PKC phosphorylation modulates PKA-dependent binding of the R domain to other domains of CFTR

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Seavilleklein G, Amer N, Evagelidis A, Chappe F, Irvine T, Hanrahan JW, Chappe V. PKC phosphorylation modulates PKA-dependent binding of the R domain to other domains of CFTR. Am J Physiol Cell Physiol 295: C1366 –C1375, 2008. First published September 24, 2008; doi:10.1152/ajpcell.00034.2008.—Activity of the CFTR channel is regulated by phosphorylation of its regulatory domain (RD). In a previous study, we developed a bicistronic construct called ΔR-Split CFTR, which encodes the front and back halves of CFTR as separate polypeptides without the RD. These fragments assemble to form a constitutively active CFTR channel. Coexpression of the third fragment corresponding to the missing RD restores regulation by PKA, and this is associated with dramatically enhanced binding of the phosphorylated RD. In the present study, we examined the effect of PKC phosphorylation on this PKA-induced interaction. We report here that PKC alone enhanced association of the RD with ΔR-Split CFTR and that binding was further enhanced when the RD was phosphorylated by both kinases. Mutation of all seven PKC consensus sequences on the RD (7CA-RD) did not affect its association under basal (unphosphorylated) conditions but abolished phosphorylation-induced binding by both kinases. Iodide efflux responses provided further support for the essential role of RD binding in channel regulation. The basal activity of ΔR-Split/7CA-RD channels was similar to that of ΔR-Split/wild type (WT)-RD channels, whereas cAMP-stimulated iodide efflux was greatly diminished by removal of the PKC sites, indicating that 7CA-RD binding maintains channels in an inactive state that is unresponsive to PKA. These results suggest a novel mechanism for CFTR regulation in which PKC modulates PKA-induced domain-domain interactions.

Cystic fibrosis; the cystic fibrosis transmembrane conductance regulator chloride channel; domain-domain interactions; protein kinase A and protein kinase C phosphorylation

THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) is an ATP-dependent, phosphorylation-activated Cl– channel that mediates cAMP-stimulated Cl– and bicarbonate secretion by epithelia and influences the activity of other membrane proteins (12, 22, 27). Mutations in the CFTR gene cause cystic fibrosis, a common fatal genetic disease characterized by abnormal electrolyte transport across exocrine epithelia (13, 23, 26). CFTR belongs to the ATP binding cassette (ABC) transporter superfamily (ABCC7) and has two transmembrane domains (TMD1 and TMD2), two nucleotide binding domains (NBD1 and NBD2), and a unique regulatory domain (RD). The RD mediates channel activation by secretagogues and has numerous potential sites for phosphorylation by PKA and PKC (25).

CFTR channel activation by PKA is increased by pretreatment with PKC (3, 6, 15, 29), and mutation of consensus PKC sites in NBD1 and the RD (9CA-CFTR) strongly inhibit PKA and abolish the small stimulation induced by PKC alone. Thus, PKC exerts this positive effect directly through the phosphorylation of CFTR (6), although there may also be indirect inhibitory effects mediated by other proteins (24).

ATP-dependent gating of the CFTR channel is inhibited by the RD under resting conditions and stimulated by RD phosphorylation (4, 7, 8, 12, 29), but the structural consequences of RD phosphorylation are not well understood. Its helical content is reduced by phosphorylation (1, 11), and this may enhance transient interaction of the RD with NBD1 (1) and promote dimerization of NBDs (19). Other interactions have also been proposed (31), for example, stimulatory and inhibitory interactions of a distal region of the RD with unidentified domain regions of CFTR (32). However, the role of PKC phosphorylation in controlling domain-domain interactions has not been explored.

In previous work, we examined PKA-dependent association of the RD with the rest of CFTR using split channels composed of three polypeptide fragments and obtained evidence that PKA phosphorylation enhances RD binding to other CFTR domains both in vitro and in live cells (7). This raised the possibility that PKC might increase the responsiveness of CFTR channels to PKA through enhanced binding of the RD. In the present study, we examined the effect of PKC phosphorylation and removal of potential PKC sites on domain-domain interactions that are induced by PKA.

MATERIALS AND METHODS

Chemicals. The type II bovine cardiac PKA catalytic subunit (PKA) was obtained from the laboratory of Dr. M. P. Walsh (University of Calgary, AB, Canada). PKC and L12B4 and M3A7 monoclonal anti-CFTR antibodies were from Upstate (Charlottesville, VA). MAB1660 monoclonal anti-CFTR RD antibody was from R&D Systems (Minneapolis, MN), and Mab 450 antibody was a generous gift from J. Riordan (University of North Carolina). Goat anti-mouse secondary antibody conjugated to peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL chemiluminescence detection kits were from GE Healthcare (Amersham, Bucks, UK). Other chemicals were from Sigma (St. Louis, MO) and were of the highest grade available.

Cell culture. Baby hamster kidney (BHK) cells were grown at 37°C with 5% CO2 in DMEM (Life Technologies, Burlington, ON, Canada) containing 5% FBS. The selecting drug methotrexate (MTX; 500 μM)
was used to ensure stable expression of wild-type (WT) CFTR or RD-expressing cells. G418 (400 μg/ml) and zeocin (250 μg/ml) were used to select ΔR-Split, ΔR-Split/RD, and ΔR-Split/7CA-RD CFTR-expressing clones. Ponasterone A (10 μM) was used as the inducer for ΔR-Split CFTR expression (see below).

ΔR-Split CFTR, ΔR-Split/RD, and ΔR-Split/7CA-RD CFTR construction and expression. As described in detail elsewhere (7), ΔR-Split CFTR was constructed by replacing the nucleotides that normally encode the RD (nt 1905-2511) with an internal ribosomal entry sequence (IRES). Two cDNA fragments were generated by PCR to allow the insertion of a stop codon at the 3′-end of the ΔR-Split CFTR front half cDNA and a Kozak translation initiation sequence at the 5′-end of the sequence encoding the back half of ΔR-Split CFTR. WT CFTR cDNA in the pNUT vector was used as the template. ΔR-Split CFTR cDNA was inserted into the pND vector and confirmed by sequencing. The Invitrogen Ecdysone-Inducible Mammalian Expression System was used to create an inducible ΔR-Split CFTR-expressing cell clone according to the manufacturer’s instructions (for details, see Ref. 7) and selected using G418 and zeocin.

ΔR-Split/RD-expressing cells were obtained by retransfecting a BHK cell clone that already expressed ΔR-Split CFTR with pNUT-RD and selecting ΔR-Split/RD cell colonies using 500 μM MTX. ΔR-Split/7CA-RD-expressing cells were obtained similarly except that 7CA-RD cDNA was obtained by PCR using full-length 9CA-CFTR cDNA as a template (see Ref. 6). GST-RD and GST-7CA-RD fusion proteins were obtained by inserting RD cDNA encoding amino acids 635-836 (obtained by PCR using WT or 9CA-CFTR cDNA as a template) into the pGEX-2T vector (Amersham Pharmacia Biotech). Constructs were verified by sequencing, and fusion proteins were expressed in E. coli BL21-codon Plus-RIL (Stratagene) and purified with glutathione-Sepharose UB beads according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Aliquots of purified glutathione-S-transferase (GST)-RD were subjected to 10% PAGE and stained with Coomassie dye to assess purity.

Immunoprecipitation. Immunoprecipitations were performed as previously described (7) using the SeizEx Protein G immunoprecipitation kit (Pierce Laboratories, Rockford, IL). Briefly, anti-CFTR antibodies M3A7 or L12B4 were first bound to ImmunoPure Immobilized Protein G and cross-linked with disuccinimidyl suberate (DSS) according to the manufacturer’s instructions. BHK cells expressing ΔR-Split, ΔR-Split/RD, or ΔR-Split/7CA-RD CFTR were induced for 48 h using ponasterone A (10 μM), harvested by scraping, and lysed on ice for 30 min in 1% Triton X-100/PBS buffer supplemented with protease inhibitor cocktail (Roche). Lysates were then centrifuged, and supernatants were used immediately for immunoprecipitation.

Cells coexpressing ΔR-Split with either RD or 7CA-RD were treated with 150 μM CPT-cAMP + 1 mM IBMX or 20 mM PMA for 3 h (or not, as a control) before being harvested, and lysates were incubated with phosphatase inhibitors.

Lysates from ΔR-Split CFTR-expressing cells were incubated with (or without, as a control) the following: 1) 1 μg of RD-GST or 7CA-RD/GST + 10 μM kinase inhibitor H7 (unphosphorylated condition); 2) 1 μg of PKA prephosphorylated RD-GST (or 7CA-RD/GST) + PKA (at indicated concentrations) + ATP (2 mM) + phosphatase inhibitors (5 μM cyclosporine A + 100 mM calcylin A); or 3) 1 μg of PKC prephosphorylated RD/GST (or 7CA-RD/GST) + PKC (5 mM) + ATP (2 mM) + phosphatase inhibitors (5 μM cyclosporine A + 100 mM calcylin A) + PKC lipid activator. Aliquots of each cell lysate were then incubated with M3A7- or L12B4-bound columns at room temperature with rotation for 10 min and spun for 1 min, and the supernatant was discarded. Another aliquot was then incubated on the same column, and this procedure was repeated until all available fractions had been subjected to the column. Columns were then washed with quenching buffer + 0.1% Triton X-100 + protease inhibitors. Immunoprecipitated proteins were eluted using low-pH (2.8) elution buffer. Elution fractions were mixed with 5 × sample buffer + 80 mM DTT for subsequent analyses by SDS-PAGE and Western blot analysis. The presence of each domain in the pulldowns was revealed by monoclonal anti-CFTR antibodies directed against an epitope located within the range of amino acid positions 386-412 for the front half (L12B4) and positions 1365-1395 in NBD2 of CFTR for the back half (M3A7), and, to detect RD, 7CA-RD, and GST-RD, we used the anti-RD monoclonal antibodies MAB1660 (amino acids 590-830, R&D Systems) or 450 (amino acids 696-705; a gift from J. Riordan, University of North Carolina).

Immunolocalization. BHK cells stably expressing ΔR-Split/7CA-RD CFTR were plated on glass coverslips at low density, and ΔR-Split/7CA-RD expression was induced by ponasterone A (10 μM) for 48 h. The medium was then removed, cultures were washed three times with PBS, and cells were fixed in 2% paraformaldehyde (in PBS) for 20 min at room temperature followed by permeabilization and antigen blockade with 0.1% Triton X-100–2% BSA in PBS for an additional 45 min at room temperature. The blocking/permeabilization solution was removed, and cells were incubated for 45 min at room temperature with anti-CFTR antibodies (1:1000) L12B4 or M3A7 diluted in PBS + 0.1% Triton X-100 + 0.2% BSA. Cells were then washed three times with PBS + 0.1% Triton X-100 for 10 min and incubated with a secondary antibody (Cy3- or Cy5-conjugated goat anti-mouse at 1:100) diluted in PBS + 0.1% Triton X-100 + 0.2% BSA for 1 h at room temperature. Cells were washed and incubated with a second anti-CFTR antibody (M3A7 or MAB1660, 1:1000) as before. Cells were washed and incubated with the second secondary antibody (Cy5- or Cy3-conjugated goat anti-mouse) diluted in PBS + 0.1% Triton X-100 + 0.2% BSA for 1 h at room temperature. After a final wash, slides were mounted, dried, and viewed using a Zeiss LSM 510 confocal microscope. Negative controls for secondary antibodies were performed by omitting the primary antibodies. L12B4, M3A7, and MAB1660 specificity has been established previously using BHK cells transfected with the empty vector (7).

Membrane localization of each domain of ΔR-Split/7CA-RD was confirmed by staining of the plasma membrane of BHK cells, which were cultured on glass coverslips and stimulated with ponasterone A for 48 h with Vibrant CM-Di cell labeling solution (from Molecular Probes, excitation: 553 nm and emission: 570 nm) according to the manufacturer’s instructions. After the plasma membrane had been labeled with CM-Dil dye, cells were fixed, permeabilized, and immunostained for each CFTR domain as above before detection by confocal microscopy.

In vitro phosphorylation of RD/GST. Purified RD-GST was incubated for 30 min at 30°C in phosphorylation buffer (140 mM NaCl, 4 mM KCl, 2 mM MgCl2, 0.5 mM CaCl2, and 10 mM Tris·HCl; pH 7.4) in the presence of 10 μM Na2-ATP and PKA at the indicated concentrations or 5 mM PKC + lipid activator. Radiolabeling was achieved with 400 nM PKA or 5 nM PKC under the same conditions except that Na2-ATP was replaced by 20μCi [γ-32P]ATP.

Immunoblot analysis. BHK cells stably expressing ΔR-Split/RD or ΔR-Split/7CA-RD CFTR were washed twice with ice-cold PBS, harvested by scraping, and lysed on ice for 30 min in RIPA buffer supplemented with protease inhibitor cocktail (Roche). Lysates were then centrifuged, and supernatants were used immediately for immunoblotting.

Cells coexpressing ΔR-Split with either RD or 7CA-RD were treated with 150 μM CPT-cAMP + 1 mM IBMX or 20 mM PMA for 3 h (or not, as a control) before being harvested, and lysates were incubated with phosphatase inhibitors.

Lysates from ΔR-Split CFTR-expressing cells were incubated with (or without, as a control) the following: 1) 1 μg of RD-GST or 7CA-RD/GST + 10 μM kinase inhibitor H7 (unphosphorylated condition); 2) 1 μg of PKA prephosphorylated RD-GST (or 7CA-RD/GST) + PKA (at indicated concentrations) + ATP (2 mM) + phosphatase inhibitors (5 μM cyclosporine A + 100 mM calcylin A); or 3) 1 μg of PKC prephosphorylated RD/GST (or 7CA-RD/GST) + PKC (5 mM) + ATP (2 mM) + phosphatase inhibitors (5 μM cyclosporine A + 100 mM calcylin A) + PKC lipid activator. Aliquots of each cell lysate were then incubated with M3A7- or L12B4-bound columns at room temperature with rotation for 10 min and spun for 1 min, and the supernatant was discarded. Another aliquot was then incubated on the same column, and this procedure was repeated until all available fractions had been subjected to the column. Columns were then washed with quenching buffer + 0.1% Triton X-100 + protease inhibitors. Immunoprecipitated proteins were eluted using low-pH (2.8) elution buffer. Elution fractions were mixed with 5 × sample buffer + 80 mM DTT for subsequent analyses by SDS-PAGE and Western blot analysis. The presence of each domain in the pulldowns was revealed by monoclonal anti-CFTR antibodies directed against an epitope located within the range of amino acid positions 386-412 for the front half (L12B4) and positions 1365-1395 in NBD2 of CFTR for the back half (M3A7), and, to detect RD, 7CA-RD, and GST-RD, we used the anti-RD monoclonal antibodies MAB1660 (amino acids 590-830, R&D Systems) or 450 (amino acids 696-705; a gift from J. Riordan, University of North Carolina).
**Iodide effluxes.** Cells were cultured in six-well plates for 3–7 days, and iodide efflux experiments were performed using confluent monolayers as previously described (6). Briefly, cells were incubated with iodide loading buffer [136 mM Na\(_7\), 3 mM K\(_2\)NO\(_7\), 2 mM Ca(NO\(_3\))\(_2\), 11 mM glucose, and 20 mM HEPES; pH 7.4] for 1 h at room temperature. Extracellular NaI solution was then removed and rapidly replaced with efflux buffer in which NaI had been replaced with NaNO\(_3\). Samples were taken and replaced at 1-min intervals. The first three samples were used to establish a stable baseline efflux [time (t) = −3 to 0 min]. Activators or inhibitors were added in the efflux buffer from t = 0 min, and collection continued at 1-min intervals for a further 12 min in the continued presence of tested drugs. The iodide concentration of each aliquot (in nmol·ml\(^{-1}\)·min\(^{-1}\)) was determined using an iodide-sensitive electrode (Thermo Electron) and plotted versus t. From these plots, the \(1^{-}\) efflux rate constant \(k\) (in min\(^{-1}\)) was calculated according to Becq et al. (Ref. 2; the European working group on CFTR expression) for every minute interval. Iodide efflux peaks (maximum efflux rate obtained during stimulation) were compared after subtraction of the efflux rate measured under control conditions of the same efflux time.

**Results.** Results are reported as means ± SE; n is the number of independent experiments. Differences were assessed using Student’s t-test, with \(P < 0.05\) considered significant.

**RESULTS**

We (7) have previously characterized \(\Delta R\)-Split CFTR lacking the RD (amino acids 635-837) expressed in stably transfected BHK cells. This inducible bicistronic construct yielded polypeptides corresponding to the two halves of CFTR (TMD1-NBD1 and TMD2-NBD2). Western blot analysis with monoclonal antibodies L12B4 and M3A7 revealed expression of the front half (amino acids 1-634) and back half (amino acids 837-1481) of \(\Delta R\)-Split CFTR, respectively, at ~62 kDa. Retransfecting the inducible \(\Delta R\)-Split CFTR cell line with pNUT-RD also yielded the RD polypeptide, as monitored by probing Western blots with MAB1660 or 450 anti-RD monoclonal antibodies. The level of RD expression was unaffected by induction of \(\Delta R\)-Split CFTR, and induction of the front and back halves was similar with and without cotransfected RD. These experiments demonstrated the expression of the entire CFTR as three polypeptide fragments that reassembled to form functional CFTR channels at the plasma membrane of BHK cells (7).

**Phosphorylation of the RD by PKA and PKC enhances its interaction with the two halves of \(\Delta R\)-Split CFTR both in vitro and in vivo.** To study the interaction of the RD with the front half and back half of CFTR, we used both \(\Delta R\)-Split/RD CFTR-expressing BHK cells and \(\Delta R\)-Split-expressing cells with a purified GST-RD fusion protein expressed in bacteria as previously described (7). Monoclonal antibodies MM13-4 or L12B4 were used to pull down the front half, and M3A7 against an epitope in NBD2 was used to pull down the back half of the Split channel. As shown previously, the two halves coimmunoprecipitated (21), and their interaction was unaffected by pretreating cell lysates with PKA (7). In the present study, we found that coimmunoprecipitation of the two halves was also unaffected by pretreating cell lysates with PKC (Fig. 1B). Thus, the presence of the RD-GST fusion protein either unphosphorylated or phosphorylated in vitro with PKA or PKC did not alter interactions between the two Split halves.

Interaction of the unphosphorylated RD-GST fusion protein with the \(\Delta R\)-Split CFTR was almost undetectable. However, PKA or PKC prephosphorylation of RD-GST strongly increased its coimmunoprecipitation with the two halves of the Split channel, and, when both kinases were used together, RD-GST was pulled down much more effectively than with PKA or PKC phosphorylation alone; this was true whether the PKA concentration was low (20 nM; compare RD-GST in lanes 1 and 3 in Fig. 1B) or high (100 nM; compare RD-GST in lanes 4 and 5 in Fig. 1B). Thus, in vitro phosphorylation by PKA or PKC increases binding of the RD to the rest of CFTR, and simultaneous phosphorylation by both kinases further enhances this interaction.

Similar results were obtained in vivo using \(\Delta R\)-Split/RD CFTR expressed in BHK cells (Fig. 2). Endogenous PKA or PKC activities were stimulated by pretreating BHK cells with 150 \(\mu\)M CPT-cAMP + 1 mM IBMX, with 20 nM PMA, or the combination of 150 \(\mu\)M CPT-cAMP + 1 mM IBMX + 20 nM PMA, by addition directly into the cell culture medium at 37°C for 3 h before immunoprecipitation experiments. As shown in Fig. 2B, stimulation of either PKA or PKC had no effect on precipitation of the front or back half of CFTR but strongly enhanced pull down of the RD with the half molecules (Fig. 2, C and D). Stimulation of both kinases yielded the strongest domain-domain interaction, as assessed by pull downs of the
RD. These results provide new insights into phosphorylation-dependent interactions of the RD and suggest a mechanism for regulation by PKC phosphorylation.

Effect of mutation of potential PKC sites on RD interactions in vitro. To further investigate the role of PKC phosphorylation on RD interactions, we prepared a RD-GST fusion protein in which all seven PKC consensus sites in the RD had been removed (Fig. 3A). PKC phosphorylation of the 7CA-RD-GST fusion protein was lost after removal of those seven potential PKC sites, whereas PKA phosphorylation was unaffected (Fig. 3B).

The expression of CFTR fragments in BHK cells was monitored by probing Western blots with L12B4 (front half), M3A7 (back half), and MAB1660 (7CA-R domain) as previously described. The two half molecules were detected as bands of ~62 kDa. A comparison with cells expressing WT RD polypeptide using densitometry indicated that expression of the half molecules was not influenced by removing PKC sites from the RD. The 7CA-RD protein was also detected at its predicted molecular weight (~25 kDa), and its expression/detection efficiency was similar to that of WT RD (Fig. 4), enabling comparisons in pulldown experiments. The anti-RD antibody MAB1660 used for immunoprecipitation and Western blot experiments was not phosphospecific since it detected RD in BHK cell lysates equally well when cells were pretreated with kinase activators or inhibitors, although retardation in electrophoretic migration of the RD was visible under phosphorylation conditions (Fig. 4C).

The front and back halves and the 7CA-RD of CFTR colocalize at the plasma membrane of BHK cells. To assess the impact of PKC site mutations on the trafficking of reassembled channels to the plasma membrane, we immu-
nostained fixed and permeabilized cells with monoclonal antibodies against each fragment (Fig. 5A). As shown previously, all three antibodies recognized heterologous WT CFTR at the plasma membrane of BHK cells. Negative controls in which all primary antibodies were omitted and untransfected parental cells confirmed that L12B4, M3A7, and MAB1660 antibodies were specific and localized Split-CFTR in BHK parental cells (Fig. 5, C and D). We detected the front and back halves of ΔR-Split/7CA-RD CFTR only after cells had been induced with 10 μM ponasterone A, and overlap of the two signals revealed that the two halves were colocalized, or very near, the plasma membrane. Immunolabeling with MAB1660 showed that cotransfected 7CA-RD also colocalized with the two halves of the Split protein. To confirm membrane localization of each domain of ΔR-Split/7CA-RD, the plasma membrane of BHK cells was stained with Vibrant CM-Dil cell membrane marker, and individual domains were detected using their specific antibodies as previously described (Fig. 5B). Overlapping signals from the membrane marker and Cy5-conjugated secondary antibodies confirmed membrane colocalization of the front and back halves of the Split channel (n = 6). A substantial fraction of the 7CA-RD fluorescent signal overlapped with the membrane dye marker, indicating close association of 7CA-RD with the membrane, presumably through interactions with the front and back halves of the Split CFTR. Neither assembly of the three polypeptide fragments of CFTR (TMD1/NBD1, TMD2/NBD2, and the RD) nor their trafficking to the membrane were affected when PKC sites were mutated.

Inhibitory role of the RD and phosphorylation-dependent activation. Xenopus oocytes expressing ΔR-Split CFTR present phosphorylation-independent activity, which was hypothesized to be due to the loss of inhibition by the RD (9). We confirmed this and demonstrated that RD inhibition was reversible by coexpressing ΔR-Split CFTR with the missing RD fragment in BHK mammalian cells (7). We have used a similar approach to investigate the role of PKC in regulating the function of assembled channels. Iodide effluxes were measured 48 h after the induction of expression of ΔR-Split/RD or ΔR-Split/7CA-RD CFTR with ponasterone A; cells expressing ΔR-Split alone or full-length WT CFTR were used for comparison. As expected, in iodide efflux experiments, NaI released from (unstimulated) cells during the first 3 min of efflux measurements was significantly lower (P < 0.032, n = 4) for ΔR-Split/RD CFTR-expressing cells than for ΔR-Split CFTR-expressing cells. Values from ΔR-Split/7CA-RD CFTR were identical to those from ΔR-Split/RD CFTR (P > 0.18, n = 10), indicating that nonmutated RD and 7CA-RD interact with, and inhibit, ΔR-Split CFTR (Fig. 6). Thus, the seven PKC conse-

Fig. 3. GST-RD phosphorylation. A: relative positions of consensus sequences for PKA and PKC phosphorylation of the RD of CFTR. All serines or threonines in PKC sites were mutated to alanine in 7CA-RD/GST. B: autoradiogram of [γ-32P]ATP-radiolabeled RD-GST [wild type (WT) or 7CA mutant] comparing PKA and PKC phosphorylation levels.

Fig. 4. Expression of ΔR-Split/RD and ΔR-Split/7CA-RD in BHK cells. A: representative Western blots showing front half, back half, and RD expression levels in ΔR-Split/RD-expressing cells (lane 1) or ΔR-Split/7CA-RD-expressing cells (lane 2) as revealed using L12B4, M3A7, and MAB1660 antibodies, respectively, after induction with 10 μM ponasterone A for 48 h. B: ratio of expression for each domain of ΔR-Split/7CA-RD versus ΔR-Split/RD after 48 h of induction with ponasterone A (10 μM). Values calculated by densitometry of scanned Western blots represent means ± SE of n = 4 different experiments. C: representative Western blot of RD present in 60 μg of total lysate protein from BHK cells expressing ΔR-Split/RD as revealed using anti-RD monoclonal antibody MAB1660. Lane 1, untreated (control) ΔR-Split/RD-expressing cells; lane 2, ΔR-Split/RD-expressing cells treated with BlisX + H89; lane 3, ΔR-Split/RD-expressing cells treated with PMA; lane 4, ΔR-Split/RD-expressing cells treated with CPT-cAMP + IBMX; lane 5, ΔR-Split/RD-expressing cells treated with CPT-cAMP + IBMX + PMA.
sus sites on the RD are not required for its negative regulation under basal conditions, and mutation of them is unlikely to cause structural alterations that strongly impact function. On the other hand, PKA stimulation by cAMP/H11001 IBMX did produce a small but significant activation ($P < 0.0177, n = 6$) of ΔR-Split/7CA-RD, although the peak $k_t$ at $t = 7$ min was $<20\%$ that observed for full-length WT CFTR ($peak k_t = 7\text{ min} -\text{basal} k_t = 7\text{ min} = 0.23 \pm 0.066 \text{ min}^{-1}$ for ΔR-Split/7CA-RD, whereas peak $k_t = 4\text{ min} -\text{basal} k_t = 4\text{ min} = 1.30 \pm 0.15 \text{ min}^{-1}$ for WT CFTR). This reduction may be due, in part, to lower

Fig. 5. Immunolocalization of ΔR-Split/7CA-RD CFTR in BHK cells. A: cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 before incubation with (1:1,000) anti-CFTR antibodies L12B4 (front half), M3A7 (back half), or MAB1660 (7CA-RD). Signals were detected using secondary goat anti-mouse antibodies conjugated with Cy3 (1:100; red) or Cy5 (1:200; green). Overlapping signals indicating colocalization of the front and back halves, front half and 7CA-RD, or back half and 7CA-RD are shown in yellow. B: the plasma membrane was labeled with the membrane dye CM-Dil (green) followed by fixation and permeabilized before being immunostained as in A with anti-CFTR antibodies L12B4 (front half), M3A7 (back half), or MAB1660 (7CA-RD) (red). Overlapping signals indicating membrane localization are shown in yellow. C: negative controls. After induction with ponasterone A for 48 h, cells were fixed, permeabilized, and then incubated with Cy3- or Cy5-conjugated secondary antibodies without primary antibodies. D: negative controls. BHK-21 cells (parental cell line) were fixed with paraformaldehyde and permeabilized before incubation with L12B4 (front half), M3A7 (back half), or MAB1660 (7CA-RD) and secondary goat anti-mouse antibodies conjugated with Cy3, as in A.
expression of the reassembled channels, although some decrease in open probability is also expected with severed channels. The more important finding was that Split channels reassembled with 7CA-RD were twofold less active compared with those with WT RD. Stimulation of PKC by PMA (20 nM) exposure for 1 h before the efflux did not modify basal or

PKA-stimulated efflux rates for 7CA-RD/RD channels (peak $k_t = 7 \text{ min}^{-1}$ - basal $k_t = 7 \text{ min}^{-1} = 0.20 \pm 0.07 \text{ min}^{-1}$). In contrast, PKC stimulation significantly lowered the basal efflux rate ($P < 0.0001$, $n = 6$) and strongly enhanced PKA-dependent stimulation of $\Delta R$/Split/RD CFTR (peak $k_t = 6 \text{ min}^{-1}$ - basal $k_t = 6 \text{ min}^{-1} = 0.37 \pm 0.07$ and $0.76 \pm 0.15 \text{ min}^{-1}$ at $t = 7 \text{ min}$ after PKA and PKC + PKA stimulation, respectively, $P < 0.003$, $n = 6$). With cells expressing WT CFTR, basal $k_t$ was unchanged during PKC stimulation, but PKA-dependent activation was enhanced (peak $k_t = 4 \text{ min}^{-1}$ - basal $k_t = 4 \text{ min}^{-1} = 1.30 \pm 0.15$ and $1.58 \pm 0.05 \text{ min}^{-1}$ after PKA and PKC + PKA stimulation, respectively, $P < 0.04$, $n = 10$; Fig. 6C). These results were consistent with previous results obtained with full-length 9CA-CFTR channels, which also lacked the seven RD PKC sites (6), and argue that functional responses to PKA and PKC + PKA are diminished by removal of consensus PKC sites. This functional dependence prompted us to examine the role of PKC sites in phosphorylation-induced binding of the RD with other CFTR domains.

Effect of removal of PKC consensus sites on regulated binding of the RD. We used coimmunoprecipitations to study the ability of the 7CA-RD to associate with the front and back halves of the Split CFTR channel expressed in BHK cells. Pull downs under basal conditions were compared with those during PKA or PKC stimulation in cells pretreated for 3 h with either cAMP + IBMX or PMA. The presence of each domain of $\Delta R$/Split/7CA-RD CFTR precipitated with MM13-4 or M3A7 antibodies was revealed by Western blot analysis with M3A7 (back half), MM13-4 (front half), and MAB1660 (7CA-RD). As shown in Fig. 7, the presence of the front and back halves of $\Delta R$/Split were detected at similar levels under all conditions examined. 7CA-RD was also present in pull downs from untreated cells or from PKA- or PKC-prestimulated cells; however, there were no changes in the levels of interaction of 7CA-RD with the rest of the channel after phosphorylation by either kinase [density (means ± SE; expressed as a percentage of control): 78.68 ± 12.76%, $P > 0.11$, and 97.8 ± 10.92%, $P > 0.74$, for cAMP- and PMA-stimulated cells, respectively], in marked contrast to the results with $\Delta R$/Split/RD CFTR (Fig. 2, C and D). The interaction of 7CA-RD was similar to that of the WT RD under control conditions [7CA-RD density (means ± SE; expressed as a percentage of WT RD density): 108.87 ± 26.61%, $P > 0.9$], indicating that PKC phosphorylation sites in the RD are essential only for the phosphorylation-stimulated interaction of the RD with the Split halves.

Taken together, these functional and biochemical results indicate that RD binding to other CFTR domains is strengthened by PKC or PKA phosphorylation and that combined phosphorylation by both kinases provides the strongest interaction. Perhaps most surprising is the finding that PKC consensus sites are needed for PKA-induced binding even though PKA phosphorylation of the mutated RD appears to be normal. Finally, the weak interaction under unstimulated conditions, which maintains the CFTR channel closed, is also preserved.
when PKC sites are removed; thus, PKC phosphorylation is only needed for phosphorylation-dependent interactions. This view of RD interactions after stimulation of PKA and PKC is in good agreement with the regulation of full-length CFTR channel activity by those two kinases observed in the same cells (5, 6, 15).

**DISCUSSION**

The present study demonstrates that phosphorylation of the RD by both PKA and PKC modulates its interaction with other parts of CFTR. Using a ΔR-Split CFTR construct and separately expressed RD in the mammalian BHK cell line, we confirmed that the RD causes ΔR-Split CFTR channels to remain closed under control conditions and restores their phosphorylation-dependent activation. Moreover, we extended our previous model of PKA and PKC regulation by showing that PKC phosphorylation increases RD binding and that the strongest binding is obtained using both kinases. Finally, the results obtained after mutagenesis of all the potential PKC sites in the RD suggest that PKC phosphorylation is essential for PKA-induced binding of the RD to the rest of the CFTR channel.

The RD has, for long time, been viewed as an inhibitory particle that becomes released upon phosphorylation by PKA, by analogy with the “ball and chain” model for fast (N-type) inactivation of voltage-gated Shaker B K⁺ channels (14). Using a ΔR-Split CFTR construct, we provided the first biochemical evidence that phosphorylation by PKA strongly increased the interaction of the RD with the other parts of CFTR. We therefore proposed that instead of being released from an inhibitory position, the phosphorylated RD binds strongly to a different part of the channel, resulting in an intermolecular rearrangement of domains that enables channel gating (7). These domain-domain interactions suggested a potential mechanism for the PKC dependence of PKA regulation. Although the reassembled Split channels were kept closed under basal, unstimulated conditions, as observed with full-length WT CFTR, PKC phosphorylation alone increased RD binding to split channels in vivo (i.e., after stimulation of endogenous kinases in BHK cells), and, in vitro (after incubation of phosphorylated GST-RD fusion protein with cell lysates), it did not induce CFTR activation. Binding of the RD greatly enhanced after phosphorylation by both kinases, and activation of ΔR-Split CFTR channels was much more robust after PKC + PKA stimulation than with PKA alone. This corresponds well with previous studies in which PKA-dependent activation was increased by PKC phosphorylation of the RD when CFTR activity was observed at the whole-cell or single-channel level (5, 6, 15, 29). Iodide efflux and patch-clamp experiments (Ref. 7 and the present report) provide further evidence that the phosphorylation-dependent interactions observed may play a key role in channel function. The RD interaction increases with PKC phosphorylation alone, PKA phosphorylation alone, and more efficiently with PKC + PKA phosphorylation, but only PKA or PKC + PKA phosphorylation induce CFTR activity. A possible interpretation of this complex regulation is that phosphorylation by PKC enables the

![Figure 7](http://ajpcell.physiology.org/)

**Fig. 7.** Coimmunoprecipitation of the front half, back half, and RD from cells expressing ΔR-Split/7CA-RD CFTR. **A:** schematic representation of the two halves of ΔR-Split CFTR and RD expressed as separate polypeptides in BHK cells. **B:** top: immunoprecipitations performed using untreated cells (lane 1) or cells pretreated for 3 h with either 150 μM CPT-cAMP + 1 mM IBMX (lane 2) or 20 nM PMA (lane 3). **Bottom:** histogram representing the density of 7CA-RD from cells treated with cAMP + IBMX or PMA expressed as a percentage of RD density from control cells. Values are means ± SE of n = 5. Immunoprecipitation was performed using M3A7 monoclonal antibody, and the presence of different domains in the pull downs were revealed by probing blots with L12B4 (front half), M3A7 (back half), or MAB1660 (RD) monoclonal antibodies. **C:** top: comparison of WT and 7CA-RD in pull downs from untreated BHK cells expressing ΔR-Split/7CA-RD-7CA-RD immuno-precipitated with M3A7 monoclonal antibody and revealed using anti-RD MAB1660. **Bottom:** histogram representing the density of 7CA-RD expressed as a percentage of WT RD density. Values are means ± SE of n = 5.
RD to interact with a nonstimulatory region of the front and/or back half of Split CFTR that is distinct from the stimulatory PKA-dependent interacting region but that results in a conformation that is more conducive to PKA-dependent gating. In this model, PKC + PKA phosphorylation stimulates the RD to interact with both unidentified regions and results in the strongest binding and optimal conformation for PKA-dependent gating.

The isolated RD does not fold into a stable, globular structure (1, 11, 20) and has been called the regulatory region for this reason (1). Nevertheless, it does have some helical content in solution that is reduced by PKA phosphorylation (11), and a NMR study (1) has indicated that the helices are dynamic and transient and may mediate RD binding to the NBD1 since PKA phosphorylation reduces both the helical propensity and interaction with NBD1. Many domain-domain interactions have been reported within CFTR that suggest possible binding partners for the RD (16–18, 21, 28, 30, 31). For example, unphosphorylated GST-RD fusion protein binds to short peptides (20-mers) from the amino terminus of CFTR, cytoplasmic loops 1 and 4, and both NBDs (31). Phosphorylation may reduce inhibitory interactions in addition to enhancing the stimulatory ones, as suggested by the present study. There is evidence for such dual regulation of CFTR by a discrete helical region in the distal RD between amino acids 817 and 838 called Neg2 (32). A negative charge in this region is required for channel inhibition, whereas its helical structure is necessary for activation. More work is needed to understand the relationship between those determinants of Neg2 function and recent NMR data indicating that negative charges (i.e., phosphyl groups) associated with channel activation reduce the helical propensity of the RD.

The mechanisms of phosphorylation regulation are probably complex since the locations of phosphorylation sites in the RD are well conserved evolutionarily even though amino acid identity is low (10). We (6) have reported previously that mutating all PKC sites in distal NBD1 and the RD (9CA-CFTR) strongly reduced PKA-dependent activation of full-length CFTR expressed in BHK cells. That study (6) confirmed that PKC regulation of CFTR occurs at least in part through direct phosphorylation. The present results are consistent with functional studies with full-length 9CA-CFTR. Assembled δR-Split/7CA-RD CFTR channels, which were inhibited under control conditions compared with δR-Split CFTR channels, did respond weakly to PKA stimulation but not to PKC alone or PKA + PKC. The present study confirms that PKC phosphorylation sites are essential to maintain the channel in a “gating-competent” conformation, which involves increased interaction of the RD with a region of CFTR that remains to be identified.

Constitutive basal phosphorylation of the RD by PKC, which enables CFTR activation by PKA alone, has been proposed in several studies using recombinant and epithelial cells (for a review, see Ref. 12). In a previous study (5), we observed significant basal phosphorylation of CFTR channels after metabolic labeling with 32PPO4 and ~30% of that basal phosphorylation was resistant to phosphatase treatment in vitro. Our functional and biochemical data are consistent with permissive basal PKC phosphorylation at one or more of the seven predicted PKC phosphorylation sites. When PKC phosphorylation was eliminated by site-directed mutagenesis of all the consensus sequences for PKC, Split CFTR channels remained unresponsive to PKA stimulation despite normal PKA phosphorylation of 7CA-RD.

S686 is a likely candidate to mediate stimulated PKC-dependent interactions since mutation of Ser686 to alanine in the full-length CFTR channel dramatically reduced CFTR activation to the level of 9CA-CFTR (5), whereas mutation of more distal PKC sites in the RD had no effect on the function (S707A/S790A/T791A/S809A). Further investigations are thus necessary to clarify the role of individual PKC sites in RD binding.

In conclusion, the present results suggest a mechanism in which weak binding of the RD under basal (unphosphorylated) conditions maintains the channel closed, perhaps through an interaction with NBD1 or another domain. Upon phosphorylation of PKC sites, the RD binds to a permissive, nonactivating region, and subsequent PKA-catalyzed phosphorylation of the RD allows release from NBD1 and stronger rebinding to another site that enables channel activation. The identity of these receptors for the phosphorylated RD remain to be determined.

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