity chromatography. RNA-binding proteins (RBP) present in extracts of alveolar epithelial cells were purified by affinity chromatography using in vitro transcribed α ENaC 3'UTR. Nonspecific RNA binding was tested with an irrelevant RNA (Irr. RNA) consisting of the negative strand of β -actin ORF. (A) The affinity-purified RBPs were separated by SDS-PAGE, and silver-stained bands were subjected to LC-MS/MS for protein identification. A representative silver-stain gel from three experiments from different rats is shown. (B) The RBPs identified by mass spectrometry were confirmed using immunoblotting. The affinity-purified RBPs were separated by SDS-PAGE followed by immunoblotting against Dhx36, hnRNPK or Tial1, showing enrichment of these proteins with α ENaC 3'UTR compared to irrelevant RNA. Representative immunoblots for each RBP are presented (original blot shown in Fig. S5). (C) The enrichment of each RBP determined by immunoblot analysis is depicted for each RBP. The data are expressed as relative enrichment \pm SEM compared to irrelevant RNA. * $P < 0.05$ by one-sample t-test compared to irrelevant RNA ($n = 4$ for each RBP).

is a protein known to interact with the polyadenylated tail of mRNA [28], we decided to focus our work on the three other proteins. To confirm the identity and interaction of these proteins with the α ENaC 3'UTR, the proteins enriched from RNA affinity chromatography were separated on denaturing gels and subjected to immunoblotting. The results show that Dhx36, hnRNP K and Tial1 were enriched by affinity chromatography using the α ENaC 3'UTR compared to a nonspecific RNA sequence (Fig. 4B).

Fig. 5. Role of Dhx36, hnRNP K or Tial1 in the modulation of α ENaC mRNA expression in alveolar epithelial cells. (A) Alveolar epithelial cells were transfected with expression vector encoding for Dhx36, hnRNP K or Tial1 RBPs. α ENaC mRNA expression was quantified by RT-qPCR 72 h posttransfection and expressed as percentage \pm SEM compared to cells transfected with an empty vector (pcDNA3) after normalization with β -actin. α ENaC mRNA



expression was significantly decreased by each RBP. * $P < 0.05$ by one-sample t-tests compared to 100; $n = 6$ for each experimental condition. (B) Alveolar epithelial cells were transfected with α ENaC-Luc construct where a 3-kb portion of the α ENaC promoter drives the expression of the Luc reporter gene in cells that were cotransfected with an expression vector coding for Dhx36, hnRNP K or Tial1 RBPs and the pRL-SV-40 coding for Renilla reniformis luciferase (RL) for normalization of the LUC signal. Luc and Renilla signals were measured 72 h posttransfection by Dual-Luciferase Reporter Assay (Promega Corporation) in an EnVision Multilabel reader. α ENaC promoter activity was expressed as percentages \pm SEM of Luc activity compared to cells transfected with an empty vector (pcDNA3) after normalization with RL. Overexpression of hnRNP K significantly inhibited α ENaC promoter activity, whereas overexpression of Dhx36 and Tial1 had no effect. * $P < 0.05$ by Kruskal-Wallis test and Dunn's post-hoc test compared to empty vector; $n \geq 3$ from different animals were tested in duplicate for each experimental condition. (C) Alveolar epithelial cells were cotransfected with the pTRE-tight plasmid encoding V5- α ENaC mRNA along with an expression vector for Dhx36, hnRNP K or Tial1 RBPs and the pTet-Off plasmid. V5- α ENaC mRNA expression was quantified by RT-qPCR 72 h posttransfection and expressed as percentage \pm SEM of V5- α ENaC mRNA compared to cells transfected with an empty vector (pcDNA3) after normalization with tTA-Ad. Overexpression of Dhx36 and Tial1 significantly inhibited V5- α ENaC mRNA expression, whereas overexpression of hnRNP K had no effect. * $P < 0.05$ by the Kruskal-Wallis tests and Dunn's post-hoc test compared to empty vector; $n \geq 3$ from different animals were tested in duplicate for each experimental condition. (D) Endogenous α ENaC mRNA expression estimated by RT-qPCR 72 h after transfection by electroporation of alveolar epithelial cells with 500 nM siRNA against Dhx36 or Tial1. α ENaC, Dhx36 and Tial1 mRNA expression \pm SEM were normalized with the expression level of alveolar epithelial cells from the same series transfected with a control siRNA (siCtrl). Inhibition of Dhx36 or Tial1 had no effect on α ENaC mRNA expression. * $P < 0.05$ by Mann-Whitney U-tests between Ctrl and siRNA treated cells; $n \geq 3$ for each experimental condition. Representative immunoblots of Dhx36 and Tial1 knockdown are presented.

Overexpression of Dhx36, hnRNPK and Tial1 decreased α ENaC mRNA expression

To study the impact of Dhx36, hnRNPK and Tial1 in the modulation of α ENaC mRNA level, the cDNAs for these proteins were cloned into the pcDNA3 vector and transfected in alveolar epithelial cells. Overexpression of Dhx36, hnRNPK or Tial1 (Fig. S1 - for all supplemental material see www.cellphysiolbiochem.com) inhibited endogenous α ENaC mRNA expression by 50% compared to cells cotransfected with the pcDNA3 control vector (Fig. 5A).

Dhx36 and Tial1 downregulate α ENaC mRNA expression by a posttranscriptional mechanism.

In addition to their role in posttranscriptional modulation, Dhx36 [29, 30], hnRNPK [31] and Tial1 [32, 33] have also been reported to modulate gene transcription and transcript splicing. For this reason, the impact of overexpression of these RBPs on α ENaC promoter activity was tested by measuring the expression of a transfected plasmid in which a 2.9-kb murine α ENaC promoter drives the expression of a luciferase (Luc) reporter gene [10-12, 14]. While Dhx36 and Tial1 overexpression had no impact on α ENaC promoter activity, hnRNPK decreased Luc expression to 25% of control values (Fig. 5B).

The effect of Dhx36, Tial1 and hnRNPK overexpression on α ENaC mRNA stability was studied using the Tet-Off system. Alveolar epithelial cells were cotransfected with the pTRE-tight plasmid (V5- α ENaC-3'UTR-comp), pTet-Off and a pcDNA3 plasmid expressing Dhx36, hnRNPK or Tial1. Both Dhx36 and Tial1 significantly downregulated V5- α ENaC mRNA

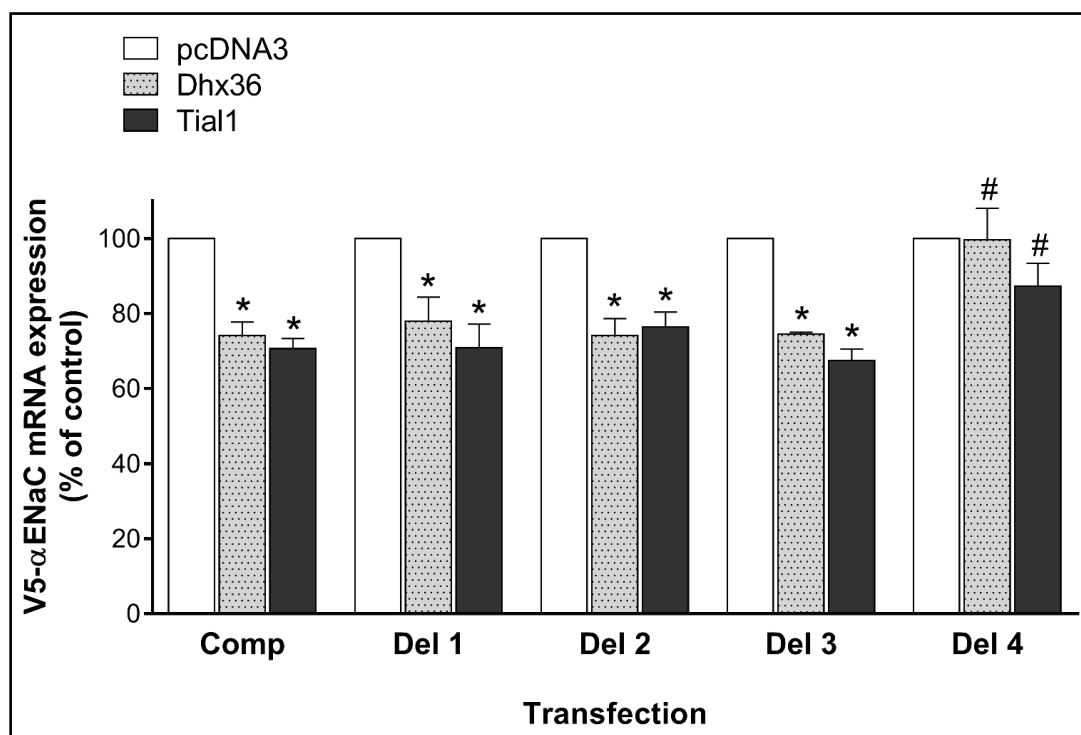


Fig. 6. Posttranscriptional modulation of V5- α ENaC deletion mutants mRNA in cells that overexpress Dhx36 and Tial1. Alveolar epithelial cells were cotransfected with the different 3'UTR deletion mutants (Del 1-4) in the pTRE-tight vector along with pTet-Off plasmid and the expression vector for Dhx36 or Tial1 RBP overexpression. V5- α ENaC mRNA expression was quantified by RT-qPCR 72 h posttransfection and expressed as percentage of V5- α ENaC mRNA compared to cells transfected with an empty vector (pcDNA3) \pm SEM after normalization with tTA-Ad. Comparison to the complete 3'UTR is shown in Fig. S6. Overexpression of Dhx36 and Tial1 significantly inhibited V5- α ENaC mRNA expression for each construction except for V5- α ENaC-Del4, which lacks the 3'UTR sequences. * $P < 0.05$ by the Kruskal-Wallis tests and Dunn's post-hoc tests compared to empty vector; # $P < 0.05$ by the Mann-Whitney U-tests compared to complete 3'UTR mutant; $n \geq 6$ for each experimental condition.

expression to 71% and 74%, respectively, compared to cells transfected with an empty vector, while hnRNPK overexpression had no effect on V5- α ENaC mRNA stability (Fig. 5C). siRNA inhibition of Dhx36 or Tial1 had no significant impact on α ENaC mRNA expression (Fig. 5D).

Dhx36 and Tial1 downregulate α ENaC mRNA expression via the proximal region of its 3'UTR

The impact of Dhx36 and Tial1 overexpression was tested in alveolar epithelial cells transfected with the α ENaC 3'UTR deletion mutants tested previously. The progressive deletion of the 3'UTR did not alleviate the downregulation of V5- α ENaC mRNA expression induced by Dhx36 and Tial1. In contrast, the construct with no α ENaC 3'UTR (deletion 4) significantly blocked the impact of these RBPs on α ENaC mRNA (Fig. 6).

The data in Fig. 6 suggest that the target of Dhx36 and Tial1 could exist in the proximal region of 3'UTR. The contribution of this region in the downregulation of α ENaC mRNA by Dhx36 and Tial1 was tested by deleting the proximal portion of the α ENaC 3'UTR (Fig. 7A). Deletion of the proximal region of α ENaC 3'UTR in deletion 5 abrogated the effect of Dhx36 and Tial1 on V5- α ENaC mRNA expression compared to V5- α ENaC-Comp (Fig. 7B). By RNA affinity chromatography, there was a loss of Dhx36 enrichment and a decrease in Tial1 binding to α ENaC 3'UTR-Del5 compared to the complete α ENaC 3'UTR (Fig. 7C).

Discussion

We reported previously that several stress conditions affecting alveolar epithelial cells downregulate α ENaC mRNA expression by affecting posttranscriptional mRNA stability [10, 11]. In the present study, we evaluated how different portions of α ENaC 3'UTR are important for modulation of transcript stability in alveolar epithelial cells. We found that α ENaC mRNA stability is a complex process that depends on multiple regions encoded in the 3'UTR that can stabilize or destabilize the transcript. Moreover, we found that Dhx36 and Tial1, two RBPs that interact with the 3'UTR, decrease α ENaC mRNA stability.

Using a Tet-Off transcriptionally controlled plasmid expression system, we estimated the half-life of the α ENaC transcript to be 99 min (Fig. 1). The results presented here show unambiguously that Actinomycin D leads to an overestimation of α ENaC mRNA half-life. Indeed, in the presence of Actinomycin D, the half-life estimated with Tet-Off increased from 99 min to 21 h (Fig. 1). For many years, Actinomycin D has been a gold standard in the determination of transcript stability. Our data with the Tet-Off system in the presence of Actinomycin D are consistent with the long half-life reported for the α ENaC transcript in the literature (Table 2). These results suggest that Actinomycin D could alter α ENaC mRNA stability by inhibiting the expression of mRNA modulating factors involved in transcript destabilization. Similar effects of Actinomycin D on mRNA stability have been reported for the MALAT1 mRNA [25], c-fos mRNA [26] and β 4-galactosyltransferase-1 mRNA [27]. However, we cannot exclude the possibility that the modulation of α ENaC mRNA stability by Actinomycin D is via factors that act outside the 3'UTR. The results presented here show that, although Actinomycin D is a standard procedure for estimating the stability of many transcripts, the half-life reported for α ENaC mRNA is overestimated with this technique. The results reported here show that the half-life of α ENaC mRNA is much shorter than what was previously thought. These data open new perspectives on how modulation of α ENaC transcript stability could play a significant role in physiological and pathophysiological modulations of α ENaC expression in alveolar epithelial cells. The Tet-Off system that we developed would be an appropriate tool for evaluating the actual half-life of the transcript and the mechanisms involved in its modulation.

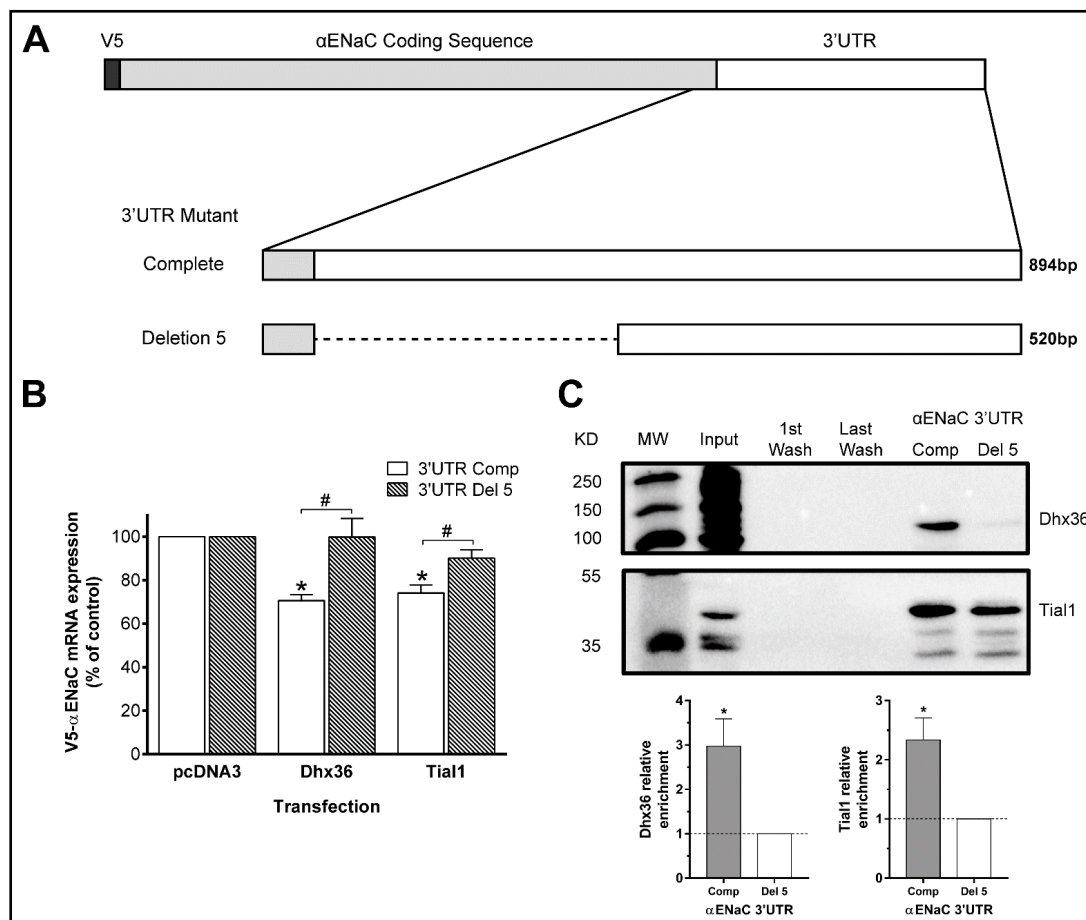


Fig. 7. Impact of the proximal region of the α ENaC 3'UTR on the posttranscriptional modulation of V5- α ENaC mRNA in cells that overexpress Dhx36 and Tial1. (A) The proximal portion of the α ENaC 3'UTR was deleted by cloning the distal region of the 3'UTR next to the α ENaC stop codon in the pTRE-tight plasmid (V5- α ENaC-Del5). (B) Alveolar epithelial cells were cotransfected with V5- α ENaC or V5- α ENaC-Del5 in the pTRE-tight vector along with pTet-Off plasmid and the expression vector for Dhx36 or Tial1 RBP overexpression. V5- α ENaC mRNA expression was quantified by RT-qPCR 72 h posttransfection and expressed as percentage \pm SEM of V5- α ENaC mRNA compared to cells transfected with an empty vector (pcDNA3) after normalization with tTA-Ad. Overexpression of Dhx36 and Tial1 had no effect on V5- α ENaC-Del5 mRNA expression. * P <0.05 by Kruskal-Wallis tests and Dunn's post-hoc tests compared experimental vectors to the empty vector; # P <0.05 by Mann-Whitney U-tests compared experimental vectors to the to complete 3'UTR mutant; $n \geq 6$ for each experimental condition. (C) RNA-binding proteins present in extracts of alveolar epithelial cells were purified by affinity chromatography using in vitro transcribed α ENaC 3'UTR or deletion 5 3'UTR. The affinity-purified RBPs were subjected to SDS-PAGE for immunoblotting detection of Dhx36 and Tial1. Less enrichment of Tial1 and Dhx36 was detected with Del5 α ENaC 3'UTR lacking the proximal region of α ENaC 3'UTR compared to the whole sequence. Representative immunoblots for each RBP are presented. (C) The enrichment of Dhx36 and Tial1 determined by immunoblot analysis is depicted for each RBP. The data are expressed as relative enrichment \pm SEM compared to Del 5. * P <0.05 by one-sample t-test compared to Del 5 ($n=4$ for each RBP).

3'UTRs are known to contain cis-elements that are typically regulatory sequences that can control the stability of an mRNA or its translation [34]. A series of mutants were designed to investigate the role of the α ENaC 3'UTR in the modulation of transcript stability by progressively deleting portions of the 3'UTR (Fig. 2). The relative expression levels of the various mutants were assessed to ensure similar mRNA expression between the constructs (Fig. S2). The half-life of the α ENaC mRNA with 3'UTR deletions 1, 2, and 4 increased

significantly compared to the complete 3'UTR, suggesting that cis-elements that destabilize the α ENaC transcript had been removed. Conversely, the deletion 3 mutant presented a drastic drop in mRNA half-life, suggesting that interplay with different portions of 3'UTR could also confer stability to the transcript. Overall, the main role of the 3'UTR sequences is to destabilize the α ENaC transcript, since removal of the complete 3'UTR sequences in Del 4 led to stabilization of the transcript (Fig. 3). These results suggest that elements in the 3'UTR portion of the transcript contribute to α ENaC expression in alveolar epithelial cells.

The nature of the transcript factors that could bind to α ENaC 3'UTR in alveolar epithelial cells and modulate transcript stability was studied. Since no conserved target sequences for miRNAs could be detected in α ENaC 3'UTR using the target-prediction tool TargetScan [35], we investigated the nature of the proteins that could bind to this sequence. The whole 3'UTR region was used as a matrix to investigate the nature of the proteins that could bind this sequence. Four RBPs, Dhx36, Pabp1, hnRNPK and Tial1 (Fig. 4), were enriched specifically from alveolar epithelial cell protein lysate by affinity-chromatography on a α ENaC 3'UTR RNA sequence compared to an irrelevant RNA. Overexpression of Dhx36, hnRNP K and Tial1 in alveolar epithelial cells all triggered a downregulation of α ENaC mRNA expression (Fig. 5A).

hnRNPK inhibited α ENaC mRNA expression most likely by downregulating promoter activity (Fig. 5B), while Dhx36 and Tial1 modulated the stability of the transcript (Fig. 5C). Although hnRNPK is mainly known to be an RNA-binding protein, it can also bind DNA and directly modulate the transcriptional activity of genes such as CD43 [31] and Cox-2 [36]. Transcriptional regulation by hnRNPK comes from the association of the factors with pyrimidine-rich sequences (CT-rich) [37-39]. Such CT-rich motifs, similar to hnRNPK DNA binding sites, can be found in the α ENaC promoter. The impact of hnRNPK overexpression on α ENaC promoter activity raises several questions, since in the present work, hnRNPK was identified through its enrichment with α ENaC 3'UTR. Although in the present report the binding of hnRNPK to α ENaC mRNA is not associated with modulation of transcript stability (Fig. 5C), it does not exclude other functions. The binding of hnRNPK to 3'UTRs has been shown to modulate translation [40, 41] and two-way RNA shuttling between the nucleus and cytoplasm [42]. How the binding of hnRNPK to the α ENaC 3'UTR affects the posttranscriptional regulation of the transcript remains to be determined.

The 3'UTR deletion mutants were used to screen for elements in this region that are important for α ENaC mRNA modulation by Dhx36 and Tial1. The deletion mutants 1-3, as for the complete 3'UTR, showed a decrease in the expression of the mRNA following Dhx36 or Tial1 overexpression. Since deletion mutant 4 abrogates the effect of Dhx36 and Tial1, it suggests that the proximal region of α ENaC 3'UTR is essential for their effects (Fig. 6). Therefore, deletion mutant 5 was created where this region was removed. Fig. 7 shows that overexpression of Dhx36 and Tial1 no longer affected the expression of α ENaC mRNA. Furthermore, immunodetection of the protein bound to α ENaC 3'UTR shows that the binding of Dhx36 is almost abolished in deletion 5 mutant, which removes the 5' part of 3'UTR (Fig. 7). Dhx36 is an RNA helicase that is an important regulator of gene expression that can reshape the RNA secondary structure and affect RNA-protein interactions that modulate mRNA stability [43]. The Dhx36 action on RNA is via recognition of G-quadruplexes (G4) corresponding to four-strand secondary structures. Interestingly, the proximal region has sequences associated with G-quadruplex formation identified with QGRS Mapper [44], by which Dhx36 could bind and modulate mRNA stability (Fig. S3) [45, 46]. However, we cannot exclude the involvement of other sequences that do not bear the G-quadruplex for Dhx36 binding [47]. Therefore, the contribution of these G4 to α ENaC mRNA stability should be tested further in the future.

Our results also suggest that Tial1 modulates α ENaC mRNA stability as seen with iNOS mRNA [48]. However, the main role of Tial1 is to modulate translation [49-51], a process that is linked to stress granule formation triggered by UVC irradiation and arsenite [52]. Unlike Dhx36, RNA-Tial1 interaction was not lost with mutant deletion 5 despite a loss of effect on mRNA stability, suggesting that Tial1 can bind other sequences on the α ENaC

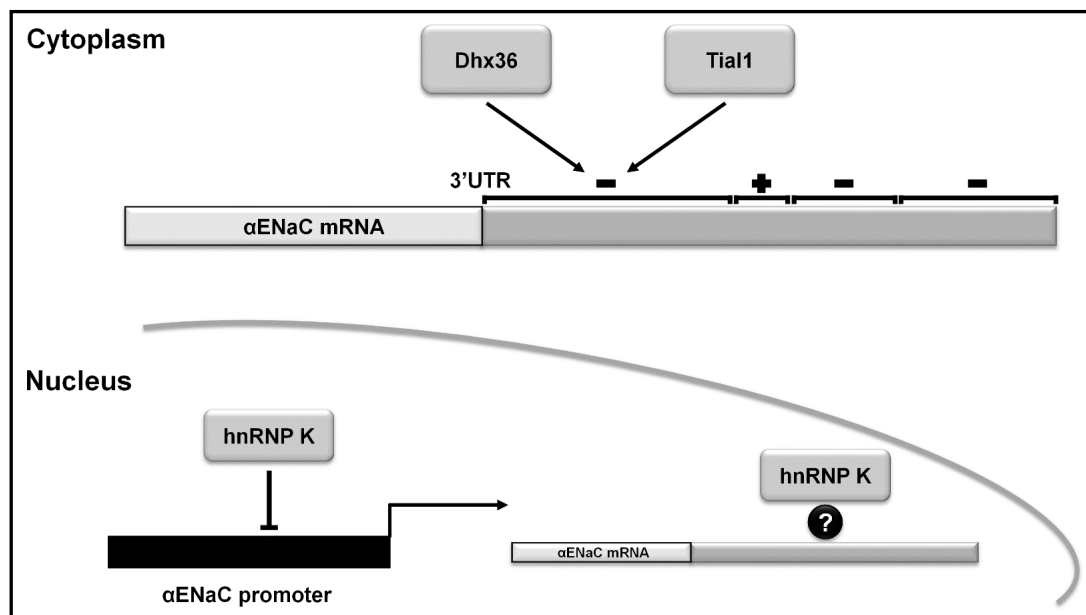


Fig. 8. Proposed mechanisms involving RNA-binding proteins in the regulation of the α ENaC mRNA expression. The α ENaC 3'UTR plays an important role in the modulation of α ENaC mRNA stability through a complex stabilizing and destabilizing interplay between different regions of the 3'UTR. The α ENaC 3'UTR can bind several RNA-binding proteins that modulate α ENaC mRNA expression. hnRNP K inhibits α ENaC mRNA expression by downregulating promoter activity. Dhx36 and Tial1 modulate the stability of the transcript through the proximal region of the α ENaC 3'UTR.

3'UTR. However, the proximal region is clearly essential for the modulation of α ENaC mRNA stability by Tial1 as seen with the sequential deletions (Fig. 7). These observations could suggest that the role of Tial1 in the modulation of α ENaC mRNA stability could indirectly involve the recruitment of other proteins. Indeed, Tial1 could recruit the transcript in stress granules [53] and lead to its degradation by protein partners such as Dhx36 [45, 54]. Further studies will clearly be necessary to elucidate the mechanism by which Dhx36 and Tial1 act on α ENaC mRNA.

Silencing Dhx36 and Tial1 did not lead to upregulation of α ENaC mRNA (Fig. 5D). The complex stabilizing/destabilizing pattern found with the different 3'UTR deletion mutants suggests that besides Dhx36 and Tial1, other RBPs could interact with α ENaC 3'UTR to modulate transcript stability. Indeed, the three proteins identified here are most likely not the only proteins that could interact with the α ENaC 3'UTR. Other experiments will be necessary to identify and study the role of other proteins that interact with the α ENaC 3'UTR. Additionally, we cannot completely exclude the possibility that the results obtained by overexpressing these RBPs might be the consequence of competition binding with endogenous regulators.

Our study also has a number of limitations concerning the α ENaC mRNA posttranscriptional modulation. First, the use of the Tet-Off system did not allow us to study endogenous transcript modulation directly. Second, despite the small size (42 nt) of the V5 epitope of the pTRE-tight-V5- α ENaC construct used to distinguish the endogenous α ENaC mRNA, we cannot rule out that it could affect the stability of the transcript. Furthermore, the V5- α ENaC transcript did not bear the α ENaC 5'UTR. Indeed, V5- α ENaC mRNA only includes 3'UTR to specifically study the impact of this sequence on the modulation of α ENaC mRNA stability. Thus, it is possible that the 5'UTR may affect posttranscriptional modulation. However, data in the literature suggest that α ENaC 5'UTR is involved in translational control of α ENaC transcript, but has no impact on the steady state mRNA level [17, 55]. Third, the high transcription rates of the artificial construct may also disrupt systems controlling

stability. This hypothesis is unlikely, since it has been shown that estimation of the half-life of a transcript with the Tet-Off system was comparable to a method involving the pulse-labeling of endogenous mRNA under nondisruptive conditions [25]. This finding suggests that the Tet-Off system is more appropriate than the use of Actinomycin D. The long-term incubation with doxycycline could have generated off-target effects on α ENaC mRNA expression. Nonetheless, treatment with doxycycline (1.0 μ g/mL) over a 24-h period did not cause any change in the expression of the α ENaC transcript in untransfected cells (Fig. S4). This concentration is widely used to completely inhibit the transcription of the Tet-Off system and is recommended for minimal cytotoxic effect [56]. Finally, the scope of this study was to evaluate the contribution of the α ENaC 3'UTR to transcript stability. However, we cannot rule out that the α ENaC mRNA coding region could also contribute to the stability of the transcript. Indeed, RBPs can elicit posttranscriptional gene regulation by interacting with coding region elements [57].

Conclusion

In conclusion, using a Tet-Off model to estimate mRNA stability, we found that the half-life of α ENaC mRNA is much shorter than what has been reported previously using transcription blockers. Our data are the first to show that α ENaC 3'UTR plays a role in the modulation of α ENaC mRNA stability, and we mapped regions involved in this regulation. Our data show that the 3'UTR is mainly involved in destabilizing the transcript. Finally, we show that α ENaC 3'UTR is able to bind several RBPs and that Dhx36 and Tial1 are involved, in part, in the decrease in α ENaC stability. The results presented here indicate that α ENaC mRNA posttranscriptional modulation is complex and could play a role in the functional regulation of ENaC expression in alveolar epithelial cells as presented in Fig. 8. In the future, it would be interesting to evaluate whether the human α ENaC 3'UTR is regulated in a similar way to our model in rats since the length is quite similar and there is a significant homology across species. The Tet-Off model developed here will also be very useful for further studying the posttranscriptional modulation of α ENaC mRNA under different lung pathophysiological conditions known to decrease ENaC function.

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Animal experiments conformed to internationally accepted standards and have been approved by the appropriate institutional review body.

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F.M., F.G., M.P., J.L., A.R., A.D., and Y.B. conceived of and designed the research. F.M., F.G., M.P., J.L. and A.R. performed experiments. F.M., F.G., M.P., J.L. and A.D. analyzed data. F.M., A.D., and Y.B. interpreted the results. F.M. prepared figures. F.M., A.D., and Y.B. drafted the manuscript. F.M., A.D., and Y.B. edited and revised the manuscript. F.M., F.G., M.P., J.L., A.R., A.D., and Y.B. approved the final version of the manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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