Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel activated by protein kinase A (PKA) phosphorylation on the regulatory (R) domain. Phosphorylation at several R domain residues stimulates ATP-dependent channel openings and closings, termed channel gating. To explore the protein segment responsible for channel potentiation and PKA-dependent activation, deletion mutations were constructed by removing one to three protein segments of the R domain including residues 708–759 (ΔR708–759), R760–783, and R784–835, each of which contains one or two PKA phosphorylation sites. Deletion of R708–759 or R760–783 had little effect on CFTR gating, whereas all mutations lacking R784–835 reduced CFTR activity by decreasing the mean burst duration and increasing the interburst interval (IBI). The data suggest that R784–835 plays a major role in stimulating CFTR gating. For ATP-associated regulation, ΔR784–835 had minor impact on gating potentiation by 2′dATP, CaATP, and pyrophosphate. Interestingly, introducing a phosphorylated peptide matching R784–835 shortened the IBI of ΔR708–783-CFTR. Consistently, ΔR708–814 had not ΔR784–814 enhanced IBI, whereas both reduced mean burst duration. These data suggest that the entirety of R784–835 is required for stabilizing the open state of CFTR; however, R815–835 through interactions with the channel, is dominant for enhancing the opening rate. Of note, PKA markedly decreased the IBI of ΔR708–783-CFTR. Conversely, the IBI of ΔR708–814–CFTR was short and PKA-independent. These data reveal that for stimulating CFTR gating, PKA phosphorylation may relieve R784–814-mediated autoinhibition that prevents IBI shortening by R815–835. This mechanism may elucidate how the R domain potentiates channel gating and may unveil CFTR stimulation by other protein kinases.

Cystic fibrosis is a genetic disease caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) \(^{2}\) (1). CFTR is an epithelial Cl\(^–\) channel composed of two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain (1, 2). It is well-known that cAMP-dependent PKA phosphorylation on the R domain activates the CFTR Cl\(^–\) channel (3). Then cycles of ATP binding and hydrolysis at one or two ATPase sites in the interface of two NBDs control CFTR openings and closings, termed channel gating (2). Gating motions are initiated by ATP-induced NBD dimerization, subsequently leading to structural rearrangements within MSDs (4–7). Finally, dephosphorylation of CFTR by phosphatases ceases channel activity (3). 10 PKA phosphorylation sites are found in the R domain (1), but how they control CFTR channel gating remains unclear.

The R domain is mostly unstructured (8) and may include residues from 634 to 835 (R634–835) (3). However, the ΔRS660A mutation that deletes a large part of the R domain (ΔR708–835) generates substantial basal activity so that this mutation greatly attenuates cAMP-stimulated CFTR current (9) and PKA-dependent CFTR activation (10). These findings suggest that R708–835 may contain inhibitory protein segments that directly prohibit CFTR activation. Interestingly, the ΔRS660A mutation also greatly reduces the open probability (\(P_\text{o}\)) of CFTR (11–13), whereas the phosphorylated peptide that matches R645–834 in the R domain evidently enhances the Cl\(^–\) current and \(P_\text{o}\) of ΔRS660A-CFTR because of an increase in the channel opening rate (11). These data suggest that the phosphorylated R domain is able to stimulate the channel activity of CFTR. In addition, previous work found that R841–838 containing no PKA phosphorylation site is also required for stimulating CFTR gating (14).

PKA phosphorylation sites Ser\(^{768}\), Ser\(^{795}\), and Ser\(^{812}\) in the R domain seemingly are important for regulating CFTR gating (2). Evidence shows that the S768A mutation enhances the

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\(^{2}\) The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; IBI, interburst interval; MBD, mean burst duration; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R domain, regulatory domain; PKA, protein kinase A; ANOVA, analysis of variance; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
stimulation of CFTR activity by isobutylmethylxanthine (15) and PKA (16). S768A also increases the $P_o$ of CFTR (16–18). By sequentially adding phosphate groups to serine or threonine residues (10, 16, 20), PKA phosphorylation enhances NBD dimerization (21) and alters interaction patterns of the R domain with NBDs (6, 22, 23), intracellular loop 3 (18, 24), front and back halves of SplitΔR-CFTR (25), and different intracellular molecules such as the SLC26A3 sulfate transporter and anti-sigma factor antagonist domain (22). These data suggest that multiple CFTR domains may mediate phosphorylation-induced gating stimulation.

Because phosphorylation alters CFTR conformation (6, 19) and the interaction sites of the R domain (6, 7, 22, 23), an R domain segment may regulate CFTR activity (e.g. inhibition or stimulation) differently before and after PKA phosphorylation. A caveat is that previous studies using site-directed mutations (10, 11, 16–18, 24) for prohibiting PKA phosphorylation at one or several serine residues may also disturb phosphorylation-induced peptide movements and interactions. In other words, these site-directed mutations may eliminate or alter the postphosphorylation function of target residues, but they may also retain or modify their prephosphorylation actions. To avoid this confounding factor, deletion mutations were used in this study for removing all pre- and postphosphorylation function of residues.

To investigate the segmental function of the R domain in regulation of CFTR gating, this study first searched which protein segment plays a major role in regulating CFTR gating. Then the study further explored whether the R domain-mediated stimulation is caused by ATP-associated gating regulation and how PKA phosphorylation elicits the gating stimulation.

**Results**

**Segmental function of the R domain in ATP-dependent CFTR gating**

Similar to previous work (26), the R domain was divided into three protein segments $R_1$ ($R_{708–759}$ residues from positions 708 to 759), $R_2$ ($R_{760–783}$), and $R_3$ ($R_{784–835}$) (Fig. 1A). All CFTR mutants in this study included a S660A mutation for data comparison (Fig. 1A).

To test segmental function of the R domain in channel gating, the single-channel activity of CFTR mutants that deleted one to three protein segments of $R_1$, $R_2$, and $R_3$ was carefully examined (Fig. 1B). All deletion mutations were without effect on the single-channel current amplitude ($i$) of the CFTR Cl$^-$ channel (Fig. 1B and C). The deletion of $R_1$ alone ($ΔR_1$) showed little or no effect on the open probability ($P_o$) (Fig. 1D) and mean burst duration (MBD) of CFTR (Fig. 1E) but appeared to decrease the interburst interval (IBI) (Fig. 1F, $p = 0.07$, one-way ANOVA). In addition, $ΔR_2$ did not alter $P_o$, MBD, and IBI (Fig. 1, D–F). By contrast, $ΔR_{12}$ that removes $R_1$ and $R_2$ together mildly reduced $P_o$ (Fig. 1D) because of a large decrease in MBD (Fig. 1E) with no change in IBI (Fig. 1F). However, the $P_o$ of those CFTR mutants with $R_3$ deleted, including $ΔR_{123}$, $ΔR_{123}$, and $ΔR_{123}$-CFTR, were greatly decreased (Fig. 1D, gray columns) by large decreases in MBD (Fig. 1E) and marked increases in IBI (Fig. 1F). Notably, $ΔR_{123}$-CFTR here is the same as $ΔR_{5660A}$-CFTR tested in early studies (9–13), and the data (Fig. 1, C–F) are consistent as previously reported (11, 13). These data suggest that $R_3$ is required for stimulating CFTR gating, whereas $R_1$ and $R_2$ might contribute to MBD regulation. It is also possible that a large deletion of $R_1$ and $R_2$ together in $ΔR_{12}$-CFTR may disturb the function of $R_3$ in the MBD prolongation.

ATP-dependent CFTR gating may consist of three primary gating motions: ATP-mediated NBD dimerization, NBD/MSD coupling, and transmembrane gate movements (2, 27). Therefore, the R domain by interacting with the intracellular side of CFTR may modulate channel gating via two major pathways: 1) it may alter properties of ATPase sites in the interface of two NBDs (11, 21, 28), and 2) it may guide NBD/MSD coupling for regulating channel gating. To test the first mechanism, three gating stimulators that may enhance ATP-associated gating regulation were used (Fig. 2), including $2'\text{dATP}$ (1 mM) as an ATP analogue (29), CaATP (1 mM) for preventing ATP hydrolysis (30), and PP$_i$ (2 mM + 1 mM ATP) that may lock the post-hydrolytic state of CFTR gating (31). If $R_3$ regulates CFTR gating through modifying ATP function in NBDs, one would expect to find that potentiation effects of stimulators on channel gating may be greatly altered in those mutants with $R_3$ deleted.

However, the data demonstrate that three gating stimulators, $2'\text{dATP}$, CaATP, and PP$_i$, largely increased the macroscopic currents (Fig. 2, A and B), $P_o$ (Fig. 2D), and $i \times P_o$ values (Fig. 2E) of all tested CFTR constructs at similar levels of $\sim 2$–$3$-fold enhancements (Fig. 2, B and E). PP$_i$ also slightly decreased the $i$ of WT and all mutant CFTRs (Fig. 2C), similar to the previous finding (32). These data suggest that $ΔR_3$ may only have minor impacts on ATP-associated gating regulation.

To further explore this finding, the effects of three stimulators on the single-channel gating kinetics of WT and $ΔR_{123}$-CFTR were examined (Fig. 3). Only membrane patches that contained a single active CFTR channel were used in this study. For WT CFTR, channel activity and $P_o$ were greatly enhanced by three stimulators (Fig. 3, A and B) because of striking increases in MBD by all stimulators (Fig. 3C) and marked decreases in IBI by $2'\text{dATP}$ and CaATP (Fig. 3D). For $ΔR_{123}$-CFTR, channel activity and $P_o$ were also largely increased by three stimulators (Fig. 3, A and B) because of the MBD prolongation by CaATP or PP$_i$ (Fig. 3F) and the IBI shortening by $2'\text{ATP}$ and possibly two other stimulators: CaATP ($p = 0.10$) and PP$_i$ ($p = 0.06$) (Fig. 3G).

Between WT and $ΔR_{123}$-CFTR, three major alterations in the potentiation of gating kinetics by three stimulators were observed. First, MBD was prolonged by PP$_i$ $\sim 7.5$-fold in WT CFTR (Fig. 3C), but only 2.1-fold in $ΔR_{123}$-CFTR (Fig. 3F) ($p < 0.05$, one-way ANOVA). Second, IBI with CaATP was reduced to $\sim 26$% of that with MgATP in WT CFTR (Fig. 3D), but to
Third, it is of interest that MBD was greatly prolonged by 2'dATP 2.7-fold in WT CFTR (Fig. 3C), but without a change in R123-CFTR (Fig. 3F). These data suggest that R domain plays an important role in the MBD prolongation by 2'dATP.

To explore the protein segment responsible for 2'dATP-mediated MBD prolongation, the single-channel kinetics of other deletion mutants were examined in the presence of 2'dATP (Fig. 4). The data demonstrate that 2'dATP stimulated the channel activity (Fig. 4A) and Po (Fig. 4B) of all deletion mutants. It also increased the MBD of WT, ΔR1-CFTR, and ΔR2-CFTR to ~2.4-, 2.2-, and 1.8-fold, respectively (Fig. 4C). However, 2'dATP did not alter the MBD of those deletion mutants with R2 removed, including ΔR1-, ΔR3-, and ΔR123-CFTR (Fig. 4C). These data suggest that R2 is required for the MBD prolongation by 2'dATP.

Moreover, the IBIs of WT and ΔR1-, ΔR2-, ΔR3-, ΔR123-, and ΔR23-CFTR were decreased by 2'dATP to ~44, 43, 49, 32, and 37%, respectively (Fig. 4D). In other words, 2'dATP enhanced the channel opening rate (1/IBI) of all constructs
Stimulation of CFTR gating by the R domain

The phosphorylated peptide matching R_{809–835} shortens the IBI of ΔR_{123}-CFTR

After phosphorylated by PKA, all peptides at 50 nM did not alter the macroscopic current (I) of WT CFTR (Fig. 5B). Similarly, four peptides P_{1–1}, P_{1–2}, P_{2}, and P_{3–1} were without effect on the I of ΔR_{123}-CFTR (Fig. 5C).

However, peptide P_{3–2} significantly enhanced the I (Fig. 5C) and single-channel activity (Fig. 5D) of ΔR_{123}-CFTR. Although P_{3–2} did not alter the i and MBD of ΔR_{123}-CFTR (Fig. 5, E and

Figure 2. Gating stimulators show similar levels of potentiation on the channel activity of WT and mutant CFTRs. A, the time course of macroscopic WT CFTR Cl− currents (I) in the presence of MgATP (1 mM) as the control and three gating stimulators, PPi, (2 mM PPi, + 1 mM MgATP), 2′dATP (1 mM), and CaATP (1 mM). Each data point was obtained from the average current of 4-s recordings. PKA was present in this and all following experiments (β–E). B, potentiation of three gating stimulators on the I of WT and mutant CFTRs. The data are presented as percentages of the control current (I_{drug}/I_{MgATP}, %). The numbers in parentheses indicate n. Circles in gray represent individual data points for each measurement (B–E). C–E, effects of three gating stimulators on the i (C), P_{o} (D), and potentiation of channel activity (E) of WT and mutant CFTRs. In E, (i × P_{o,drug})/(i × P_{o,MgATP}) (%) is used to measure potentiation of the single-channel activity of CFTR by three gating stimulators. In C, the numbers in parentheses indicate n. In B–E, the data of the columns are the means ± S.D.; *, p < 0.05 compared with the control (black columns; Student’s paired t test).

~2.5-fold (1/0.4). The data suggest that all tested R domain deletions had minor effects on the IBI shortening by 2′dATP. Without a significant contribution to CFTR gating potentiation by three stimulators (Figs. 2–4), R_{3} might stimulate CFTR gating by modulating coupling of NBDs and MSDs.

To support this hypothesis, R_{3} may provide essential peptide interactions for potentiating CFTR gating, as shown in previous work (11, 14). Therefore, five synthesized peptides were tested in the following experiment, each matching a short segment of the R domain (Fig. 5A).
it evidently enhanced $P_o$ (Fig. 5F) by reducing IBI (Fig. 5H). The data suggest that R$_{809–835}$, covered by P$_{3–2}$ (Fig. 5A), may generate crucial interactions for the IBI shortening or, in other words, for accelerating the channel opening rate of CFTR, consistent with previous findings (11, 14). To search specific small segments contributing to the IBI shortening by P$_{3–2}$ (Fig. 5H) and gating regulation by R$_3$ (Figs. 1–4), CFTR mutants that remove the whole, one-half, or one-seventh of R$_3$ were examined (Fig. 6, A and B).

R$_{815–835}$ in R$_3$ dominantly stimulates the IBI shortening

In the presence of ATP (1 mM) and PKA, all deletion mutations did not alter $t$ (Fig. 6B and data not shown). By contrast, the $P_o$ (Fig. 6C) and MBD (Fig. 6D) of these mutants were all lower than that of WT CFTR. The data suggest that the entirety of R$_3$ regulates the MBD of CFTR.

For the IBI regulation by R$_3$ (Fig. 1F), ΔR$_3$ and ΔR$_{815–835}$, but not ΔR$_{784–814}$, apparently increased the IBI of CFTR (Fig. 6E). These data and the data of P$_{3–2}$ (Fig. 5H) suggest that in R$_3$, R$_{815–835}$ plays a major role in stimulating the IBI shortening. By using mutations that remove both R$_1$ and R$_2$ (ΔR$_{12}$), further investigation demonstrates that the IBI of CFTR was not changed by ΔR$_{12}$ (Fig. 1F) but was slightly increased by ΔR$_{208–814}$ that deletes R$_{784–814}$ from ΔR$_{12}$ (Fig. 6E) and markedly prolonged by ΔR$_{123}$ that deletes both R$_{784–814}$ and R$_{815–835}$ (Fig. 6E). Moreover, the IBI of ΔR$_{123}$-CFTR was much longer than that of

Figure 3. ΔR$_{123}$ variously modulates potentiation of 2’dATP, CaATP, and PPi on CFTR gating. A, representative recordings show effects of MgATP and three gating stimulators, 2’dATP, CaATP, and PPi, on the single-channel activity of a WT and ΔR$_{123}$-CFTR in the presence of PKA. Each trace is 30 s long. $B$–$G$, effects of 2’dATP, CaATP, and PPi, on the $P_o$, MBD, and IBI of WT ($B$–$D$) and ΔR$_{123}$-CFTR ($E$–$G$). The data of the columns are the means ± S.D. Circles in gray represent individual data points. In $B$ and $E$, numbers in parentheses indicate $n$. $^*$, $p < 0.05$ compared with the control (Student’s paired $t$ test).
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Figure 4. R2 is required for the MBD prolongation by 2’dATP. A, representative recordings show effects of MgATP and 2’dATP on the single-channel activity of a ΔR1-, ΔR2-, and ΔR3-CFTR in the presence of PKA. Each trace is 10 s long. B–D, effects of MgATP and 2’dATP on the Po, MBD, and IBI of indicated CFTR mutants. The data of the columns are the means ± S.D. Circles in gray represent individual data points. In B, the numbers in parentheses indicate n. * , p < 0.05 compared with the control (Student’s paired t test).

ΔR708–814–CFTR (Fig. 6E , p < 0.05, one-way ANOVA). These data suggest that R815–835–mediated IBI shortening is dominant for stimulating CFTR gating but may need the assistance of R784–814 for achieving the full effect of R3.

Moreover, ΔR829–835 in R815–835, but not ΔR815–821 and ΔR822–829, evidently increased the IBI of CFTR (Fig. 6E, gray columns). The data suggest that R829–835 is important for eliciting IBI shortening.

Two PKA phosphorylation sites Ser795 and Ser813 are present in R784–814 but none in R815–835 (Fig. 6A). In addition, site-directed mutations S795A and S813A that abolish PKA phosphorylation at these two serine residues both greatly increase IBI (11). These data suggest that PKA phosphorylation of R784–814 may control R815–835–mediated IBI shortening.

To test this hypothesis, this study further compared the effects of PKA on channel gating of ΔR12(708–783)– and ΔR708–814–CFTR (Fig. 7). In these two constructs, only ΔR12–CFTR contains R784–814 (Fig. 7A).

Phosphorylation of R784–814 relieves strong autoinhibition of IBI shortening

After a patch of the cell membrane was excised from a HeLa cell, CFTR Cl− currents across the patch membrane were recorded at 1 mM ATP for 3 min initially (Fig. 7B). After PKA was added directly into the chamber, the currents were continually recorded for 6 min or more (Fig. 7B). The number of active channels (N) was increased by PKA in three of seven membrane patches for ΔR12-CFTR (Fig. 7C) and
one of seven membrane patches for $\Delta R_{708-814}$–CFTR (Fig. 7C). Because $N$ may be underestimated at 1 mM ATP because of short recording time, these data suggest that the majority of both mutants in membrane patches were already partially active before PKA was added, especially for $\Delta R_{708-814}$–CFTR.

For each experiment, $N$ in Fig. 7C was used to calculate the $P_o$ of two mutants in the absence and presence of PKA. The data demonstrate that PKA greatly stimulated the channel activity (Fig. 7B) and $P_o$ (Fig. 7D) of $\Delta R_{123}$–CFTR by slightly increasing MBD (Fig. 7E) but markedly decreasing IBI (Fig. 7F). Note that the effects of PKA on the $P_o$ and IBI of $\Delta R_{123}$–CFTR (Fig. 7, D and F) might be less marked because of possible underestimation of $N$ at 1 mM ATP for three patches (Fig. 7C). The data suggest that $\Delta R_{123}$–CFTR retains the PKA-dependent activation mechanism.

Conversely, PKA showed no apparent effect on the channel activity (Fig. 7B) and gating kinetics (Fig. 7, D–F) of $\Delta R_{708-814}$–CFTR, suggesting that by removing $R_{784-814}$ from $\Delta R_{123}$–CFTR, the mutant $\Delta R_{708-814}$–CFTR exhibited PKA-independent channel activity. However, the IBI of $\Delta R_{708-814}$–CFTR (Fig. 7F) at 1 mM ATP with or without PKA were all greatly shorter than that of $\Delta R_{123}$ (708–835)–CFTR (for ATP, IBI $= 2943 \pm 1276$; for ATP+PKA, IBI $= 2782 \pm 1353$; $n = 6$; $p < 0.05$, one-way ANOVA). These data suggest that by adding $R_{815-835}$ into $\Delta R_{123}$–CFTR, the mutant $\Delta R_{708-814}$–CFTR exhibited a marked decrease in IBI. This finding further supports the notion that $R_{815-835}$ is required for the IBI shortening.
Importantly, the $P_o$ of $\Delta R_{12}$-CFTR was lower than that of $\Delta R_{708-814}$-CFTR at 1 mM ATP but became higher after adding PKA (see # in Fig. 7D). Two alterations in gating kinetics were observed. First, the MBD of $\Delta R_{12}$-CFTR was slightly longer than that of $\Delta R_{708-814}$-CFTR after adding PKA (Fig. 7E). Second, the dominant change is that after PKA was added, the IBI of $\Delta R_{12}$-CFTR was altered from strikingly longer to mildly shorter than that of $\Delta R_{708-814}$-CFTR (Fig. 7F). These data suggest that unphosphorylated R784–814 may strongly inhibit the IBI shortening by R815–835. Moreover, the data also suggest that phosphorylated R784–814 may not only lose its inhibition on R815–835 but may also simultaneously stimulate CFTR gating by slightly increasing MBD and decreasing IBI.

Discussion

Among three protein segments of the R domain, $R_1$ and $R_2$ play a minor role in regulation of CFTR gating. It is of great interest that $R_2$ is required for the MBD prolongation by 2‘dATP. By contrast, $R_3$ predominantly stimulates CFTR gating by greatly increasing MBD and markedly decreasing IBI. Moreover, the data suggest that $R_3$ contributes little to gating potentiation by three stimulators: CaATP, PPi, and 2‘dATP, but generates peptide interactions to decrease the IBI of CFTR. Notably, in $R_3$, unphosphorylated R784–814 may inhibit the IBI shortening mediated by R815–835. PKA phosphorylation of R784–814 may relieve this autoinhibition.

Figure 6. The entirety of $R_3$ maintains MBD, whereas $R_{815–835}$ is primarily responsible for the IBI shortening. A, $R_3$ is divided into two segments, $R_{784–814}$ and $R_{815–835}$. B, representative recordings show the single-channel activity of indicated CFTR mutants in the presence of MgATP (1 mM) and PKA. Each mutant deletes a part of $R_3$. Each trace is 10 s long. C–E, the $P_o$, MBD, and IBI of indicated CFTR mutants are shown. The data of the columns are the means $\pm$ S.D. Circles in gray represent individual data points. In C, numbers in parentheses indicate $n$. *, $p < 0.05$ compared with that of WT CFTR (one-way ANOVA).
and further mildly stimulate CFTR gating. These findings reveal an activation mechanism by which PKA phosphorylation at Ser795 and Ser813 in R784–814 elicits a great stimulation of CFTR gating by permitting R815–835–mediated IBI shortening.

**Segmental function of the R domain in CFTR gating**

By site-directed mutations (10, 11, 16–18, 33, 34) or deletions (11) of the R domain, previous work and this study demonstrate that PKA phosphorylation of the R domain modulates CFTR gating. By contrast, the previous study (35) that deletes the R domain by co-expression of the N- and C-terminal half of CFTR, including FLAG-cut-ΔR (N-terminal RFLAG3–633 + C-terminal R837–1480) and cut-ΔR-CFTR (R1–633 + R837–1480) in Xenopus oocytes, demonstrates that the $P_o$ of these split CFTRs are only moderately reduced. When expressed in Chinese hamster ovary cells, cut-ΔR-CFTR exhibits phosphorylation-independent $P_o$ with channel activity and gating kinetics similar to that of WT CFTR (36). Therefore, studies on split CFTR (35, 36) may suggest that the R domain is not important for regulating CFTR gating.

The discrepancies among these studies may result from confounding variations among different CFTR constructs. For this...
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study, one may argue that the peptide chain deletion in the R domain could generate structural constraints that significantly disrupt CFTR activity. However, a deletion of 52 residues in $\Delta R_1$-CFTR or 24 residues in $\Delta R_2$-CFTR showed little effect on CFTR gating (Fig. 1). $\Delta R_{12}$-CFTR with a deletion of 76 residues only reduced MBD but without effect on IBI (Fig. 1, E and F). Conversely, $\Delta R_1$, $\Delta R_{15-832}$, and $\Delta R_{29-835}$ with deletions of 52, 21, and 7 residues, respectively, all significantly altered MBD and IBI (Fig. 1, D and E). Moreover, among all synthesized peptides (Fig. 1E), only peptide P$_{3-2}$ matching R$_{3809-815}$ was able to stimulate the channel activity of $\Delta R_{123}$-CFTR. These data suggest that disruption of CFTR gating by $\Delta R_2$ is mainly caused by removing function of specific residues, rather than the peptide deletion or deletion-induced structural constrains. Thus, it is speculated that two half-split polypeptides of cut-$\Delta R$-CFTR might allow specific structural arrangements, possibly around the interaction region of R$_3$, overcoming the loss of R$_3$-mediated gating stimulation.

This study demonstrates that R$_2$ plays a minor role in MgATP-mediated CFTR gating (Fig. 1). However, PKA phosphorylation at Ser$^{768}$ in this segment is thought to be important for inhibiting CFTR activity. By abolishing phosphorylation at Ser$^{768}$, the site-directed mutation S768A enhances the stimulation of CFTR activity by isobutylmethylxanthine (15) and PKA (16). S768A also increases the $P_c$ of CFTR (16–18) possibly by increasing MBD (16–18). The reason for this discrepancy is unclear. It is noted that Ser$^{768}$ and its neighboring residues may interact with NBD1 (23) or intracellular loop 3 (18), whereas phosphorylation also alters intra- and interdomain interactions of the R domain, including the region around Ser$^{768}$ (6, 22, 23). Because the R$_2$ function is seemingly associated with the MBD regulation (Figs. 1E and 4C), one possible mechanism is that without phosphorylation at Ser$^{768}$, R$_2$ in S768A-CFTR may interact with the site differently from that of WT CFTR, permitting stimulation on channel gating. Future work is required to test this possibility.

Finally, the mutation S737A that abolishes the major PKA phosphorylation in R$_2$ appears to reduce the closed time of CFTR (17), consistent with the data of $\Delta R_1$-CFTR (Fig. 1F). Overall, this study indicates that R$_2$ is not important for regulating CFTR gating because $\Delta R_1$ appeared to have only minor impact on MBD and IBI (Fig. 1, E and F).

Allosteric regulation of R$_3$ on MgATP-dependent channel gating

Previous studies demonstrate that the phosphorylated R domain enhances the NBD dimerization (21) and ATPase activity of CFTR (5, 28), whereas ATP binding to CFTR is phosphorylation-independent (37). Enhanced NBD dimerization and ATPase activity by PKA phosphorylation might be due to relieving the R domain inhibition that blocks gating motions (5).

The constitutive channel activity of $\Delta R_{12}$-CFTR at 1 mM ATP alone (Fig. 7, B–F) is consistent with the previous finding that R$_2$ predominantly blocks CFTR activation (26). The remaining large PKA-dependent channel activity of $\Delta R_2$-CFTR (26) is likely achieved by R$_3$-mediated gating stimulation (Fig. 7). It is of interest that $\Delta R_3$ greatly disrupted CFTR gating (Figs. 1 and 4) but showed little or no effect on the gating stimulation of 2'dATP (Fig. 4, C and D). The data suggest that R$_3$ may potentiate channel gating by the mechanism different from that of 2'dATP.

Because the hydrolysis rate of 2'dATP ($V_{max} = 8.5$ nmol/mg-min) is slower than that of ATP ($V_{max} = 47$ nmol/mg-min) (29), it is suggested that 2'dATP increases MBD by slowing the hydrolysis rate. However, without R$_2$, 2'dATP-mediated MBD was similar to that by ATP (Fig. 4C), and $\Delta R_2$ did not significantly alter ATP-mediated MBD (Fig. 1E). These data suggest that in R$_2$-deleted mutants, the ATP hydrolysis rate might not be strictly coupling to the channel closing rate.

How the ATPase activity of NBDs regulates CFTR gating remains unclear (2, 38). The major argument focuses on whether there is a strict correlation between ATP hydrolysis and channel closing (2, 38). An unmatched correlation was observed in this study because ATP- and 2'dATP-mediated MBDs of R$_2$-deleted CFTR mutants were similar (Fig. 4C). Our recent study (13) suggests that the Michaelis–Menten relationship of the ATP concentration and $P_c$ of CFTR, conventionally used for simulating ATP dependence of channel gating, may just denote the transitions of gating states. By contrast, channel opening is likely triggered by ATP (2, 38), whereas the rate-limiting step of channel opening likely occurs after ATP binding to NBDs, e.g. the NBD dimerization (2, 38).

Here, an interesting finding is that 2'dATP increased the channel opening rate ~2.5-fold in WT and other mutant CFTRs (Fig. 4D), despite the IBI of $\Delta R_3$, $\Delta R_{23}$, and $\Delta R_{123}$-CFTR being greatly increased to ~5-fold longer than that of WT CFTR (Fig. 4D). Biochemical evidence suggests that 2'dATP may have higher binding affinity ($K_m = 0.2$ mM) to CFTR than that of ATP ($K_m = 1.1$ mM) (29). However, 2'dATP simply removes the 2'-hydroxyl (-OH) group in the ribose of ATP. Most electrostatic interactions between ATP and NBDs come from phosphate groups of ATP (6). Therefore, it seems unlikely that 2'dATP can generate the binding force 2.5-fold higher than that by ATP in the NBD dimer.

A plausible explanation for above findings is that 2'-OH group in the ribose of ATP may generate steric hindrance for the NBD dimerization. Thus, for shortening IBI, 2'dATP might allow NBDs to dimerize faster than that by ATP. Consequently, the NBD dimer induced by 2'dATP might be formed differently from that by ATP, permitting R$_2$ to stabilize the dimer and prolong MBD.

In contrast to 2'dATP, R$_3$ may stimulate CFTR activity by modulating coupling of NBDs and MSDs (2, 27), based on the data in this study. This mechanism is consistent with recent findings that in phosphorylated CFTR, the C-terminal half of R$_3$ (R$_{825-835}$) may interact with the region between NBD and MSD, away from ATPase sites in CFTR (7). Following this idea, R$_3$ may serve as a platform providing essential peptide interactions that reduce the energy barrier required for channel openings and also lower the energy level of the bursting state for prolonging MBD (39).

Moreover, this mechanism suggests that $\Delta R_3$ may disturb NBD/MSD coupling, meant slowing and destabilizing the gating motion that contains the tight and synchronous coordination between NBD dimerization and NBD/MSD coupling (27).
Consequently, 2’dATP may still slowly accelerate the NBD dimerization at a speed 2.5-fold higher than that by ATP for shortening the IBI of those CFTR variants with R3 removed (Fig. 4D). This mechanism might also allow R3 to stimulate CFTR gating by allosterically regulating NBD dimerization (21) and ATP dependence of channel gating (11). A caveat for this proposed mechanism is that CFTR structural changes accompanying with 2’dATP-induced NBD dimerization remain unclear.

**Autoinhibition and self-stimulation of CFTR gating by R3**

Phosphorylation is a key protein modification that activates various signaling molecules in a cell (40). However, the phosphorylation-induced activation process for most of these molecules is not well-understood. Phosphorylation may cause charge–charge interactions and regional conformational changes, which may further modulate protein–protein interactions (40). For example, PKA phosphorylation on the protein kinase A–anchoring protein smAKAP may alter the helicity of the domain, subsequently disrupting interactions between smAKAP and the type I regulatory subunit of PKA (41).

Similarly, PKA phosphorylation greatly alters the conformation of the R domain (6, 19). Therefore, the autoinhibition of the IBI shortening by R784–814 (Fig. 7F) may be relieved by phosphorylation-induced structural changes (Fig. 8). Consistently, mutations S795A and S813A that prevent phosphorylation on R784–814 both markedly prolong the IBI of CFTR (11). Moreover, providing R815–835–mediated peptide interactions by the peptide P1–2 (R809–835) in this study (Fig. 5H) and NEG2 (R817–838) in previous work (14) all stimulate the IBI shortening. Furthermore, the N-ethylmaleimide modification at the Cys832 residue also reduces IBI (42). Recent structural evidence reveals that in phosphorylated CFTR, R825–843 may be relocated from the region wedged by transmembrane helices 9, 10, and 12 (5, 7) to the position interacting with helices 10 and 11 and residues 34–39 of the lasso motif (7). Therefore, unphosphorylated R784–814 might disrupt interactions of R815–835 with the above helices 10 and 11 and the lasso motif for blocking the IBI shortening.

It is unclear whether unphosphorylated R784–814 directly interacts with R815–835 or binds to the interaction sites of R815–835 to inhibit IBI shortening (Fig. 8). A direct interaction between R784–814 and R815–835 is feasible because R815–835 contains many negatively charged residues, and PKA phosphorylation sites in R784–814 comprise positively charged dibasic residues, including lysine and arginine residues (1). Then added phosphate groups at Ser795 and Ser813 after PKA phosphorylation may produce negative-to-negative repulsive forces that prevent the inhibition of R815–835 by R784–814.

It is intriguing that deletions of R12, R3 (Fig. 1E), or a part of R3 (Fig. 6D) all decreased MBD to a similar value, suggesting that the R domain may adopt a common mechanism to regulate MBD. It could be a short protein segment in R3 essential for prolonging MBD, whereas any small deletions in R3 or the large deletion like ΔR12 may be sufficient to disrupt the MBD regulation by this responsible segment.

This study addressed a major CFTR activation mechanism, namely how PKA phosphorylation of the R domain stimulates channel gating. The data suggest that PKA phosphorylation on R784–814 relieves an autoinhibition that blocks the IBI shortening by R815–835 (Fig. 8). Phosphorylated R3 may induce the protein segment relocation (7) in which R815–835 may provide essential peptide interactions for potentiating CFTR gating (Fig. 8). This stimulatory mechanism advances our understandings of PKA-dependent CFTR activation. Similar mechanisms may be adopted by other protein kinases, such as PKC (43), cGMP-dependent protein kinase II (44) and tyrosine kinases (45) for regulating CFTR gating.

**Experimental procedures**

**Mutagenesis and CFTR expression**

Human CFTR mutants were constructed using the pTM1-CFTR4/S660A plasmid (26), in which the T7 promoter is recognized by the bacteriophage T7 RNA polymerase. Some CFTR mutants were made in the previous study (26), and others were created by the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and verified by DNA sequencing.

WT and all mutant CFTRs were expressed in HeLa cells by the vaccinia virus/bacteriophage T7 hybrid expression system as described previously (10). In brief, HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Gibco, Thermo Fisher Scientific) with 10% FBS at 37 °C and 5% CO₂. For transiently expressing CFTR in the cell membrane, HeLa cells were infected for 1 h with recombinant vaccinia viruses that encode the T7 RNA polymerase gene. After that, virus-infected cells were transfected with CFTR plasmids by Lipofectamine 2000 (Invitrogen) and then used for experiments within 12–48 h.

**Electrophysiology**

The patch-clamp technique with excised inside-out membrane patches was adopted for studying the channel activity of CFTR as described previously (13). The CFTR activity was activated and maintained by 1 mM ATP and 75 nM PKA in the bath (intracellular) solution at room temperature. To amplify CFTR
currents, membrane voltage was clamped at $-50$ mV. The pipette (extracellular) solution contained 140 mM $N$-methyl-d-glucamine, 140 mM aspartic acid, 5 mM CaCl$_2$, 2 mM MgSO$_4$, and 10 mM Tricine, pH 7.4, with Tris ([Cl$^-]$), 10 mM). The bath solution contained 140 mM $N$-methyl-d-glucamine, 3 mM MgCl$_2$, 1 mM CsEGTA, 10 mM Tricine, pH 7.4, with HCl.

ATP was prepared fresh in the bath solution before each experiment. For making the CaATP solution, MgCl$_2$ in the bath solution was replaced by CaCl$_2$, and EGTA was removed. The stock solution of synthesized R domain peptides (AnaSpec, Fremont, CA) contained 0.005 mM peptides, 10 mM KH$_2$PO$_4$, 1 mM EDTA, and 1 mg/ml BSA, pH 6.7, with NaOH. Peptides (5 $\mu$l) were diluted in the bath solution (50 $\mu$l) and phosphorylated by PKA (750 nm) for 10 min before being added into the recording chamber (~500 $\mu$l; Brook Industries, Lake Villa, IL) with a final concentration of 50 nM at room temperature. Recording of the CFTR current was started within 30 s after adding peptides and lasted for 10–15 min.

CFTR currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Union City, CA), filtered by an eight-pole Bessel filter (Frequency Devices, Inc., Ottawa, IL) at a corner frequency of 500 Hz and digitized by a Digidata 1322 interface (Molecular Devices) at 10 kHz. The software pCLAMP (Molecular Devices) was used for data acquisition and analysis.

For membrane patches that contain many channels, the average of the macroscopic CFTR currents was measured. Moreover, the basal current in the absence of ATP and PKA was subtracted from the total current, as described previously (12). For membrane patches that contained five or less channels, single-channel analysis was performed. The number of active channels ($N$) in a membrane patch was determined by the maximum number of channels that opened simultaneously at one time during the entire experiment. To measure the single-channel current amplitude ($i$), Gaussian distributions were fit to current amplitude histograms. To measure the open probability ($P_o$), event lists of open- and closed-times were created using a half-amplitude crossing criterion, whereas transitions with durations less than 1 ms were excluded (eight-pole Bessel filter rise time ($T_{10-90}$) was ~0.73 ms at a corner frequency of 500 Hz).

To measure mean burst duration (MBD), interburst interval (IBI), and $P_o$ within a burst ($P_o$-burst), burst analysis was performed. The delimiter time that separates intraburst closures from intraburst closures was determined from the point of intersection between the two exponential curves fitting fast and slow populations of channel closures in the closed-time histogram, as described previously (46). Event lists and values of the delimiter time were used to derive MBD and $P_o$-burst with pCLAMP software. To obtain burst durations, opening bursts formed by only one active channel were measured. Finally, IBI was calculated using the following equation:

$$P_o = \frac{\text{MBD} \times P_o}{\text{MBD} + \text{IBI}} \quad (\text{Eq. 1})$$

To obtain $N$ accurately, the time required to observe an event that all active channels open simultaneously was estimated by the following equation: $(3 \times \tau_o/N)/(P_o)^N$ (47), where the open time $\tau_o$ could be estimated by MBD. For WT CFTR with $P_o = 0.41$ and MBD = 504 ms (Fig. 1, D and E), it requires 26 s for observing all five channels opening at the same time and 53 s for six channels. Therefore, after PKA was added, CFTR currents were recorded at each intervention for 6 min or more for WT CFTR and 10–30 min for CFTR mutants in most experiments. PKA was regularly added into the bath solution again after 15-min recordings. However, $N$ would be underestimated for mutant CFTR with large gating anomalies. Therefore, the reported $P_o$ values of these CFTR mutants should be considered maximum $P_o$ values, and their IBI values should be considered minimal IBI values.

To reduce above uncertainty in $N$, the majority of our data were obtained from recordings with three or fewer channels in a membrane patch. For experiments tested with gating stimulators 2’mATP, CaATP, and PP, that greatly prolong the MBD of CFTR (29–31), only membrane patches that contained a single active channel were used for kinetic analysis. For the purpose of illustration, recordings were further digitized at 1 kHz, in which current tracings of 10 s could clearly demonstrate the gating kinetics of CFTR with reduced data points, noise, and some very brief current transitions (e.g. those of <1 ms).

Reagents and chemicals

PKA catalytic subunits purified from bovine heart (Calbiochem) were used. Other chemicals were purchased from the Sigma–Aldrich or in the reagent grade.

Statistical analysis

The data are presented as means ± S.D. of $n$ observations. Student’s paired $t$ test or one-way ANOVA values were used to test statistically significant differences between sets of data. The differences were considered statistically significant when $p < 0.05$.

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References

1. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., and Chou, J. L. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245, 1066–1073 CrossRef Medline
2. Hwang, T. C., and Kirk, K. L. (2013) The CFTR ion channel: gating, regulation, and anion permeation. Cold Spring Harb. Perspect. Med. 3, a009498 Medline
3. Ostergedgaard, L. S., Baldursson, O., and Welsh, M. J. (2001) Regulation of the cystic fibrosis transmembrane conductance regulator Cl$^-$ channel by its R domain. J. Biol. Chem. 276, 7689–7692 CrossRef Medline
22. Bozoky, Z., Csányd, L., Gadbry, D. C., and Chen, J. (2017) Molecular structure of the human CFTR ion channel. *Cell* **169**, 85–95.e8 CrossRef Medline

23. Baker, J. M., Henderson, R. P., Kanelis, V., Choy, W. Y., Thibodeau, P. H., Thomas, P. I., and Forman-Kay, J. D. (2007) CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices. *Nat. Struct. Mol. Biol.* **14**, 738–745 CrossRef Medline

24. Wang, G. (2010) State-dependent regulation of cystic fibrosis transmembrane conductance regulator (CFTR) gating by a high affinity Fe$^{3+}$ bridge between the regulatory domain and cytoplasmic loop 3. *J. Biol. Chem.* **285**, 40438–40447 CrossRef Medline

25. Chappe, Y., Irvine, T., Liao, J., Evagelidis, A., and Hanrahan, J. W. (2005) Phosphorylation of CFTR by PKA promotes binding of the regulatory domain. *EMBO J.* **24**, 2730–2740 CrossRef Medline

26. Baldursson, O., Ostedgaard, L. S., Rokhlina, T., Cotten, J. F., and Welsh, M. J. (2001) Cystic fibrosis transmembrane conductance regulator CI$^{-}$ channels with R domain deletions and translocations show phosphorylation-dependent and -independent activity. *J. Biol. Chem.* **276**, 1904–1910 CrossRef Medline

27. Hwang, T. C., Yeh, J. T., Zhang, J., Yu, Y. C., Yeh, H. I., and Destefano, S. (2018) Structural mechanisms of CFTR function and dysfunction. *J. Gen. Physiol.* **150**, 539–570 CrossRef Medline

28. Li, C., Ramjesingh, M., Wang, W., Garami, E., Hewryk, M., Lee, D., Rommens, J. M., Galley, K., and Bear, C. E. (1996) ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **271**, 28463–28468 CrossRef Medline

29. Aleksandrov, A. A., Aleksandrov, L., and Riordan, J. R. (2002) Nucleotide triphosphate pentose ring impact on CFTR gating and hydrolysis. *FEBS Lett.* **518**, 183–188 CrossRef Medline

30. Ikuma, M., and Welsh, M. J. (2000) Regulation of CFTR Cl$^{-}$ channel gating by ATP binding and hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8675–8680 CrossRef Medline

31. Jih, K. Y., Sohma, Y., Li, M., and Hwang, T. C. (2012) Identification of a novel post-hydrolytic state in CFTR gating. *J. Gen. Physiol.* **139**, 359–370 CrossRef Medline

32. Scott-Ward, T. S., Cai, Z., Dawson, E. S., Doherty, A., Da Paula, A. C., Davidson, H., Porteous, D. J., Wainwright, B. J., Amaral, M. D., Sheppard, D. N., and Boyd, A. C. (2007) Chimeric constructs endow the human CFTR Cl$^{-}$ channel with the gating behavior of murine CFTR. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 16365–16370 CrossRef Medline

33. Seibert, F. S., Tabcharani, J. A., Chang, X. B., Dulhanty, A. M., Mathews, C., Hanrahan, J. W., and Riordan, J. R. (1995) cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J. Biol. Chem.* **270**, 2158–2162 CrossRef Medline

34. Chang, X. B., Tabcharani, J. A., Hou, Y. X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W., and Riordan, J. R. (1993) Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* **268**, 11304–11311 Medline

35. Csányd, L., Chan, K. W., Cenciarelli, C., Angel, B. B., Qin, J., McCauley, A., Kruchinsky, A. N., Chait, B. T., Nairn, A. C., and Gadbry, D. C. (2005) Preferential phosphorylation of R-domain serine 768 damps activation of CFTR channels by PKA. *J. Gen. Physiol.* **125**, 171–186 CrossRef Medline

36. Bompadre, S. G., Ai, T., Cho, J. H., Wang, X., Sohma, Y., Li, M., and Gadbry, D. C. (2000) Diphosphorylation of serine 752 and 768 activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* **276**, 11304–11311 Medline

37. Wang, G. (2011) The inhibition mechanism of non-phosphorylated Ser$^{768}$ in the regulatory domain of cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **286**, 2171–2182 CrossRef Medline

38. Grimard, V., Li, C., Ramjesingh, M., Bear, C. E., Goormaghtigh, E., and Ruysschaert, J. M. (2004) Phosphorylation-induced conformational changes of cystic fibrosis transmembrane conductance regulator monitored by attenuated total reflection-Fourier transform IR spectroscopy and fluorescence spectroscopy. *J. Biol. Chem.* **279**, 5528–5536 CrossRef Medline

39. Auerbach, A. (2013) The energy and work of a ligand-gated ion channel. *J. Gen. Physiol.* **139**, 294–296 Medline

40. Ikuma, M., and Welsh, M. J. (2000) Cystic fibrosis transmembrane conductance regulator (CFTR) gating by a high affinity Fe$^{3+}$ bridge between the regulatory domain and cytoplasmic loop 3. *J. Biol. Chem.* **285**, 40438–40447 CrossRef Medline

41. Auerbach, A. (2013) The energy and work of a ligand-gated ion channel. *J. Gen. Physiol.* **139**, 294–296 CrossRef Medline

42. Kitchen, J., Saunders, R. E., and Warwicker, J. (2008) Charge environments around phosphorylation sites in proteins. *BMC Struct. Biol.* **8**, 19 CrossRef Medline

Stimulation of CFTR gating by the R domain

4. Vergani, P., Lockless, S. W., Nairn, A. C., and Gadsby, D. C. (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature* **433**, 876–880 CrossRef Medline

5. Liu, F., Zhang, Z., Csányd, L., Gadbry, D. C., and Chen, J. (2017) Molecular structure of the human CFTR ion channel. *Cell* **169**, 85–95.e8 CrossRef Medline

6. Zhang, Z., Liu, F., and Chen, J. (2018) Molecular structure of the ATP-binding, phosphorylated human CFTR. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 12757–12762 CrossRef Medline

7. Ostedgaard, L. S., Baldursson, O., Vermeer, D. W., Welsh, M. J., and Robertson, A. D. (2000) A functional R domain from cystic fibrosis transmembrane conductance regulator is predominantly unstructured in solution. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5657–5662 CrossRef Medline

8. Rich, D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E., and Welsh, M. J. (1991) Effect of deleting the R domain on CFTR-generated chloride channels. *Science* **253**, 205–207 CrossRef Medline

9. Rich, D. P., Berger, H. A., Cheng, S. H., Travis, S. M., Saxena, M., Smith, A. E., and Welsh, M. J. (1993) Regulation of the cysic fibrosis transmembrane conductance regulator CI$^{-}$ channel by negative charge in the R domain. *J. Biol. Chem.* **268**, 20239–20267 CrossRef Medline

10. Winter, M. C., and Welsh, M. J. (1997) Stimulation of CFTR activity by its phosphorylated R domain. *Nature* **389**, 294–296 CrossRef Medline
Stimulation of CFTR gating by the R domain

41. Burgers, P. P., Bruystens, J., Burnley, R. J., Nikolaev, V. O., Keshwani, M., Wu, J., Janssen, B. J., Taylor, S. S., Heck, A. J., and Scholten, A. (2016) Structure of smAKAP and its regulation by PKA-mediated phosphorylation. FEBS J. 283, 2132–2148 CrossRef Medline

42. Cotten, J. F., and Welsh, M. J. (1997) Covalent modification of the regulatory domain irreversibly stimulates cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. 272, 25617–25622 CrossRef Medline

43. Chappe, V., Hinkson, D. A., Howell, L. D., Evangelidis, A., Liao, J., Chang, X. B., Riordan, J. R., and Hanrahan, J. W. (2004) Stimulatory and inhibitory protein kinase C consensus sequences regulate the cystic fibrosis transmembrane conductance regulator. Proc. Natl. Acad. Sci. U.S.A. 101, 390–395 CrossRef Medline

44. French, P. J., Bijman, J., Edixhoven, M., Vaandrager, A. B., Scholte, B. J., Lohmann, S. M., Nairn, A. C., and de Jonge, H. R. (1995) Isotype-specific activation of cystic fibrosis transmembrane conductance regulator-chloride channels by cGMP-dependent protein kinase II. J. Biol. Chem. 270, 26626–26631 CrossRef Medline

45. Billet, A., Jia, Y., Jensen, T., Riordan, J. R., and Hanrahan, J. W. (2015) Regulation of the cystic fibrosis transmembrane conductance regulator anion channel by tyrosine phosphorylation. FASEB J. 29, 3945–3953 CrossRef Medline

46. Carson, M. R., Travis, S. M., and Welsh, M. J. (1995) The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. J. Biol. Chem. 270, 1711–1717 CrossRef Medline

47. Venglarik, C. J., Schultz, B. D., Frizzell, R. A., and Bridges, R. J. (1994) ATP alters current fluctuations of cystic fibrosis transmembrane conductance regulator: evidence for a three-state activation mechanism. J. Gen. Physiol. 104, 123–146 CrossRef Medline