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The Hypertonic Environment Differentially Regulates Wild-type CFTR and TNR-CFTR Chloride Channels

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Key Words

CFTR • TNR-CFTR • Sodium chloride • Urea • Sucrose • MDCK cells • Hypertonic environment • Plasma membrane

Abstract

This study tested the hypotheses that the hypertonic environment of the renal medulla regulates the expression of cystic fibrosis transmembrane conductance regulator protein (CFTR) and its natural splice variant, TNR-CFTR. To accomplish this, Madin-Darby canine kidney (MDCK) stable cell lines expressing TNR-CFTR or CFTR were used. The cells were treated with hypertonic medium made with either NaCl or urea or sucrose (480 mOsm/kg or 560 mOsm/kg) to mimic the tonicity of the renal medulla environment. Western blot data showed that CFTR and TNR-CFTR total cell protein is increased by hypertonic medium, but using the surface biotinylation technique, only CFTR was found to be increased in cell plasma membrane. Confocal microscopy showed TNR-CFTR localization primarily at the endoplasmic reticulum and plasma membrane. In conclusion, CFTR and TNR-CFTR have different patterns of distribution in MDCK cells and they are modulated by a hypertonic environment, suggesting their physiological importance in renal medulla.

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Introduction

The cystic fibrosis transmembrane conductance regulator protein (CFTR) is an ABC (ATP-binding cassette) transporter [1]. This protein was first identified as a chloride channel but it can also function as a conductance regulator of other channels such as ENaC and ROMK [2-5]. Mutations in the CFTR gene cause disorder in secretory epithelia of the lungs, pancreas, liver, epididymis and sweat ducts, leading to a genetic disease called cystic fibrosis (CF). Although CFTR is expressed abundantly in the kidney [1], CF is not associated with major renal dysfunction perhaps suggesting that CFTR's function is redundant in the kidney.

Wild-type CFTR is composed of two transmembrane domains (TMD), two nucleotide domains (ND) and a regulatory domain [6, 7]. The maintenance of this structure was thought to be important for CFTR function.

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In the renal medulla a truncated splice variant of CFTR was found with 145 base pairs deleted in exons 13 and 14. The deleted portion results in a frame shift and a premature stop codon [8] leading to a new protein that includes only the first half of CFTR wild-type called TNR-CFTR. TNR-CFTR contains only the first TMD, the first NBD and 92% of the regulatory domain. Even with the only first part of CFTR, TNR-CFTR is functional when its mRNA is expressed in *Xenopus laevis* oocytes and mammalian cells, with single-channel properties similar to full-length CFTR but with a reduced amount of expression at the plasma membrane [8]. TNR-CFTR mRNA and protein are expressed in renal medulla but not in the cortex; CFTR is expressed in both regions but much less expressed in medulla compared with TNR-CFTR [8]. An unanswered question regarding TNR-CFTR is where in the cell does it function and can it be modulated by natural changes in the extracellular milieu commonly occurring in the renal medulla?

The renal medulla has a high osmolality (as high as 1200 mOsm/kg in humans) compared with plasma. The high osmolality in the medulla is important not only for providing the driving force for reabsorption of water in the collecting duct but it can also affect expression of transporters along the nephron. For example, surface aquaporin-2 (AQP2) protein expression increases significantly in LLC-PK1 cells expressing AQP2 subjected to a hypertonic environment [9]. Renal outer medullary potassium channel 1 (ROMK1) mRNA expression is enhanced by hypertonic shock induced by NaCl but not by urea in medullary thick ascending limb mouse cells [10]. The same response was observed for Na^+:K^+:$2Cl^-$ co transporter 2 (NKCC2) in gill fish under a hypertonic environment [11].

It has been shown previously that adaptation of a high salt environment induces an increase in CFTR abundance in the salt glands of marine birds, and also alters glycosylation [12]. After abrupt exposure to seawater, CFTR expression in the gills of killifish previously adapted to fresh water increased several fold [13]. These data suggest that changes in CFTR expression controlled by differences in osmolality are important adaptive responses to high salinity in marine birds and fish. On the other hand, in colon-derived HT-29 cells (human colon adeno-carcinoma grade II cell line) increasing the medium osmolality by 150 mOsm/kg using NaCl, urea, or mannitol reduces the amount of CFTR mRNA [14].

The purpose of this study was to investigate if CFTR and its renal medulla splice variant (TNR-CFTR) expressed in Madin–Darby canine kidney (MDCK) cells can be regulated by a hypertonic environment.

Materials and Methods

Plasmid construction

In this study, chimeric proteins of CFTR or TNR-CFTR with green fluorescent protein (GFP) were used (GFP-CFTR and GFP-TNR-CFTR). The plasmid pcDNA5/FRT/CFTR-GFP used for this purpose was the kind gift of Dr Garry R. Cutting (School of Medicine, The Johns Hopkins University). The mutagenesis polymerase chain reaction (PCR) was used to construct pcDNA5/FRT/TNR-CFTR-GFP. Primers were used in the PCR mutagenesis to delete the specific 145 base pairs characterizing TNR-CFTR. The sequences of these primers were: GTC ACT GGC CCC TCA GGC AAA CTT CGA TAT ATT ACT GTC CAC (forward) and GTG GAC AGT AAT ATA TCG AAG TTT GCC TGA GGG GCC AGT GAC (reverse). DNA sequencing was carried out to confirm the pcDNA5/FRT/TNR-CFTR-GFP plasmid construction.

Stable cell line expressing CFTR and TNR-CFTR

The MDCK type-II FRT cell line, which contains pcDNA5/FRT/CFTR-GFP but expresses no endogenous CFTR, was the kind gift of Dr Garry R. Cutting [15] (The Johns Hopkins School of Medicine, Baltimore, MD).

The Flp-In System was used to make the stable cell line for TNR-CFTR according to the manufacturer's instructions (Invitrogen catalog No. K6010-01). MDCK cells stably transfected with pFRT/lacZeo (gift from Greg Germino, The Johns Hopkins University) were co-transfected with pOG44 and pcDNA5/FRT/TNR-CFTR-GFP using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The co-transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 20 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% fetal calf serum and 2 µg/µl of hygromicin once these cells becomes hygromicin resistant.

During the cell culture was possible to evaluate the cell viability and growth. There was no difference regarding time shape of cell growing between MDCK wild type and the both stable lines used in this paper (data not shown).

Hypertonic medium preparation

Hypertonic media (480 mOsm/kg or 560 mOsm/kg) were prepared by adding sodium chloride (NaCl), urea or sucrose to increase the normal osmolality (320 mOsm/kg) of the DMEM + hygromicin medium. The osmolality of the media was confirmed by osmometer (Vapor Pressure Osmometer, Model 5100C, WESCOR). The stable MDCK cell lines (GFP-TNR-CFTR and GFP-CFTR) were treated for 18 h with normal medium or 480 mOsm/kg or 560 mOsm/kg medium made hyperosmotic with each of the solutes. Summarizing the groups: control (CTRL, treated with regular medium, 320 mOsm/kg), 480 mOsm/kg NaCl (480 NaCl), 480 mOsm/kg urea (480 Urea), 480 mOsm/kg sucrose (480 Suc), 560 mOsm/kg NaCl, (560 NaCl) 560 mOsm/kg urea (560 Urea) and 560 mOsm/kg sucrose (560 Suc).

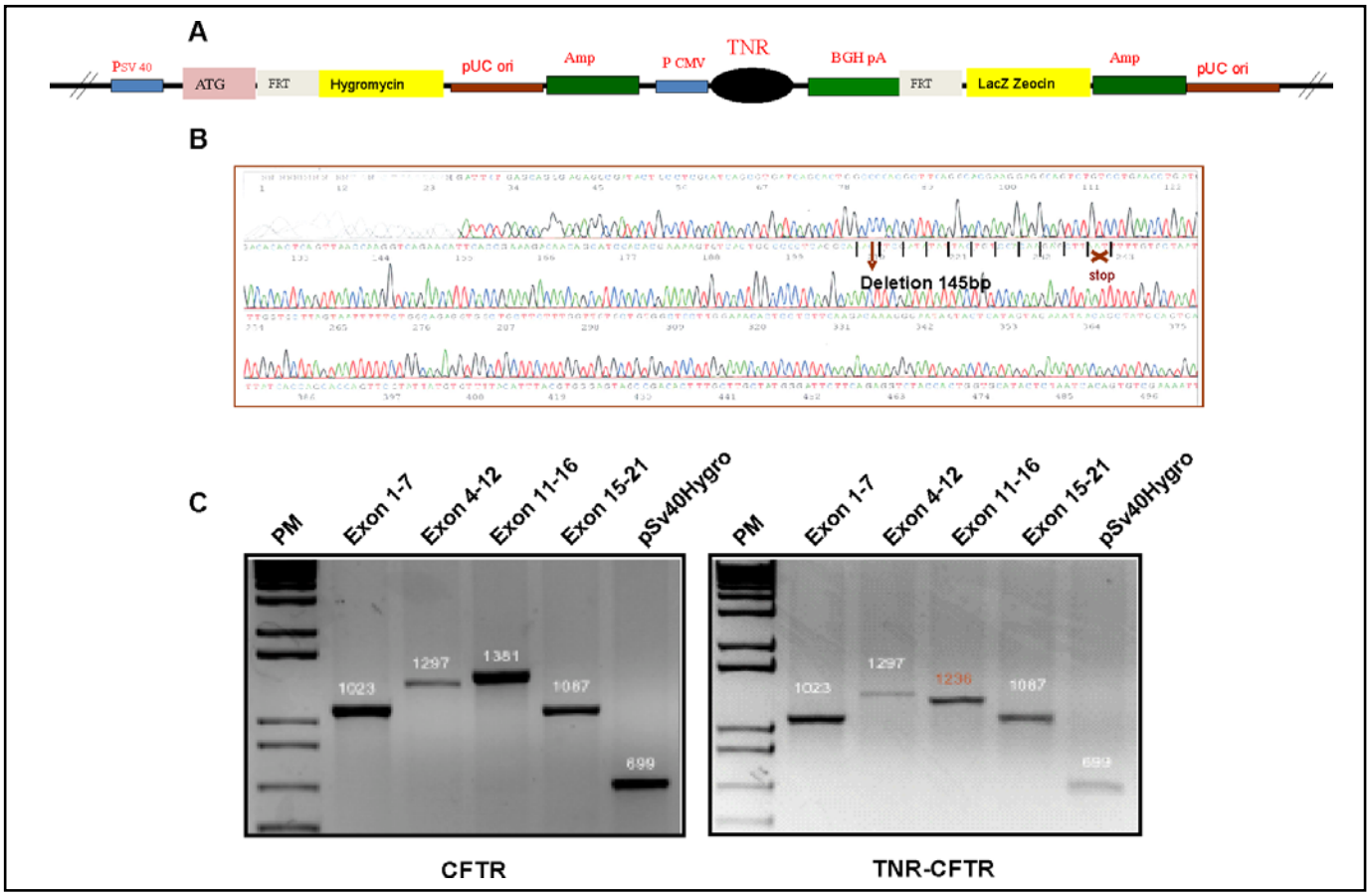

Fig. 1. Stable cell line characterization. A - Schematic sequence of genomic DNA integration modified from the Flp-In System. Integration of the specific TNR site inserted to make the stable line. B - DNA sequencing of pcDNA5/FRT-TNR-GFP plasmid construction made by PCR mutagenesis of pcDNA5/FRT-CFTR-GFP. This figure shows the 145 bp deletion and the frame shift creating nine new amino acids and a premature stop codon. C - Confirmation of GFP-CFTR and GFP-TNR-CFTR cDNA integration by PCR analysis. Photographs of agarose gels with amplified DNA stained with ethidium bromide and visualized by ultraviolet (UV) trans-illumination. The gels show amplification of genomic DNA from a hygromycin-resistant, zeocin-sensitive cell line. The amplification of the 699-bp product using primers psV40 and hygromicin verify the cDNA into the FRT site. Pairs of primers were used to cover the GFP-CFTR wt and GFP-TNR-CFTR genes.

Verification of pcDNA5/FRT/GFP-TNR-CFTR integration into the FRT site in MDCK cells

To confirm that TNR-CFTR integrated into the FRT site, genomic DNA was extracted from the stable TNR-CFTR cell lines and subjected to a PCR-based test as previously described [16]. Briefly, genomic DNA was extracted from TNR-CFTR cells using a Puregene kit (D5500; Gentra, Minneapolis, MN) and PCR primers specifically for exons 1-7 (AGA GGT CGC CTC TGG AAA AG and TGC TCC AAG AGA GTC ATA CC) amplified a 1023-bp region; for exons 4-12 (CAC ATT GGA ATG CAG ATG AG and GTG TTA AAA CAT CTA GGT ATC) amplified a 1297 bp region; for exons 11-16 (CAT CTC CAA GTT TGC AGA GA and GTC AAA TAT GGT AAG AGG C) amplified a 1236 bp region; and for exons 15-21 (CAC CTA TGT CAA CCC TCA AC and CAT CTG CAA CTT TCC ATA TTT C) amplified a 1087 bp region. In addition, a pair of primers was used to confirm the integration of the hygromycin gene (699 bp; forward: pSv40 CCT AAC TGA CAC ACA TTC CAC A and reverse: hygromycin TCA GCG AGA GCC TGA CCT AT) [15] (Fig. 1).

Immunoblotting

The cells were harvested and processed as described previously [17]. Briefly, the cells were solubilized in lysis buffer (50 mM NaCl, 150 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and complete protease inhibitor, Roche Applied Science). The cell lysates were spun at 14,000×g for 15 min at 4°C. The protein concentrations of the supernatants were quantified with a BCA protein assay kit (Pierce) and Bradford kit (Bio-Rad). The normalized supernatants were subjected to SDS-PAGE and Western blotting, followed by Super Signal (Amersham Biosciences). The chemiluminescence signal on the polyvi nylidene difluoride membrane was directly captured by a FujiFilm LAS-1000 plus system with a 1,300,000-pixel cooled CCD camera with a linearity of 3.7 orders of magnitude. GFP-tagged proteins were detected with a rabbit polyclonal anti-GFP antibody (1:1000; BD Biosciences, Boston, MA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins were detected with a mouse monoclonal GAPDH antibody (1:10,000,

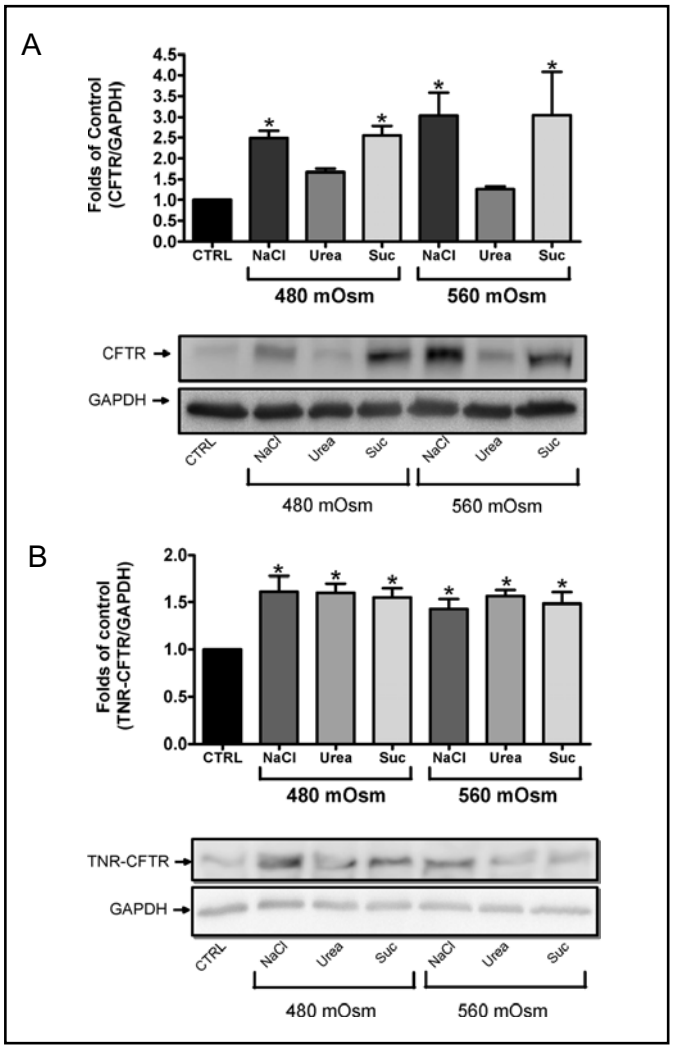

Fig. 2. Western blot of CFTR and TNR-CFTR total protein cell content from MDCK cells. A - Total protein expression of CFTR in MDCK cells under hypertonic shock. B - Total protein expression of TNR-CFTR in MDCK cells under hypertonic shock. GAPDH total protein was used as an internal control. *Significantly different from the CTRL group ($p < 0.05$). CTRL, controls; NaCl, cells under hypertonic shock with sodium chloride; Urea, cells under hypertonic shock with urea; Suc, cells under hypertonic shock with sucrose.

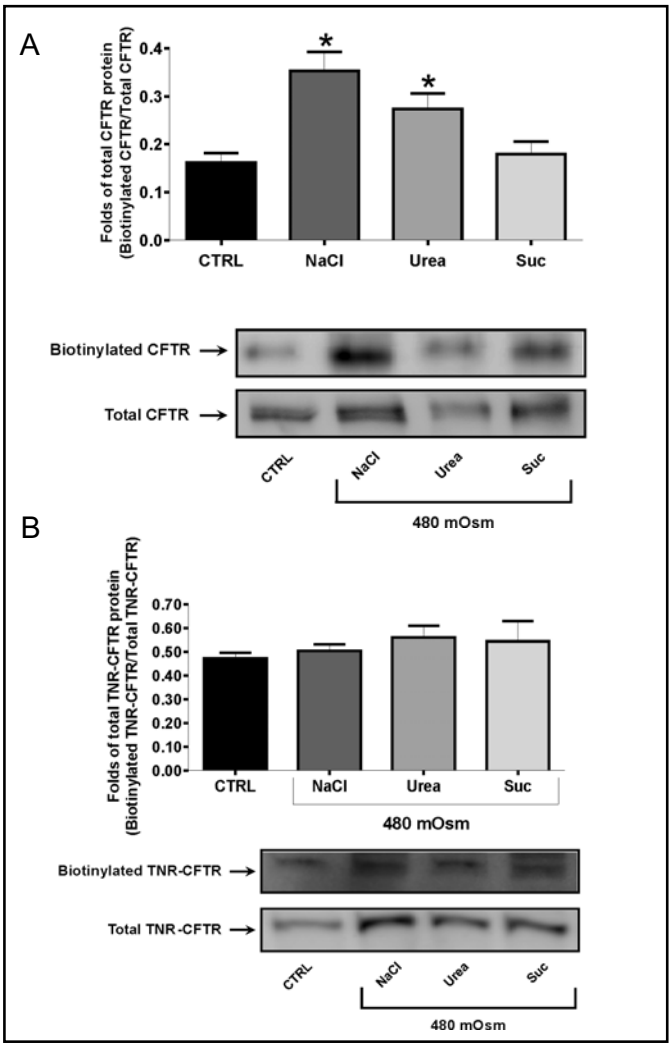

Fig. 3. Surface byotinylation analysis showing the ratio of membrane protein expression per total protein expression of each group. A - Membrane protein expression of CFTR in MDCK cells treated with hypertonic medium. B - Membrane protein expression of TNR-CFTR in MDCK cells treated with hypertonic medium. *Significantly different from the CTRL group ($p < 0.05$). CTRL, controls; NaCl, cells under hypertonic shock with sodium chloride; Urea, cells under hypertonic shock with urea; Suc, cells under hypertonic shock with sucrose.

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Surface biotinylation

Only the hypertonic medium of 480 mOsm/kg was used for surface byotinylation once both hypertonic media (480 mosm/kg and 560 mosm/kg) presented the same pattern of results using the confocal microscopy analyses method. Surface biotinylation of CFTR at the plasma membrane was described previously [17] and the same protocol was performed for the TNR-CFTR gene. Briefly, the cell surface proteins were labeled with cell-impermeable EZ-Link™ Sulfo-NHS-SSBiotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce) at 4°C for 15 min. The cell surface proteins were isolated from lysate by incubating with immobilized NeutrAvidin beads at 4°C for 2 h (Pierce; catalog no. 53151). The bound proteins were eluted with 2× Laemmli sample buffer supplemented with 100 mM dithiothreitol at 42°C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blotting followed by Super Signal (Amersham Biosciences). CFTR and TNR-CFTR (both constructed in a plasmid containing GFP) were detected with a rabbit polyclonal GFP antibody (1:1000; BD Biosciences).

Confocal microscopy

MDCK GFP-CFTR and GFP-TNR-CFTR stable line cells were 100% confluent and polarized before the treatment

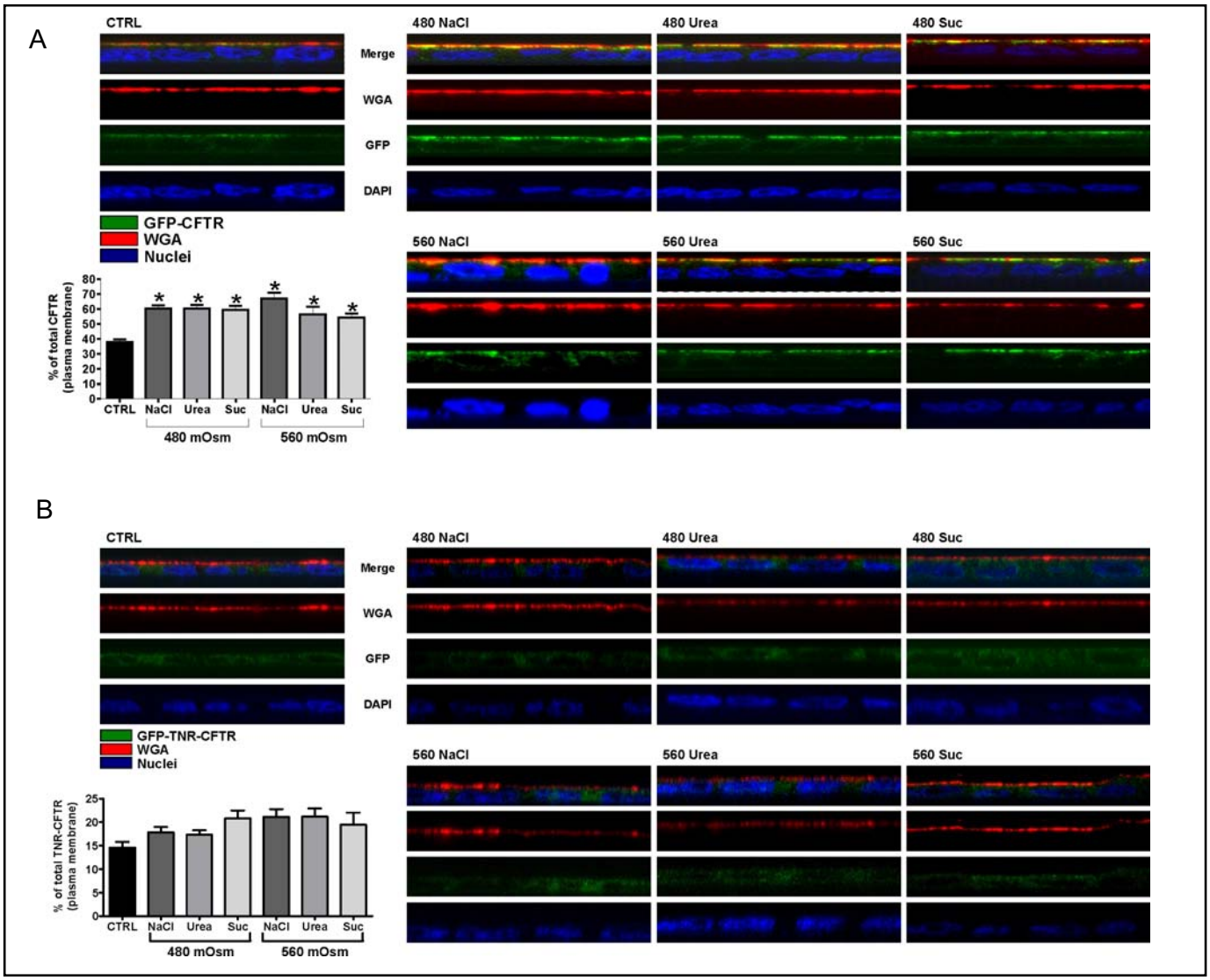

Fig. 4. Confocal microscopy of CFP-CFTR and CFP-TNR-CFTR in MDCK cells. A - CFTR and WGA staining in polarized MDCK cells treated with hypertonic medium. A, graphic - Percentage of CFTR co-localized with WGA showing CFTR expression at the plasma membrane. B - TNR-CFTR and WGA staining in polarized MDCK cells treated with hypertonic medium. B, graphic - Percentage of TNR-CFTR co-localized with WGA showing TNR-CFTR expression at the plasma membrane. *Significantly different from the CTRL group ($p<0.05$). CTRL, controls; NaCl, cells under hypertonic shock with sodium chloride; Urea, cells under hypertonic shock with urea; Suc, cells under hypertonic shock with sucrose; WGA, wheat germ agglutinin plasma membrane marker.

with hypertonic medium. The cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Nonidet P-40, 18 h after treatment. Nonspecific binding sites were blocked with 5% normal goat serum. The cells were stained with anti-calnexin (BD Bioscience), which is an ER marker, or anti-GM130 (BD Bioscience), which is a Golgi marker monoclonal antibody (1:400 and 1:200 respectively) in 5% normal goat serum, and then washed with 1% bovine serum albumin and incubated with goat anti-mouse Cy3 secondary antibodies in 1% normal goat serum (1:200; Jackson ImmunoResearch, West Grove, PA). Wheat germ agglutinin (Invitrogen, 1:500) was used for plasma membrane staining without cell permeabilization; 4',6-diamidino-2-phenylindole (DAPI, Roche) was used to stain the nucleus (1:1000). To observe the green stain from GFP in GFP-TNR-CFTR and GFP-CFTR stable cell lines, the antibody stain was not necessary; it was possible to analyze the green fluorescence stain with only the GFP tag. The specimens were mounted and viewed on an UltraVIEW spinning disk confocal microscope (PerkinElmer Life Sciences).

Measurement of green fluorescence signal (GFP-CFTR and GFP-TNR-CFTR) and red fluorescence signal (wheat germ agglutinin) (Invitrogen, Carlsbad, California) in dual color confocal images was performed with the "Colocalization features" in Volocity v4.2 (Perkin Elmer, Waltham, MA) based

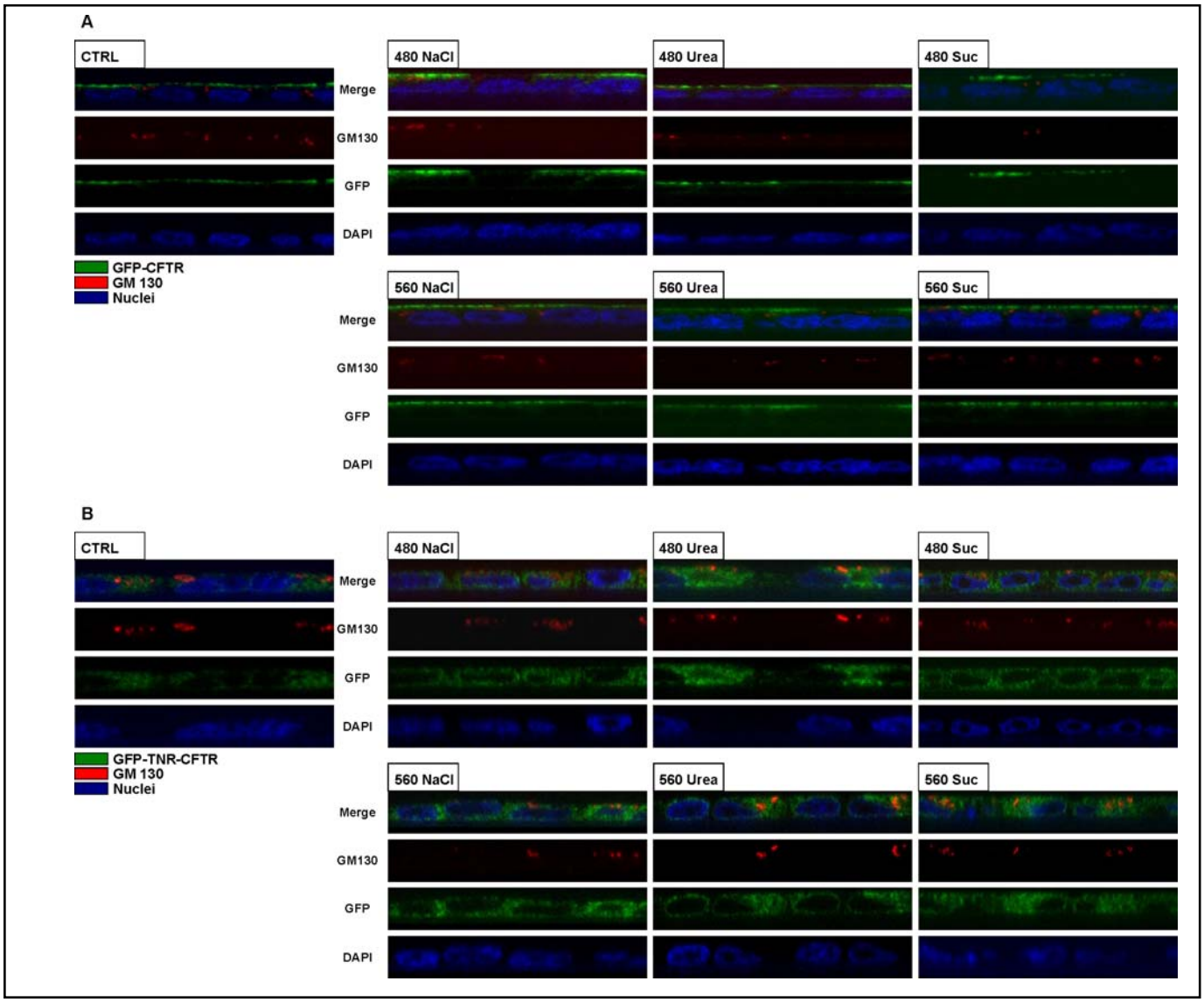

Fig. 5. Confocal microscopy of GFP-CFTR and GFP-TNR-CFTR in MDCK cells. A - CFTR and MG 130 Golgi antibody staining in polarized MDCK cells treated with hypertonic medium. B - TNR-CFTR and MG 130 Golgi antibody staining in MDCK cells treated with hypertonic medium.

on the method previously describe [16].

Statistical analyses

GraphPad Prism 5.0 statistical software package was used. Differences among the groups were assessed by one-way ANOVA followed by the Bonferroni test when required. A p value < 0.05 was considered significant.

Results

Stable cell lines expressing CFTR or TNR-CFTR

The success of PCR mutagenesis to construct pcDNA5/FRT-TNR-CFTR-GFP was observed by DNA sequencing (Fig. 1B). The deletion of 145 bp in exons 13 and 14 causes a frame shift translating into nine new amino acids and a premature stop codon.

The creation of GFP-CFTR and GFP TNR-CFTR stable cell lines was confirmed by showing the expected size of PCR products using primers covering all CFTR gene exons (Fig. 1C). In addition, PCR showed the expected size difference between CFTR and TNR-CFTR, corresponding with the TNR-CFTR 145 bp deletion in exons 13 and 14 (Fig. 1C).

Although CFTR half molecules have been created previously, this is the first study focusing on the TNR-CFTR protein with the unique characteristics occurring in the natural splice variant present in the renal medulla.

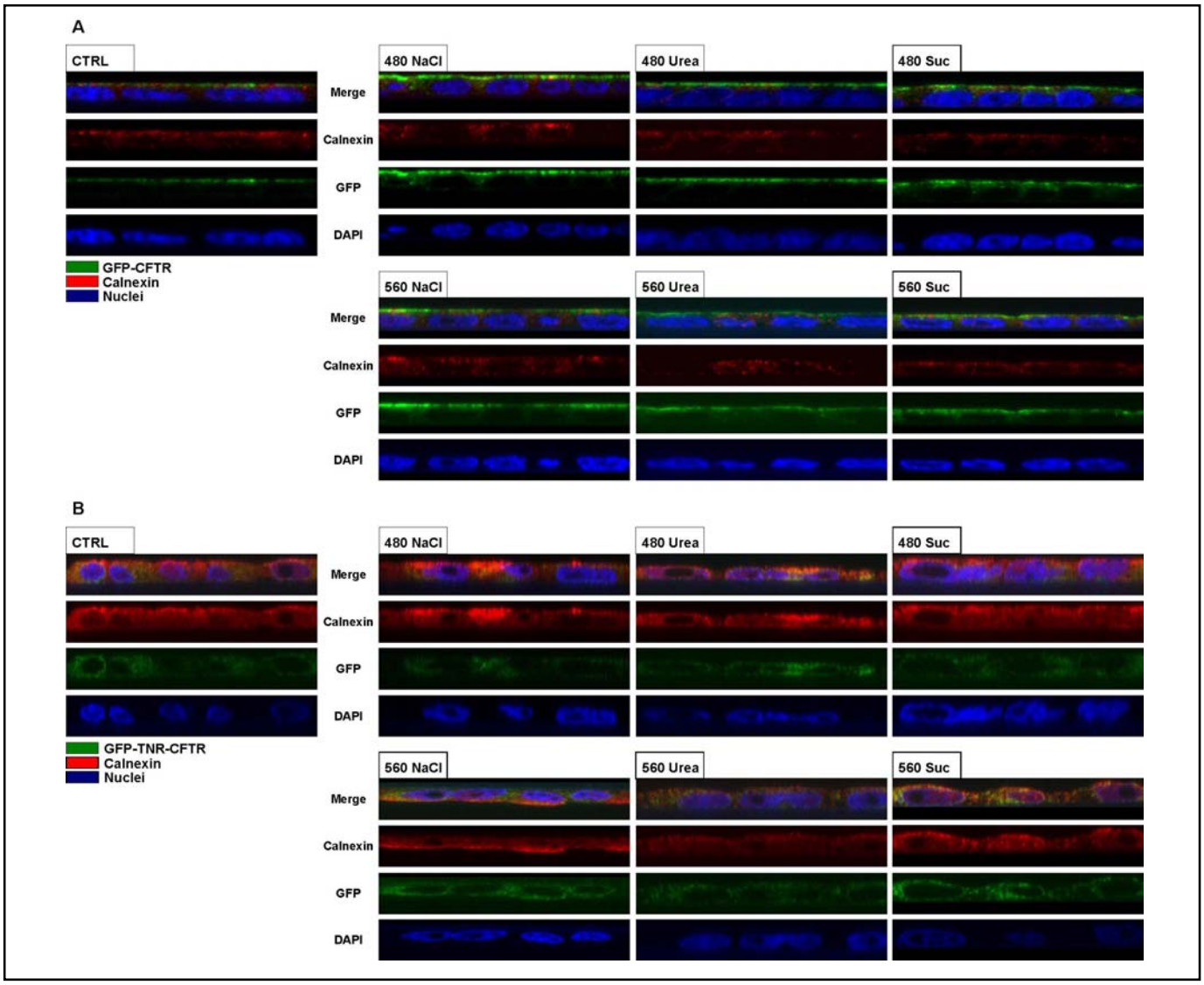

Fig. 6. Confocal microscopy of GFP-CFTR and GFP-TNR-CFTR in MDCK cells. A - CFTR and calnexin ER antibody staining in polarized MDCK cells treated with hypertonic medium. B - TNR-CFTR and calnexin ER antibody staining in polarized epithelial cells treated with hypertonic medium.

Total CFTR and TNR-CFTR protein expression under hypertonic shock

GFP-CFTR and GFP-TNR-CFTR MDCK stable cell lines were treated for 18 h with hypertonic medium at 480 mOsm/kg or 560 mOsm/kg using NaCl, urea or sucrose as osmotic particles. CFTR total protein was increased in cells where the osmolality of the medium was increased to 480 mOsm/kg or 560 mOsm/kg with NaCl (2.5±0.18 or 3.0±0.54 fold of control) and sucrose (2.6±0.23 or 3.1±1.0 fold of control) (Fig. 2A). In contrast, when the medium was hypertonic (both 480 mOsm/kg and 560 mOsm/kg) using urea, CFTR total protein expression did not change (1.7±0.084 or 1.3±0.068 fold of control) compared with the control group (Fig. 2A). TNR-CFTR total protein expression was increased in cells subjected to hypertonic media of 480 mOsm/kg or 560 mosm/kg using NaCl (1.6±0.17 or 1.4±0.10 fold of control), urea (1.6±0.99 or 1.6±0.067 fold of control) or sucrose (1.6±0.10 or 1.5±0.12 fold of control), when compared with cells kept in regular osmolar medium (Ctrl group) (Fig. 2B).

Plasma membrane expression of CFTR and TNR-CFTR under hypertonic shock

The plasma membrane expression of CFTR and TNR-CFTR was studied in cells exposed to hypertonic media. Surface biotinylation experiments detected an increase of CFTR at the plasma membrane when cells

were treated with 480 mOsm/kg medium using NaCl (0.31±0.023 fold of total CFTR protein) or urea (0.23±0.040 fold of total CFTR protein) but not when sucrose was used (0.13±0.034 fold of total CFTR protein) compared with control (0.13±0.022 fold of total CFTR protein) (Fig. 3A). TNR-CFTR did not show any difference in plasma membrane protein expression when cells were treated with 480 mOsm/kg medium using NaCl (0.47±0.036 fold of total TNR-CFTR protein), urea (0.57±0.051 fold of total TNR-CFTR protein) or sucrose (0.51±0.12 fold of total TNR-CFTR protein) compared with the control cell group (0.51±0.057 fold total TNR-CFTR protein) (Fig. 3B).

Cellular localization of CFTR and TNR-CFTR

Consistent with surface biotinylation data, confocal microscopy experiments also showed that CFTR expression is increased in plasma membrane when treated with hypertonic medium (480 mOsm/kg or 560 mOsm/kg) using NaCl (60±2.2% or 67±4.0% of total CFTR), urea (60±2.3% or 56±5.1% of total CFTR) or sucrose (59±2.6% or 54±2.9% of total CFTR) as osmotic particles (Fig. 4A). In the other words, the co-localization of GFP and WGA was higher in the cells treated with hypertonic medium compared with the control group (38±1.8% of total CFTR) (Fig. 4A). In the GFP-TNR-CFTR stable cell line there were no significant differences among the groups (Fig. 4B).

Co-localization of TNR-CFTR and calnexin was observed by confocal microscopy suggesting that TNR-CFTR is localized mainly in the ER (Fig. 6). Using the same technique we also observed that neither CFTR nor TNR-CFTR was detected in Golgi cells (Fig. 5).

Discussion

Stable cell lines were used to evaluate the regulation of CFTR and TNR-CFTR expression under hypertonic shock. Both surface biotinylation and confocal studies showed that CFTR is located mainly at the cell surface but TNR-CFTR is localized primarily within the cells. The observation that TNR-CFTR co-localizes with the calnexin protein, a specific marker of ER, strongly suggests that TNR-CFTR is localized primarily within the ER (Fig. 6). The different pattern of distribution of the two channels in MDCK cells indicates their differential role in renal cell physiology. In addition, we found a significant amount of CFTR protein at the plasma membrane at the baseline condition of 320 mOsm/kg using surface biotinylation and confocal techniques and its plasma membrane protein expression was significantly increased when the cells were subjected to hypertonic shock using urea, NaCl or sucrose (480 mOsm/kg and 560 mOsm/kg) (Figs. 3A and 4A). However, the surface biotinylation showing an increase in CFTR in MDCK cells were consistent with confocal microscopy data except with some differences (e.g. sucrose 480 mOsm/kg) (Figs 3A and 4A). These apparent inconsistencies between surface biotinylation and confocal microscopy may be correlated with the differences in sensibility of the two techniques used. Urea, NaCl and sucrose were used to perform the hypertonic shock once they can interfere with cell physiology differently. It is well known that urea is permeable to cell membrane [17] and has less impact in cell volume decrease [14] compared to NaCl and sucrose . In contrast, NaCl solution could change ion cell concentrations and it has been shown to be able to change cell gene expression in the kidney [18, 19]. CFTR is a chloride channel as well as a modulator of other conductance and its increased expression in cell membrane under hypertonicity could modify the cell ion transport protecting cells from shrinkage stress induced by different osmolytes. Western blot technique showed that total cell content of CFTR expression increased with hypertonic shock except when urea was used (Fig. 2). These results are consistent with other publications where the use of NaCl and mannitol in hypertonic medium, but not urea, increases the expression of cell Na+/K+-ATPase classically involved in cell protection from hypertonicity [20].

Moreover, we showed enhanced CFTR expression in the plasma membrane of cells subjected to urea (480 mOsm/kg) suggesting that this osmolyte can induce CFTR translocation to the plasma membrane (Fig. 3A and 4A).

In our previous studies we showed that CFTR mRNA is present in thick ascending limb of Henle´s loop (TAL), a portion of the nephron involved in the formation and regulation of renal medulla hypertonicity [8, 21, 22]. CFTR can inhibit epithelia sodium channel (ENaC) activity[23, 24] and during hypertonic shock could prevent an excessive increase of intracellular sodium helping the adaptation of cells in renal medulla. In addition, it is well established that CFTR is able to up regulate the activity of the well known channel involved in the maintenance of renal medullary hypertonicity: the Renal Outer Medullary Potassium channel (ROMK) [4, 5, 25, 26].

The increase of CFTR protein found in the plasma membrane of the MDCK cells under hypertonic shock could suggest that CFTR plays a role in the renal medulla

probably by helping in the maintenance of renal medulla hypertonicty and/or adapting tubular cells to a hypertonic environment.

We have shown in the present study that CFTR is 5-fold less expressed in MDCK cell plasma membrane under basal conditions (320 mOsm/kg) compared with TNR-CFTR expression, and under hypertonic shock CFTR increases but cannot achieve the values of expression of TNR-CFTR. In contrast to CFTR, TNR-CFTR plasma membrane cell surface expression was unchanged under hyperosmotic stress using different osmotic particles but total cell protein expression was increased using the same procedure, probably by increasing TNR-CFTR expression in intracellular compartments such as the ER and Golgi cells (Fig. 2). This is the first report of a different pattern of expression between CFTR and TNR-CFTR besides they share similar electrophysiological characteristics [8]. We proposed previously that TNR-CFTR is an alternative splicing of wild-type CFTR pre-mRNA by homology of cDNA molecules forming TNR-CFTR and CFTR, and this hypothesis was supported by Souza-Menezes et al. [27] who showed that small nuclear RNAs U11 and U12 (small nuclear RNAs present in the composition of spliceosomes that participate in alternative splicing mechanisms) are probably involved in the production of TNR-CFTR mRNA using a primary transcript from the CFTR gene. In this way, it is possible to suggest that TNR-CFTR misses some portion of its protein that is important for its plasma membrane expression regulation in a hypertonic environment.

In conclusion, we have shown an increase in total CFTR and TNR-CFTR expression in cells under hypertonic shock and only surface CFTR protein expression increased under these conditions. This up-regulation in surface CFTR expression can be important in the adaptation of tubular cells to the hypertonic environment and can be involved in the regulation of renal medullary hypertonicity.

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