Cystic Fibrosis Transmembrane Conductance Regulator Differentially Regulates Human and Mouse Epithelial Sodium Channels in Xenopus Oocytes*

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The cystic fibrosis transmembrane conductance regulator (CFTR), in addition to its well defined Cl⁻ channel properties, regulates other ion channels. CFTR inhibits murine or rat epithelial Na⁺ channel (ENaC) currents in many epithelial and non-epithelial cells, whereas murine or rat ENaC increases CFTR functional expression. These regulatory interactions are reproduced in Xenopus oocytes where both the open probability and surface expression of wild type CFTR Cl⁻ channels are increased when CFTR is co-expressed with αβγ ENaC, and conversely the activity of mENaC is inhibited after wild type CFTR activation. Using the Xenopus oocyte expression system, differences in functional regulatory interactions were observed when CFTR was co-expressed with either αβγ mENaC or αβγ human ENaC (hENaC). Co-expression of CFTR and αβγ mENaC or hENaC resulted in an ~3-fold increase in CFTR Cl⁻ current compared with oocytes expressing CFTR alone. Oocytes co-injected with both CFTR and mENaC or hENaC expressed an amiloride-sensitive whole cell current that was decreased compared with that observed with the injection of mENaC or hENaC alone before CFTR activation with forskolin/3-isobutyl-1-methylxanthine. CFTR activation resulted in a further 50% decrease in mENaC-mediated currents, an ~20% decrease in α-T663-hENaC-mediated currents, and essentially no change in α-A663-hENaC-mediated currents. Changes in ENaC functional expression correlated with ENaC surface expression by oocyte surface biotinylation experiments. Assessment of regulatory interactions between CFTR and chimeric mouse/human ENaCs suggest that the 20 C-terminal amino acid residues of α ENaC confer species specificity regarding ENaC inhibition by activated CFTR.

Cystic fibrosis (CF)* results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (1). In addition to functioning as a cAMP-activated, ATP-dependent Cl⁻ channel, CFTR influences the transepithelial transport of other solutes, including Na⁺ via the epithelial sodium channel (ENaC), Cl⁻ via an outwardly rectifying Cl⁻ channel, K⁺ via Kir1.1, HCO₃⁻, and ATP (2–5).

Functional interactions between CFTR and rodent ENaC have been observed in epithelial as well as non-epithelial cells (6–12). The activation of CFTR is generally associated with an inhibition of ENaC (7, 8, 10, 11, 13, 14), although activation of CFTR leads to activation of ENaC in the sweat duct (12) suggesting that the regulatory interactions between these two transporters are complex. The co-expression of CFTR and rodent ENaC in Xenopus oocytes results in regulatory interactions that mimic the airway where there is a decrease in ENaC-mediated Na⁺ transport in the presence of CFTR (9–11, 14). Furthermore CFTR-mediated Cl⁻ conductance is increased in presence of ENaC in oocytes (4, 7–11, 14). In contrast, the ∆F508-CFTR mutation, the most prevalent mutation found in North American Caucasian patients with CF, does not inhibit the functional expression of rat (15) or mouse ENaC (mENaC) (11), and mENaC does not enhance the functional expression of ∆F508-CFTR in Xenopus oocytes (11). The isoflavone genistein, which activates the Cl⁻ conductance of both wild type and mutant CFTRs (16–19), can restore these interactions (11).

The mechanism by which this interregulation of CFTR and ENaC occurs in oocytes is unclear and somewhat controversial. Others have suggested that the decrease in ENaC-mediated current after CFTR activation may result from a series resistor error (20) and have presented data in abstract form that suggest that activation of CFTR in oocytes does not result in a further decrease in hENaC-mediated current (21). Our previous data (11) are inconsistent with the hypothesis that the apparent decrease in mENaC-mediated current with CFTR activation is due to a series resistor error. Furthermore such a mechanism cannot account for the increased CFTR functional expression observed with co-expression of rodent ENaC (9–11, 22).

The present studies were aimed at better understanding the regulatory interactions between CFTR and ENaC and whether there are species-specific differences in these interactions. In this regard, our data are consistent with the decrease in mENaC functional expression that occurs with CFTR activa-
tion being due, in part, to decreased mENaC surface expression, while hENaC (with Ala in position 663) functional and surface expression does not decrease following CTFR activation. These observations led us to test two additional hypotheses. First we tested the hypothesis that the C terminus of the ENaC α subunit, which has limited homology between mouse and human, may influence the species-related differences in the amiloride-sensitive currents. We also tested the hypothesis that a naturally occurring polymorphism in the C terminus of an hENaC, substitution of Ala at residue 663 for Thr (T663A), which we have recently shown to decrease the functional and surface expression of hENaC in oocytes, would influence regulatory interactions between CTFR and hENaC. Our data suggest that the C terminus of α hENaC confers species specificity regarding ENaC inhibition by activated CTFR, whereas the αT663A polymorphism has a modest effect on the response of hENaC to activated CTFR.

EXPERIMENTAL PROCEDURES

Materials—Forskolin and IBMX were purchased from Sigma. All other reagents were purchased from Fisher.

Expression of Human CTFR and Mouse and Human ENaC in Xenopus Oocytes—Human CTFR (wild type and ΔF508) and mouse and human ENaC were expressed in Xenopus oocytes as described previously (10, 11). Briefly human CTFR and mouse and human α, β, and γ ENaC cRNAs were prepared using a cRNA synthesis kit (mMESSAGE mMACHINE, Ambion Inc., Austin, TX) according to the manufacturer’s protocol. cRNA concentrations were determined spectroscopically. Oocytes were placed in a 1-ml chamber containing modified ND96 (96 mM NaCl, 1 mM CaCl2, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 15 mM Heps, pH 7.6, supplemented with 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin sulfate). Each batch of oocytes obtained from an individual frog was injected (50 nl/oocyte) using a Nanjoeck II microinjector (Drummond Scientific, Broomall, PA) with α, β, and γ subunits of either mENaC (0.33 ng/subunit) or hENaC (2 ng/subunit), CTFR (wild type or ΔF508, 10 ng), or a combination of ENaC and CTFR cRNAs dissolved in RNase-free water.

Electrophysiological Analyses—Whole cell current measurements were performed 24–48 h after injection using the two-electrode voltage clamp method as described previously (10, 11). Oocytes were placed in a 1-ml chamber containing modified ND96 (96 mM NaCl, 1 mM CaCl2, 0.2 mM CaCl2, 5.8 mM MgCl2, 10 mM Heps, pH 7.4) and micropipetted with micropipettes of 0.5–1.0 mS in resistance, impaled with micropipettes of 0.5–1.0 mS in resistance, and placed in a 1-ml chamber containing modified ND96 (96 mM NaCl, 1 mM CaCl2, 0.2 mM CaCl2, 5.8 mM MgCl2, 10 mM Heps, pH 7.4). The bath solution contained 140 mM NaCl, 5 mM CaCl2, 1 mM MgCl2, 10 mM Heps, pH 7.4, and 0.1 mM amiloride. Oocyte membrane potentials were monitored using an Axoclamp 500B (Axon Instruments, Foster City, CA). To reduce error due to series resistance, the voltage clamp (Axon Genecamp 500B) was configured to clamp the bath potential to 0 mV. In this configuration, we independently monitored the oocyte membrane potential during our clamp protocol and routinely observed membrane potentials that were <5% depolarized from our target holding potentials.

The difference in whole cell currents measured in the absence and presence of 10 μM amiloride was used to define the amiloride-sensitive Na+ current that was carried by ENaC. CTFR was activated by perfusion of the oocyte with buffer containing 10 μM forskolin and 500 μM IBMX (forskolin/IBMX) for 25 min (10, 11). CTFR Cl– current was defined as the difference between amiloride-insensitive current measured before and after perfusion with forskolin/IBMX. Whole cell currents were recorded at a clamp potential of −100 mV for comparisons. All measurements were performed at room temperature.

Assessment of ENaC Surface Expression—β and δ hENaC-V5 epitope C-terminal fusion protein (β-V5) was constructed in pcDNA6/V5-HisA (Invitrogen) as described for β mENaC-V5 (mβ-V5) (23). cRNA for β-V5 was prepared and co-injected into oocytes with αγ ENaC with or without CTFR as described above. After 48 h, oocytes were treated with forskolin/IBMX for 20 min and cell surface proteins of oocytes that were mechanically stripped of their vitellin membrane using hypotonic medium (300 mM sucrose in modified Barth’s saline without penicillin, streptomycin, and gentamicin) were labeled with sulfo-N-hydroxysuccinimide-biotin (Pierce) as we have described previously.2 Oocytes (10/group) were subsequently lysed in 0.1% NaOAc, 0.01% Trit-CI, pH 8.0, 0.01% EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1.0 mM phenylmethylsulfonfloure, 0.1 mM N’-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM t-tosylamide-2-phenylethyl-chloro methyl ketone, and 2 μg/ml aprotinin for 1 h at 4 °C and centrifuged at 13,000 × g for 15 min at 4 °C, and labeled proteins in the supernatant were precipitated with streptavidin-agarose (Pierce).

Immunoblot Analysis—Streptavidin−precipitated proteins or whole oocyte lysates (prepared as above) were incubated in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose as described previously by our group (24, 25). Fluorogram density was quantitated using an Alphamax 2200 system and version 5.5 software (Alpha Innotech, San Leandro, CA), and the intensity of β-V5 was expressed relative to that of oocytes injected with ENaC alone without forskolin/IBMX stimulation. As in our previous work,2 less than 20% of intracellular β-V5 epitope was biotin-labeled in oocytes expressing only β-V5 and γ ENaC subunits (data not shown).

Statistical Analyses—All data are presented as mean ± S.E. Statistical comparisons were performed using the Student’s t test. A pairwise t test was used for pre/post-treatment in experiments using an individual oocyte. A two-tailed t test was used when comparing currents obtained from oocytes injected with a cRNA for a single transporter (i.e. ENaC or CTFR versus oocytes co-injected with cRNAs for both ENaC and CTFR). p values ≤0.05 were accepted to indicate statistical significance. Statistical analyses were performed using SigmaStat version 2.03 software.

RESULTS

Regulatory Interactions between mENaC and CTFR in Xenopus Oocytes—Several groups have reported that when wild type CTFR and rodent (murine or rat) ENaC were co-expressed in Xenopus oocytes, ENaC-mediated Na+ currents were inhibited in response to CTFR activation in two-electrode voltage clamp (TEV) experiments (7–9, 11). Furthermore co-expression of either of these rodent ENaCs enhanced forskolin/IBMX-stimulated CTFR Cl– currents (7, 9–11). In agreement with these previous observations (7, 10, 11, 15, 22), oocytes injected with mENaC alone had similar amiloride-sensitive currents before (−3.23 ± 0.75 μA) and after (−3.61 ± 0.85 μA, n = 10, p = ns) treatment with forskolin and IBMX (Fig. 1A). Oocytes co-injected with mENaC and CTFR had reduced amiloride-sensitive current (−1.36 ± 0.20 μA, n = 10) compared with oocytes injected with mENaC alone (−3.23 ± 0.75 μA, p = 0.03), and this amiloride-sensitive current was further reduced in co-injected oocytes upon activation of CTFR with forskolin/IBMX (−0.89 ± 0.20 μA, p = 0.01, Fig. 1A). Also in agreement with our previous data (10, 11), co-injection of mENaC with CTFR increased the functional expression of CTFR (−10.08 ± 1.07 μA, n = 10) compared with oocytes injected with CTFR alone (−2.12 ± 0.42 μA, n = 10, p < 0.001, Fig. 1B). As the mechanism by which these alterations in mENaC functional expression upon co-expression and activation of CTFR occur is not clear, we performed surface biotinylation experiments to assess the amount of mENaC at the oocyte surface. In these experiments, we used mENaC where the β subunit contained a C-terminal V5 epitope tag to increase sensitivity of our immunoblots; control experiments suggested that this β mENaC modification did not influence regulatory interactions between CTFR and mENaC as measured by TEV (data not shown). As shown in Fig. 1C, mENaC (β-V5) expression at the oocyte surface was unlabeled by forskolin/IBMX in oocytes injected with mENaC alone. In contrast, mENaC surface expression decreased when mENaC was co-expressed with

2 Samaha, F. F., Rubenstein, R. C., Yan, W., Ramkumar, M., Levy, D. I., Ahn, Y. J., Sheng, S., and Kleyman, T. R. (April 6, 2004) J. Biol. Chem. 10.1074/jbc.M401941200
CFTR and was further decreased by activation of CFTR in oocytes expressing both channels. There was direct correspondence of these changes in surface expression of mENaC(β-V5) and the mENaC-mediated currents by TEV (Fig. 1A), suggesting that alterations in mENaC functional expression are, in part, a result of changes in mENaC expression at the oocyte surface (or “N”).

We also assessed whole oocyte expression of mENaC(β-V5) (Fig. 1D). Expression of mENaC(β-V5) was decreased by coinjection of CFTR but was not further decreased upon activation of CFTR with forskolin/IBMX. These data are consistent with CFTR activation causing an acute decrease in mENaC(β-V5) surface and functional expression without altering the total amount of mENaC(β-V5) present in the oocyte.

**Co-expression of CFTR and hENaC**—We next assessed regulatory interactions between CFTR and hENaC as well as the potential influence of the T663A functional polymorphism of α-hENaC described recently by our group on these interactions. Similar to our data with mENaC (Fig. 1A), forskolin/IBMX did not alter hENaC functional expression in oocytes injected with hENaC alone as is shown in Fig. 2A for α-T663-hENaC (−1.97 ± 0.27 versus −1.94 ± 0.30 μA, n = 21, p = ns) and in Fig. 2B for α-A663-hENaC (−1.85 ± 0.28 versus −1.95 ± 0.31 μA, n = 21, p = ns). Again co-injection of CFTR reduced hENaC functional expression prior to activation of CFTR (α-T663-hENaC: −1.97 ± 0.27 μA, n = 21 versus −1.81 ± 0.09 μA, n = 24, p < 0.001; α-A663-hENaC: −1.85 ± 0.28 μA, n = 21 versus −1.78 ± 0.15 μA, n = 25, p = 0.001). However, activation of CFTR in co-injected oocytes resulted in a further, more modest decrease in α-T663-hENaC functional expression (−0.81 ± 0.09 versus −0.65 ± 0.08 μA, n = 24, p = 0.02, Fig. 2A) compared with mENaC (Fig. 1A), and CFTR activation did not significantly decrease α-A663-hENaC functional expression (−0.78 ± 0.15 versus −0.69 ± 0.14 μA, n = 25, p = ns, Fig. 2B), although the lack of significance of this decrease for α-A663-hENaC may be related to the larger standard errors in these data compared with those of α-T663-hENaC. CFTR functional expression was enhanced by co-injection of α-T663-hENaC (co-injected: −6.32 ± 1.14 μA, n = 24, versus injected with CFTR alone: −2.83 ± 0.50 μA, n = 19, p = 0.009) or α-A663-hENaC...
FIG. 2. Expression of CFTR and hENaC in Xenopus oocytes. A, B, and C, CFTR and hENaC were expressed separately or together in oocytes, and TEV was performed as described under “Experimental Procedures.” A, whole cell currents that were sensitive to inhibition by 10 μM amiloride (−100 mV holding potential) were determined in oocytes expressing α-T663-hENaC or co-expressing α-T663-hENaC and CFTR prior to (closed bars) and following (open bars) stimulation with 10 μM forskolin, 500 μM IBMX. B, whole cell currents that were sensitive to inhibition by...
Species-specific Regulation of ENaC by CFTR

The next sought to correlate the functional expression of α-A663-hENaC by TEV with its surface expression by surface biotinylation of oocytes expressing α-A663-hENaC (where the hENaC β subunit contained a C-terminal V5 epitope tag). Again, there was direct correlation of α-A663-hENaC (V5) surface expression in response to CFTR activation.

We performed a similar experiment with ENaC chimeras containing murine α human β γ ENaC and human α(A663) murine β γ ENaC. Oocytes injected with murine α human β γ ENaC alone displayed amiloride-inhibited currents that were slightly but significantly stimulated by forskolin/IBMX (−1.40 ± 0.33 versus −1.85 ± 0.45 μA, n = 20, p = 0.003, Fig. 4A) and were not decreased by co-expression of CFTR in the absence of forskolin/IBMX (−1.40 ± 0.33 μA, n = 20 versus −1.13 ± 0.22 μA, n = 20, p = ns, Fig. 4A). However, activation of CFTR in co-injected oocytes decreased the functional expression of murine α human β γ ENaC (−1.13 ± 0.22 versus −0.68 ± 0.32 μA, n = 20, p = 0.002), and co-expression of CFTR with this ENaC chimera enhanced CFTR functional expression compared with oocytes injected with CFTR alone (−6.78 ± 1.43 μA, n = 20 versus −2.36 ± 0.62 μA, n = 19, p = 0.009, Fig. 4B). This behavior is largely consistent with that of mouse αβγ ENaC (Fig. 1).

In contrast, human α(A663) murine β γ ENaC behaved more like human α(A663)β γ ENaC. Human α(A663) murine β γ ENaC functional expression was not altered by forskolin/IBMX in oocytes injected with chimeric ENaC alone (−1.29 ± 0.25 versus −1.22 ± 0.23 μA, n = 21, p = ns, Fig. 4C) and was decreased by co-expression of CFTR prior to CFTR activation (−1.29 ± 0.25 μA, n = 21 versus −0.70 ± 0.12 μA, n = 24, p = 0.05, Fig. 4C). Also, as was the case for α-A663-hENaC (Fig. 2), activation of CFTR in co-injected oocytes did not alter functional expression of α(A663) murine β γ ENaC (−0.70 ± 0.12 versus −0.70 ± 0.10 μA, n = 24, p = ns, Fig. 4C), and co-expression of this ENaC chimera enhanced CFTR functional expression compared with oocytes injected with CFTR alone (−4.42 ± 1.12 μA, n = 24 versus −0.81 ± 0.16 μA, n = 21, p = 0.003, Fig. 4D). These data suggest that the α subunit of ENaC is an important determinant in the regulation of ENaC functional expression by activated CFTR.

C-terminal 20 Amino Acid Residues of an ENaC Determine Species Differences in ENaC Functional Response to CFTR Activation—The distal C-terminal amino acid sequences of the α subunits of mouse and rat ENaC are quite similar but diverge from that of the human α subunit (Fig. 5). We recently observed that the C-terminal 20 amino acid residues (residues 650–669) of α ENaC, when expressed at the C terminus of a murine/human α ENaC chimera in place of the 21 C-terminal amino acids of α mENaC (residues 679–699), were sufficient to confer functionality of the T663A polymorphism of α hENaC.2 Given that mENaC (Fig. 1 and Ref. 11) and rat ENaC (9), but not α-A663-hENaC (Fig. 2), have decreased functional expression in response to activation of CFTR in oocytes and that the determinant of this effect appears to be within α ENaC (Fig. 4), we examined regulatory interactions between CFTR and an ENaC chimera consisting of α-(murine 1–678, human 650–669, A663) murine β γ ENaC (Fig. 6). The functional expression of this chimeric ENaC was not influenced by forskolin/IBMX in oocytes injected with chimeric ENaC alone (−1.29 ± 0.36 versus −1.50 ± 0.41 μA, n = 17, p = ns, Fig. 6A), and the activity of

10 μM amiloride (−100 mV holding potential) were determined in oocytes expressing α-A663-hENaC or co-expressing α-A663-hENaC and CFTR prior to (closed bars) and following (open bars) stimulation with 10 μM forskolin, 500 μM IBMX. C, changes in whole cell currents (−100 mV holding potential) after stimulation with 10 μM forskolin, 500 μM IBMX that were not inhibited by 10 μM amiloride were determined in oocytes expressing CFTR alone (closed bars) and oocytes co-expressing CFTR and hENaC (open bars). Data obtained from the same CFTR/hENaC-co-injected oocytes are presented in A, B, and C. Means ± S.E. are illustrated. D and E, α-A663-βγ hENaC, where the β subunit contained a C-terminal V5 epitope tag, was expressed in oocytes either alone or together with CFTR. 24–48 h after injection surface biotinylation was performed (D) or whole oocyte lysates were prepared from oocytes (E) prior to or following treatment with 10 μM forskolin, 500 μM IBMX for 20 min as described under "Experimental Procedures." D, immunoblot of streptavidin-agarose-precipitated proteins probed with anti-V5 antisera to detect hENaC β-V5. A representative immunoblot and densitometry (mean ± S.E. of n = 3 independent experiments) are shown. Data were normalized to levels of V5 surface expression in oocytes expressing hENaC alone and not treated with forskolin/IBMX. E, immunoblot of whole oocyte lysate from equal numbers of solubilized oocytes probed with anti-V5 antisera to detect hENaC β-V5. A representative immunoblot of n = 3 independent experiments is shown. Forsk, forskolin.
expressing CFTR alone (4.71 enhanced CFTR functional expression compared with oocytes ns, Fig. 6). Co-injection of CFTR and the ENaC chimera A669, A663) murine/H9252/H9253 0.88/H11002 experiments) are shown. Data were normalized to levels of/H9252 23188 A 312). Data obtained from the same CFTR/chimeric ENaC-co-injected oocytes are presented in H9252 1.29 (increased by co-injection of CFTR prior to CFTR activation this ENaC chimera was modestly but not significantly decreased by co-injection of CFTR prior to CFTR activation (−1.29 ± 0.36 μA, n = 17 versus −0.83 ± 0.17 μA, n = 27, p = ns, Fig. 6A). Co-injection of CFTR and the ENaC chimera enhanced CFTR functional expression compared with oocytes expressing CFTR alone (−4.71 ± 1.12 μA, n = 27 versus −0.47 ± 0.11 μA, n = 15, p < 0.001, Fig. 6B). Most interestingly, functional expression of α-(murine 1–678, human 650–669, A663) murine β ENaC in co-injected oocytes was unchanged following activation of CFTR (−0.83 ± 0.17 versus −0.88 ± 0.15 μA, n = 27, p = ns, Fig. 6A). These data suggest that the C-terminal 20 residues of α ENaC are an important determinant of the regulation of ENaC by activated CFTR in Xenopus oocytes.

Co-expression of ΔF508-CFTR and hENaC—We (11) and others (15) have demonstrated an absence of regulatory interactions between ΔF508-CFTR and murine and rat ENaC, respectively, in Xenopus oocytes. However, regulatory interactions between ΔF508-CFTR and hENaC in oocytes have not been described. As our data suggest differences between the interregulation of wild type CFTR and hENaC versus wild type CFTR and mENaC, we sought to characterize regulatory interactions between ΔF508-CFTR and hENaC as well as the potential influence of the T663A functional polymorphism on these interactions (Fig. 7). Similar to our data of Fig. 2, forskolin/IBMX did not alter hENaC functional expression in oocytes injected with hENaC alone as is shown in Fig. 7A for α-T663-hENaC (−0.91 ± 0.18 versus −1.09 ± 0.26 μA, n = 15, p = ns) and Fig. 7B for α-A663-hENaC (−0.69 ± 0.10 versus −0.76 ± 0.11 μA, n = 15, p = ns). Similar to our observations with mENaC (11), co-injection of ΔF508-CFTR did not alter hENaC functional expression prior to activation of ΔF508-CFTR (α-T663-hENaC: −0.91 ± 0.18 μA, n = 15 versus −0.75 ± 0.11 μA, n = 27, p = ns; α-A663-hENaC: −0.69 ± 0.10 μA, n = 15 versus −0.55 ± 0.08 μA, n = 26, p = ns). Activation of ΔF508-CFTR in co-injected oocytes slightly but significantly increased α-A663-hENaC functional expression (−0.55 ± 0.08 versus −0.70 ± 0.10 μA, n = 27, p = 0.007, Fig. 7B) but did not significantly increase α-T663-hENaC functional expression (−0.75 ± 0.11 versus −0.93 ± 0.18 μA, n = 27, p = ns, Fig. 7A).

This ENaC chimera was modestly but not significantly decreased by co-injection of CFTR prior to CFTR activation (−1.29 ± 0.36 μA, n = 17 versus −0.83 ± 0.17 μA, n = 27, p = ns, Fig. 6A). Co-injection of CFTR and the ENaC chimera enhanced CFTR functional expression compared with oocytes expressing CFTR alone (−4.71 ± 1.12 μA, n = 27 versus −0.47 ± 0.11 μA, n = 15, p < 0.001, Fig. 6B). Most interestingly, functional expression of α-(murine 1–678, human 650–669, A663) murine β ENaC in co-injected oocytes was unchanged following activation of CFTR (−0.83 ± 0.17 versus −0.88 ± 0.15 μA, n = 27, p = ns, Fig. 6A). These data suggest that the C-terminal 20 residues of α ENaC are an important determinant of the regulation of ENaC by activated CFTR in Xenopus oocytes.

Co-expression of ΔF508-CFTR and hENaC—We (11) and others (15) have demonstrated an absence of regulatory interactions between ΔF508-CFTR and murine and rat ENaC, respectively, in Xenopus oocytes. However, regulatory interactions between ΔF508-CFTR and hENaC in oocytes have not been described. As our data suggest differences between the interregulation of wild type CFTR and hENaC versus wild type CFTR and mENaC, we sought to characterize regulatory interactions between ΔF508-CFTR and hENaC as well as the potential influence of the T663A functional polymorphism on these interactions (Fig. 7). Similar to our data of Fig. 2, forskolin/IBMX did not alter hENaC functional expression in oocytes injected with hENaC alone as is shown in Fig. 7A for α-T663-hENaC (−0.91 ± 0.18 versus −1.09 ± 0.26 μA, n = 15, p = ns) and Fig. 7B for α-A663-hENaC (−0.69 ± 0.10 versus −0.76 ± 0.11 μA, n = 15, p = ns). Similar to our observations with mENaC (11), co-injection of ΔF508-CFTR did not alter hENaC functional expression prior to activation of ΔF508-CFTR (α-T663-hENaC: −0.91 ± 0.18 μA, n = 15 versus −0.75 ± 0.11 μA, n = 27, p = ns; α-A663-hENaC: −0.69 ± 0.10 μA, n = 15 versus −0.55 ± 0.08 μA, n = 26, p = ns). Activation of ΔF508-CFTR in co-injected oocytes slightly but significantly increased α-A663-hENaC functional expression (−0.55 ± 0.08 versus −0.70 ± 0.10 μA, n = 27, p = 0.007, Fig. 7B) but did not significantly increase α-T663-hENaC functional expression (−0.75 ± 0.11 versus −0.93 ± 0.18 μA, n = 27, p = ns, Fig. 7A).
Again the lack of significance of the increase for \( \alpha \)-T663-hENaC may be related to the larger standard errors in these data compared with those of \( \alpha \)-A663-hENaC.  

\( \Delta F508 \)-CFTR functional expression was enhanced by co-injection of \( \alpha \)-T663-hENaC (co-injected: \(-0.73 \pm 0.13 \mu A, n = 27\), \textit{versus} injected with \( \Delta F508 \)-CFTR alone: \(-0.30 \pm 0.05 \mu A, n = 20, p = 0.008\)) but was not significantly enhanced by \( \alpha \)-A663-hENaC (co-injected: \(-0.66 \pm 0.15 \mu A, n = 26\), \textit{versus} injected with \( \Delta F508 \)-CFTR alone: \(-0.43 \pm 0.08 \mu A, n = 23, p = ns\), Fig. 7C). These data for \( \alpha \)-A663-hENaC are similar to our published data with mENaC and \( \Delta F508 \)-CFTR (11) where co-expression of mENaC did not enhance \( \Delta F508 \)-CFTR functional expression, but the data for \( \alpha \)-T663-hENaC contrast with those for mENaC. While there was an \(-2.5\)-fold statistically significant enhancement of \( \Delta F508 \)-CFTR-mediated current in \( \alpha \)-T663-hENaC-co-injected oocytes, which is similar to the \(-1\)-fold increase in wild type CFTR expression with co-injection of hENaC (Fig. 2), the physiologic significance of this increase is not clear as the magnitude of this enhanced \( \Delta F508 \)-CFTR functional expression remains only \(-1/10\) of that of wild type CFTR in hENaC-co-injected oocytes.

We also assessed the effect of co-injection of \( \Delta F508 \)-CFTR on the whole oocyte expression of \( \alpha \)-A663-hENaC (\( \beta \)-V5). Unlike for wild type CFTR (Fig. 2E), co-injection of \( \Delta F508 \)-CFTR did not decrease the whole oocyte expression of hENaC (\( \beta \)-V5). Furthermore forskolin/IBMX stimulation did not alter hENaC (\( \beta \)-V5) whole oocyte expression (Fig. 7D). These data suggest that the decrease in hENaC (\( \beta \)-V5) whole oocyte expression with co-injection of wild type CFTR (Fig. 2E) is not a result of competition for cellular translational or biosynthetic machinery as injection of a similar amount of \( \Delta F508 \)-CFTR cRNA did not decrease hENaC (\( \beta \)-V5) whole oocyte expression.

**DISCUSSION**

Defects in or the absence of CFTR leads to increased ENaC activity in the CF airway epithelia, resulting in increased solute and liquid absorption, decreased airway surface volume, and decreased mucociliary clearance (26–28). Regulatory interactions between CFTR and ENaC are complex, and our data address a number of issues regarding these interactions. We
observed that CFTR-induced inhibition of ENaC functional expression correlates with changes in ENaC surface expression, suggesting that an element of the regulatory interactions between CFTR and ENaC occurs at the level of plasma membrane expression of ENaC and presumably at the level of intracellular trafficking of this channel. Furthermore the extent of ENaC inhibition following CFTR activation is species-specific, and the distal C terminus of the α subunit of ENaC has a key role in determining species specificity.

Correlation of ENaC Functional and Surface Expression in Oocytes—A number of groups have suggested that regulatory interactions between CFTR and ENaC in airway epithelia are replicated in Xenopus oocytes. Functional expression of CFTR has been associated with an inhibition of functional ENaC expression (7, 9–11, 15) that was due, in part, to a decrease in ENaC open probability (29, 30). We observed that co-expression of CFTR decreased both the total cellular pool and cell surface pool of mouse and human ENaC, suggesting that CFTR may alter ENaC biosynthesis, trafficking to the oocyte membrane, or retrieval from the plasma membrane. A further decrease in mouse ENaC currents was observed following CFTR activation that correlated with a decrease in mENaC surface expression. One group has suggested that the inhibition of ENaC functional expression with CFTR activation may be due to a series resistor error that is potentially inherent to TEV experiments in oocytes (20). However, our previous data (11, 22) and experiments presented here (Fig. 1) demonstrating that decrease in mENaC currents following CFTR activation correlate with mENaC surface expression suggest that the decrease in mENaC activity following CFTR activation does not reflect a series resistor error.

Species-specific Regulatory Interactions between CFTR and ENaC and the Role of the C Terminus of α ENaC—Our data suggest that there are differences in the regulation of mouse and human ENaC by activated CFTR in oocytes. Co-expression of CFTR with mouse or human ENaC was associated with a reduction in ENaC activity and surface expression. Although CFTR activation led to a further decrease in functional activity of mouse ENaC, either no decrease (α-T663-hENaC) or a modest decrease (α-T663-hENaC) in human ENaC-mediated currents was observed following CFTR activation. Furthermore, when mouse ENaC was modified by replacing the C-terminal 21 residues of the mouse α subunit with the corresponding distal C terminus of the human α (murine 1–678, human 650–669, A663), the resulting chimera was not inhibited following CFTR activation. These data suggest that the C terminus of α ENaC is a critical determinant for the differential responses of mouse and human ENaC to activated CFTR and complement previous findings indicating that the C terminus of the β subunit and the N terminus of the γ subunit of rat ENaC are required for functional interactions between CFTR and ENaC.

Human αT663A is a common polymorphism (31, 32), and our recent data suggest that it is associated with differences in functional activity in oocytes.2 However, the corresponding mouse αT692A polymorphism was not associated with differences in mENaC functional activity.2 Replacement of the C-terminal 21 residues of the mouse α subunit with the corresponding distal C-terminal 20 residues of the human α (murine 1–678, human 650–669, A663) restored the functional differences that were observed with the human αT663A polymorphism.2 With the increasing interest in potential modifier genes for the CF phenotype and as ENaC function is clearly implicated in CF airway pathophysiology, hENaC functional polymorphisms are candidates for modifying the CF phenotype (26–28, 33). One study presented in abstract form examined the prevalence of the α-A663-hENaC polymorphism in a group of 80 CF patients and found an allele frequency that was similar to the general Caucasian population. However, differences in pulmonary function between patients with α-A663- and α-T663-hENaC were not observed (34), although this study may have been underpowered to detect small differences.

Regulatory Interactions between ΔF508-CFTR and ENaC—We observed that co-expression of an appropriately folded and trafficked CFTR, prior to its activation by forskolin/IBMX, decreases whole cell, surface, and functional expression of mouse and human ENaC. In contrast, co-expression of ΔF508-CFTR did not decrease hENaC whole oocyte or functional expression.
Species-specific Regulation of ENaC by CFTR

**Procedures.**

A representative immunoblot of prior to (closed bars) and following (open bars) stimulation with 10 \( M \) forskolin, 500 \( M \) IBMX. A, whole cell currents sensitive to inhibition by 10 \( M \) amiloride (−100 mV holding potential) were determined in oocytes expressing α-T663-hENaC or co-expressing α-T663-hENaC and ΔF508-CFTR prior to (closed bars) and following (open bars) stimulation with 10 \( M \) forskolin, 500 \( M \) IBMX. B, whole cell currents sensitive to inhibition by 10 \( M \) amiloride (−100 mV holding potential) were determined in oocytes expressing α-A663-hENaC co-expressing α-A663-hENaC and ΔF508-CFTR prior to (closed bars) and following (open bars) stimulation with 10 \( M \) forskolin, 500 \( M \) IBMX. C, changes in whole cell currents (−100 mV holding potential) after stimulation with 10 \( M \) forskolin, 500 \( M \) IBMX that were not inhibited by 10 \( M \) amiloride are illustrated (gray bars). Data obtained from the same ΔF508-CFTR/hENaC co-injected oocytes are presented in A, B, and C. Means ± S.E. are illustrated. D, α-A663-β-V5 hENaC, where the β subunit contained a C-terminal V5 epitope tag, was expressed in oocytes either alone or together with ΔF508-CFTR. 24–48 h after injection, whole cell lysates from equal numbers of oocytes were prepared from oocytes prior to or following treatment with 10 \( M \) forskolin, 500 \( M \) IBMX for 20 min and then subjected to immunoblot analysis with anti-V5 antiserum to detect hENaC β-V5 as described under “Experimental Procedures.” A representative immunoblot of \( n = 3 \) independent experiments is shown. Forsk, forskolin.

ΔF508-CFTR is poorly trafficked to the cell surface and likely does not effectively compete for trafficking machinery (in distinction from translational and biosynthetic machinery) with ENaC. These data are consistent with a model in which CFTR decreases ENaC functional expression, in part, by reducing ENaC trafficking to the plasma membrane, while absent or poorly trafficked CFTR (like ΔF508-CFTR) does not hinder ENaC trafficking.

**Summary**—Our results and previous studies suggest that regulation of ion transport in the airway epithelia is complex. Understanding the regulatory interactions between wild type or mutant CFTR and ENaC will likely impact strategies designed to improve mutant CFTR function in CF. That hENaC behaves differently than mENaC in oocytes in response to activated CFTR and that a region of α hENaC responsible for this differential response is within its C-terminal 20 amino acid residues will likely, with future investigations, provide additional insight into this critical interaction of epithelial ion transporters.

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