Identification of the Cystic Fibrosis Transmembrane Conductance Regulator Domains That Are Important for Interactions with ROMK2*

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In addition to functioning as a cAMP-activated chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR) plays an important role in conferring regulatory properties on other ion channels. It is known, with respect to CFTR regulation of ROMK2 (renally derived K\textsubscript{ATP} channel), that the first transmembrane domain and the first nucleotide binding fold domain (NBF1) of CFTR are necessary for this interaction to occur. It has been shown that under conditions that promote phosphorylation, the ROMK2-CFTR interaction is attenuated. To elucidate the complex nature of this interaction, CFTR constructs were co-expressed with ROMK2 in Xenopus oocytes, and two microelectrode voltage clamp experiments were performed. Although the second half of CFTR can act as a functional chloride channel, our results suggest that it does not confer glibenclamide sensitivity on ROMK2, as does the first half of CFTR. The attenuation of the ROMK2-CFTR interaction under conditions that promote phosphorylation is dependent on at least the presence of the R domain of CFTR. We conclude that transmembrane domain 1, NBF1, and the R domain are the CFTR domains involved in the ROMK2-CFTR interaction and that NBF2 and transmembrane domain 2 are not essential. Lastly, the R domain of CFTR is necessary for the attenuation of the ROMK2-CFTR interaction under conditions that promote phosphorylation.

The CFTR\textsuperscript{1} gene encodes for a multifunctional transmembrane protein that is both a cAMP-activated chloride channel and a regulator of other ion channels (1, 2). It is a member of the ATP binding cassette superfamily, and like other members of this group it is comprised of transmembrane spanning domains and nucleotide binding folds (NBFs); however, it has a regulatory domain (R domain), which is unique to CFTR (3). The domains of CFTR involved in its regulation of other ion channels may be functionally distinct from those involved in its Cl\textsuperscript{−} channel function.

CFTR expression has been linked to protein kinase A regulation of the outwardly rectifying chloride channel (ORCC), the epithelial sodium channel (ENaC), and a K\textsubscript{ATP} channel, ROMK2 (4, 5, 6). The first nucleotide binding domain of CFTR and the R domain are important for its regulation of ORCC and ENaC (7, 8). For CFTR to regulate ROMK2 channels, \textit{in vitro}, an intact NBF1 is required (9). Although many groups are investigating the role of NBF1 and NBF2 with regard to channel gating, their role with regard to regulation of other ion channels has yet to be elucidated. For instance, it is not known if NBF2 can have the same effect as NBF1 with respect to regulation of ROMK2 channels.

Many of the disease-producing mutations are located in the NBF domains of CFTR, predominantly within NBF1. These mutations are often associated with abnormal CFTR channel activity, as well as abnormal kinase regulation of ORCC and ENaC. For example, the protein kinase activation of ORCCs depends on CFTR expression and is affected by mutations in NBF1, as is the cAMP-dependent protein kinase-dependent inhibition of ENaC (4, 10). The majority of cAMP-dependent protein kinase phosphorylation sites are located in the R domain of CFTR (11). It is not known what role the phosphorylation-rich R domain plays in these processes. With respect to the ROMK2-CFTR interaction, it has been shown that this interaction is attenuated under conditions that promote phosphorylation. It is not known if the R domain of CFTR is important in this phosphorylation-dependent attenuation (6).

To examine further the structure-function relationships of the NBFs and the R domain, with respect to the ability of CFTR to act as a regulator of ROMK2 channels, CFTR constructs (see Fig. 1) and ROMK2 were co-expressed in Xenopus oocytes, and two-microelectrode voltage clamp techniques were applied. First, to determine if the R domain of CFTR is involved in the phosphorylation process that attenuates ROMK2-CFTR coupling, we co-expressed ROMK2 with CFTR constructs containing truncations before and after the R domain. Next, we substituted SUR1 for CFTR in our ROMK2-CFTR co-expression experiments to learn more about the dephosphorylation/phosphorylation-dependent ROMK2-CFTR interaction. SUR1 is an ABC transporter that does not contain an R domain and is known to regulate another K\textsubscript{ATP} channel (12). Lastly, we investigated if CFTR constructs containing NBF2 alone can confer glibenclamide on ROMK2.

**EXPERIMENTAL PROCEDURES**

Preparation of Oocytes for Voltage Clamp Experiments—Stage V-VI Xenopus laevis oocytes were isolated and injected with RNA as described previously (6). RNA concentrations were adjusted such that an

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§ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBF, nucleotide binding folds; ORCC, outwardly rectifying chloride channel; ENaC, epithelial sodium channel; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthene; SUR1, sulfonyl-urea receptor 1.
Membrane level with 0.278 the second half of CFTR forms a functional chloride channel (18). In addition, it has been demonstrated that D835X and CFTR-K593X are expressed at membrane level, in varying expression systems (9, 19). In addition, it has been demonstrated that D835X and CFTR-K593X are expressed at membrane level, in varying expression systems (9, 16). CFTR, the CFTR clone pBQ4.7 by standard oligonucleotide-directed mutagenesis kit as described previously (16, 17). The oligonucleotides used for mutagenesis were CFTR-K593X, 5'AGCAAACTAGG-3' and CFTR-D835X, 5' TTTGTGC-3'. 9

To address the possibility that the current observed under conditions that promote phosphorylation, was stimulated Cl current and not K current, a subset of experiments were subjected to an alternate protocol. It contained an additional 3-min barium pulse after the initial control period. These experiments were performed on oocytes expressing ROMK2/CFTR-K593X and ROMK2/CFTR-D835X and showed that the current was Ba2+-sensitive K+ current and not stimulated Cl current.

For oocytes injected with ROMK2 cRNA, only cells expressing >500 nA whole-cell current were selected. Experiments were discounted if a stable whole-cell current was not obtained (>10% variation in control current). Data were compared using a paired Student’s t test within a single experiment or with an analysis of variance (ANOVA) when comparing multiple conditions and/or groups. A p value <0.05 was considered significant.

**CFTR Constructs and Method of Mutagenesis—**Cells were injected with ROMK2 alone, ROMK2/CFTR-WT, ROMK2/SUR, ROMK2/CFTR-D835X (a CFTR construct with an intact nucleotide binding fold and a functional R domain), ROMK2/CFTR-K593X (a CFTR construct with an intact nucleotide binding fold but no R domain), or ROMK2/RT2N2CFTR (a CFTR construct containing the R domain, the second nucleotide binding fold domain, the second transmembrane domain, and the first 159 bases of CFTR-WT so as to include the Kozak methionine for translation initiation) (Fig. 1). Site-directed mutagenesis was performed as described by Kunkel et al. (15). Mutations were created in the CFTR clone pBQ4.7 by standard oligonucleotide-directed mutagenesis of single-stranded DNA using the Muta-Gen Phagemid in vitro mutagenesis kit as described previously (16, 17). The oligonucleotides used for mutagenesis were CFTR-K593X, 5'-CTGTTAATCTGATGCT-AGCAAATCGA-3' and CFTR-D835X, 5'-CACGAAAAATCTCATCGT-GCCCTCTACTGATAGGAGKCCAGACCCCTTTTGTGC-3'. The RT2N2CFTR mutation was constructed as outlined by Devidas et al. (18). The mutations were confirmed by DNA sequencing. To prepare cRNA, the plasmid was linearized with appropriate restriction enzymes and transcribed in vitro using T7 RNA polymerase (mMessage mMachine, Ambion) as described previously (9, 16). CFTR-D835X and CFTR-K593X are expressed at membrane level, in varying expression systems (9, 19). In addition, it has been demonstrated that the second half of CFTR forms a functional chloride channel (18). These experiments were repeated, and confirmed RT2N2CFTR was expressed at membrane level with 0.278 ± 0.041 μM of current (V_HOLD = −40 mV using a two-microelectrode voltage clamp) (p < 0.05 when compared with an un.injected oocyte) (Fig. 2).

**RESULTS**

**Treatment with FSK/IBMX, a Mixture That Increases Intracellular cAMP, Attenuates the ROMK2-CFTR Interaction—**To assess how conditions that promote phosphorylation affect the ROMK2-CFTR interaction, ROMK2 and CFTR-WT were co-expressed in Xenopus oocytes and exposed to 100 μM/1 mM FSK/IBMX. Under these conditions the majority of observed current was Ba2+-sensitive K+ current. This current was insensitive to glibenclamide (9 ± 2.9% inhibition, n = 9) (Fig. 3). This finding was similar to the glibenclamide insensitivity observed when ROMK2 was expressed alone (17 ± 4.3% inhibition under basal conditions, n = 11, 26 ± 6% inhibition with FSK/IBMX, n = 6) (Table I, Fig. 4). These data support the previous observation made in excised patches that cAMP-dependent protein kinase phosphorylation can uncouple the ROMK2-CFTR interaction. This finding can be contrasted to the enhanced glibenclamide sensitivity of the resultant K+ current when ROMK2 is co-expressed with CFTR-WT in the absence of FSK/IBMX (55 ± 5.7% inhibition, n = 19) (p < 0.0001) (Table I, Fig. 4). These data suggest that phosphorylation of CFTR, ROMK, or both uncouples this interaction and led us to investigate which domains of CFTR participate in this process.

**The Presence of an R Domain Is Required for Attenuation of the ROMK2-CFTR Interaction under Conditions That Promote Phosphorylation—**To investigate, under conditions that maximize intracellular cAMP, which domain of CFTR is important for the attenuated ROMK2-CFTR interaction, CFTR constructs with mutations involving the R domain were chosen to be co-expressed with ROMK2. When ROMK2 was co-expressed with a CFTR construct containing transmembrane domain 1 and NBF1 without an R domain (CFTR-K593X) and exposed to FSK/IBMX, the resultant Ba2+-sensitive K+ current was in-
transporter that does not contain the R domain (20, 21), was substituted for CFTR in the ROMK2-ABC transporter interaction. SUR1 can substitute for CFTR in imparting sulfonylurea sensitivity on ROMK2 potassium current with 55 ± 9% inhibition, (n = 10) (Table I, Fig. 4), in agreement with previous findings (22). After stimulation with FSK/IBMX the ROMK2/SUR interaction retained sulfonylurea sensitivity, that is, 55 ± 6.5% of the Ba²⁺-sensitive K⁺ current was inhibited by glibenclamide (n = 7). This suggests that the ROMK2/SUR proteins remain functionally interactive under conditions that increase cAMP-dependent phosphorylation unlike the ROMK/KFTR-WT and ROMK2/CFTR-D835X proteins. However, in contrast to the ROMK2/CFTR-K593X currents, glibenclamide inhibition is notenhanced after cAMP stimulation.

Can the Second Half of CFTR Substitute for the First Half in Conferring Channel Regulation upon ROMK2?—To assess if the second half of the CFTR protein can confer properties of channel regulation on ROMK2, a CFTR construct comprised of the second half of the molecule was substituted for CFTR-WT in the ROMK2-CFTR interaction. When RT2N2CFTR was co-expressed with ROMK2, the resultant Ba²⁺-sensitive K⁺ current remained glibenclamide insensitive (19 ± 3.1% inhibition, n = 10) (Table I, Fig. 5). This suggests that co-expression of the RT2N2CFTR construct with ROMK2 does not alter the sulfonylurea sensitivity of the K⁺ current. Conditions that promote phosphorylation did not alter the glibenclamide insensitive

To further assess the effect of an R domain on this interaction, SUR1 (pancreatic β cell sulfonylurea receptor), an ABC

A

B

C

D

E

Fig. 2. Effect of glibenclamide on whole cell Ba²⁺-sensitive K⁺ currents for ROMK2 when co-expressed with CFTR-D835X, CFTR-K593X, or RT2N2CFTR under basal conditions (in the absence of FSK/IBMX) (A, B, and E) in Xenopus oocytes, using two-microelectrode voltage clamp techniques. Shown are outward currents elicited at V̅_HOLD = 60 mV plotted against time. After an initial control equilibration period (5 min), the oocyte was perfused with control solution containing 0.5 mM glibenclamide (Glib). After 14 min of glibenclamide exposure the oocyte was then perfused with 2 mM barium (to determine total K⁺ current), and this was followed with a wash off period with control solution. An identical protocol was followed for each condition with 100 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine added to all solutions, including preincubation. In a subset of experiments (C and D), to determine that the majority of current after FSK/IBMX application was still K⁺ current and not Cl⁻ current, generated from activated CFTR, a barium pulse is applied and subsequently washed off with control solution before the application of glibenclamide.

hhibited by glibenclamide (66 ± 4.3% inhibition, n = 18) (Table I, Figs. 2–4). Without cAMP stimulation glibenclamide inhibited 46 ± 4.5% (n = 6) of K⁺ current when ROMK2 and CFTR-K593X were co-expressed, which is similar to previous findings. These data suggest that CFTR constructs without the R domain or NBF2 can influence ROMK2 currents. In addition the data suggest there is no attenuation of the interaction by cAMP stimulation when the R domain is absent. Furthermore, after cAMP stimulation there is a significant enhancement of glibenclamide effect on ROMK2/CFTR-K593X currents (Table I).

However, when ROMK2 and CFTR-D835X (a CFTR construct with an intact nucleotide binding fold and a functional R domain) were co-expressed and stimulated with the cAMP-enhancing mixture, the resultant K⁺ current demonstrated an attenuated sulfonylurea response (26 ± 5% inhibition, n = 16) (Fig. 4). In the absence of FSK/IBMX there was a 48 ± 10% inhibition of K⁺ current when ROMK2 and CFTR-D835X were co-expressed (n = 9) (Table I, Figs. 2–4). Taken together, these data suggest that phosphorylation uncouples the ROMK2/CFTR interaction in the presence of an R domain.

To further assess the effect of an R domain on this interaction, SUR1 (pancreatic β cell sulfonylurea receptor), an ABC

Fig. 3. A representative family of whole cell currents from oocytes injected with either ROMK2/CFTR-D835X or ROMK2/CFTR-K593X, and traces under basal conditions (in the absence of FSK/IBMX) and after stimulation with FSK/IBMX (100 μM/1 mM) are compared. Oocytes were held at −60 mV and thereafter pulsed for 20 ms from −100 to 40 mV in 20-mV increments, and currents elicited are shown for control, after glibenclamide and during barium application for each condition. It demonstrates that in ROMK2/CFTR-D835X-injected oocytes, glibenclamide inhibition of whole cell potassium current when ROMK2 and CFTR-D835X were co-expressed, which is similar to previous findings (22). After stimulation with FSK/IBMX the ROMK2/SUR interaction retained sulfonylurea sensitivity, that is, 55 ± 6.5% of the Ba²⁺-sensitive K⁺ current was inhibited by glibenclamide (n = 7). This suggests that the ROMK2/SUR proteins remain functionally interactive under conditions that increase cAMP-dependent phosphorylation unlike the ROMK/KFTR-WT and ROMK2/CFTR-D835X proteins. However, in contrast to the ROMK2/CFTR-K593X currents, glibenclamide inhibition is notenhanced after cAMP stimulation.

Can the Second Half of CFTR Substitute for the First Half in Conferring Channel Regulation upon ROMK2?—To assess if the second half of the CFTR protein can confer properties of channel regulation on ROMK2, a CFTR construct comprised of the second half of the molecule was substituted for CFTR-WT in the ROMK2-CFTR interaction. When RT2N2CFTR was co-expressed with ROMK2, the resultant Ba²⁺-sensitive K⁺ current remained glibenclamide insensitive (19 ± 3.1% inhibition, n = 10) (Table I, Fig. 5). This suggests that co-expression of the RT2N2CFTR construct with ROMK2 does not alter the sulfonylurea sensitivity of the K⁺ current. Conditions that promote phosphorylation did not alter the glibenclamide insensitive
for each condition. Percent inhibition is barium-sensitive whole-cell current inhibited by the application of 500 nM glibenclamide, whole-cell current (% Inhibition with glibenclamide) injected with ROMK2 alone, ROMK2 co-expressed with a designated CFTR construct or co-expressed with SUR. n is the number of experiments for each condition. Percent inhibition is barium-sensitive whole-cell current inhibited by the application of 500 μM glibenclamide.  

| Construct          | Basal conditions | Whole cell current | % Inhibition with glibenclamide | FSK/IBMX | Whole cell current | % Inhibition with glibenclamide |
|--------------------|------------------|--------------------|---------------------------------|----------|--------------------|---------------------------------|
| ROMK2              | ±5.17 ± 0.94 (n = 11) | 17 ± 4.3           | 4.27 ± 1.2 (n = 6)              | ROMK2    | ±3.45 ± 0.86 (n = 9) | 55 ± 5.7 | 26.5 ± 6 |
| R2/CFTR-WT         | 4.22 ± 1.28 (n = 19)  | 55 ± 5.7           | 4.21 ± 1.17 (n = 16)            | R2/CFTR-WT | ROMK2/CFTR-D835X   | 48 ± 10  | 8.8 ± 2.9 |
| R2/D835X           | 3.68 ± 1.16 (n = 9)   | 46 ± 10           | 1.74 ± 0.257 (n = 6)            | R2/T2N2CFTR | ROMK2/K593X      | 19.6 ± 3.1 | 29.5 ± 5.3 |
| R2/K593X           | 3.18 ± 0.36 (n = 10) | 46 ± 4.5          | 2.0 ± 0.28 (n = 18)             | R2/SUR   | ±2.02 ± 0.84 (n = 10) | 55 ± 5.9 | 65.9 ± 4.3 |
| R2/SUR             | ±2.02 ± 0.84 (n = 10) | 55 ± 5.9          | 1.99 ± 0.65 (n = 9)             | Uninjected | ±0.12 ± 0.02 (n = 8) | 0 | 55 ± 6.4 |

a Average whole cell current (V_m = −60 mV), under basal conditions, the using two-microelectrode voltage clamp technique, observed in oocytes injected with ROMK2 alone, ROMK2 co-expressed with Suggested construct or co-expressed with with SUR. n is the number of experiments for each condition. Percent inhibition is barium-sensitive whole-cell current inhibited by the application of 500 μM glibenclamide.

b Average whole cell current (V_m = −60 mV), after stimulation with FSK/IBMX (100 μM/1 μM).

c p value <0.05 when comparing % glibenclamide inhibition of whole cell K+ current under basal conditions and after stimulation with FSK/IBMX for R2/CFTR-WT where there is an attenuation of inhibitory response with FSK/IBMX.

d p value <0.05 when comparing % glibenclamide inhibition of whole cell K+ current under basal conditions and after stimulation with FSK/IBMX for R2/D835X where there is an enhancement of inhibitory response with FSK/IBMX.

DISCUSSION

Both the first and second halves of the CFTR protein can act as functional chloride channels (18, 23); however, they do not have the same function when it comes to conferring properties of channel regulation on ROMK2 channels. The first nucleotide binding fold along with the first transmembrane domain is necessary for the ROMK2-CFTR interaction to occur (9); however, the attenuation of the ROMK2-CFTR interaction, under conditions of phosphorylation, only occurs in the presence of an R domain. It suggests that the R domain is essential for this coupling to take place; however, it is necessary for the regulation of this interaction. The data presented also suggest that NBF2 is not involved in the ROMK2-CFTR interaction.

It is unknown whether the ability of CFTR to act as a channel regulator is because of a direct protein-protein interaction or another process. Studies on the CFTR regulation of ORCC suggest that the NBF1 of CFTR is an essential domain.
for this interaction (7). Investigators postulate that there is direct protein-protein interaction involving NBF1 and ORCC, but no direct evidence has been provided. Evidence of a direct interaction between CFTR and other channel proteins is provided by the analysis of CFTR domains and recombinant ENaC subunits in yeast two hybrid assays. These assays demonstrate that NBF1 and the R domain of CFTR interact directly with the C-terminal tail of α-recombinant ENaC (8).

CFTR may be coupled directly with ROMK2 in the cell membrane, providing a missing domain to the cloned K⁺ channel and thus restoring the sulfonylurea sensitivity seen in native tissue (25). Such a coupling process would be similar to the and thus restoring the sulfonylurea sensitivity seen in native type, it has been suggested that the severity of pulmonary disease in cystic fibrosis may be primarily associated with the otype, it has been suggested that the severity of pulmonary disease in cystic fibrosis may be primarily associated with the regulatory properties of CFTR (29); thus understanding the regulatory properties of CFTR is crucial to potential future interventions for cystic fibrosis.

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