Phosphorylation-dependent Block of Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel by Exogenous R Domain Protein*

The cystic fibrosis transmembrane conductance regulator (CFTR) constitutes a linear conductance chloride channel, which is regulated by cAMP-dependent protein kinase phosphorylation at multiple sites located in the intracellular regulatory (R) domain. Studies in a lipid bilayer system, reported here, provide evidence for the control of CFTR chloride channel by its R domain. The exogenous R domain protein (encoded by exon 13 plus 85 base pairs of exon 14) interacted specifically with the CFTR molecule and inhibited the chloride conductance in a phosphorylation-dependent manner. Only the unphosphorylated R domain protein blocked the CFTR channel. Such functional interaction suggests that the putative gating particle of the CFTR chloride channel resides in the R domain.

CFTR belongs to a family of ABC transporters (1), each of which contains two transmembrane domains and, usually, two nucleotide binding folds. CFTR contains, in addition, a large intracellular regulatory (R) domain (2). The CFTR protein constitutes a linear conductance Cl⁻ channel, the opening of which requires both phosphorylation of the R domain by cAMP-dependent protein kinase (PKA) (3, 4) and ATP binding and hydrolysis by the nucleotide binding folds (5, 6). The working hypothesis for CFTR activation by the R domain has been that phosphorylation by PKA presumably induces a conformational change (7), which relieves the inhibitory action of the R domain on the Cl⁻ conductance, as CFTR with its R domain removed generates a constitutively open Cl⁻ channel (without requiring PKA phosphorylation) (8, 9).

Some voltage-dependent ion channels, such as the Shaker K⁺ channel, inactivate or close by the "ball and chain" mechanism (10). The amino-terminal portion of the Shaker K⁺ channel serves as the "ball" that occludes the conductance pathway of the open K⁺ channel (11). It is not known, however, whether the R domain of CFTR functions as a gating particle for the Cl⁻ channel. In favor of this possibility is the observation that deletion of 130 amino acids from the R domain results in a constitutively open channel (8). On the other hand, the R domain of CFTR is quite large (over 250 amino acids) compared with the putative "ball" for the Shaker K⁺ channel, which is less than 25 amino acids in length.

We took a reconstitution approach in an attempt to search for the putative gating particle of the CFTR Cl⁻ channel. First, the R domain protein (RDP) was expressed as a peptide in vitro in a rabbit reticulocyte lysate system. Second, the wild type CFTR cDNA was subcloned into an eukaryotic expression vector pCEP4 and expressed in a human embryonic kidney cell line (HEK 293), from which the microsomal membrane vesicles were isolated and incorporated into lipid bilayers where the CFTR channel activity can be directly assessed. Alternatively, CFTR channels were incorporated into the planar lipid bilayer from vesicles prepared from T84 cells, a human colon carcinoma cell line that expresses abundant CFTR. The effect of RDP on the CFTR channel was then tested in the lipid bilayer reconstitution system. We reasoned that if the R domain, or portions of it, function as a "ball" or gating particle for CFTR, then exogenous R domain protein might enter the CFTR pore or vestibule when the channel is open and block it. Alternatively, if the open configuration of CFTR is stabilized by intramolecular interactions of the R domain with other portions of the molecule, then exogenous R domain might hold the channel in the closed state by occupying this putative intramolecular binding site and preventing the endogenous R domain from assuming the open configuration. Based on the function of intact CFTR, in which phosphorylation at a single site in the R domain is sufficient to induce channel opening in the presence of ATP (9), we postulated that the open channel block model, in which R domain protein blocks the conduction pore, would most likely require unphosphorylated R domain to block, whereas block of the closed channel by the R domain protein occupying an intramolecular stabilization site would probably be favored by phosphorylation of the exogenous R domain.

EXPERIMENTAL PROCEDURES

In Vitro Expression of R Domain Protein—A pseudogene was constructed by polymerase chain reaction to amplify the R domain of CFTR. The pseudogene contains the entire exon 13 plus 85 bases of exon 14, with AGCTTATGGGAGG added at the 5' end and TGATCTAGGAG at the 3' end for ease of cloning and to provide translational initiation and termination sites. The R pseudogene was ligated 5' to 3' into the HindIII and BamHI sites of pBluescript SKII (Stratagene). The HindIII-BamHI orientation allowed for sense transcription of the R domain pseudogene from the T7 promoter. The RDP was made in vitro using the TNT-coupled reticulocyte lysate system (Promega). SDS-PAGE was performed on the translation products with electrophoresis of the gel onto polyvinylidene difluoride membrane. The polyvinylidene difluoride membranes were blocked with phosphate-buffered saline plus 0.5% Tween 20 (PBS-T) and 1% bovine serum albumin and then

* This work was supported by National Institutes of Health Grants P30DK27651, RO1DK/HL49003, and RO1DK45965 (to M. L. D.), a Cystic Fibrosis Foundation grant (to P. B. D.), and an Established Investigatorship from the American Heart Association (to J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| To whom correspondence should be addressed: Dept. of Pediatrics, 10900 Euclid Ave., Case Western Reserve University, Cleveland, OH 44106. Tel.: 216-368-4370; Fax: 216-368-4223.

1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; R domain, regulatory domain; PKA, protein kinase A; RDP, R domain protein; PAGE, polyacrylamide gel electrophoresis; pS, picosiemen; PKI, protein kinase inhibitor.
incubated with antibody (mouse monoclonal anti-human CFTR exon 13) against the R domain (Genzyme). A secondary peroxidase-conjugated affinity-purified goat antibody to mouse IgG was added to allow visualization of the RDP-antibody complex. For every in vitro translation, production of RDP was verified by either incubating an aliquot of the translation mix with $^{35}$S-methionine and subsequent SDS-PAGE and autoradiography to identify the labeled 35-kDa band or by Western blotting of an aliquot of unlabeled translation product with antibody against the R domain of CFTR to identify the R domain protein.

Two control translation mixes were prepared. For one, no exogenous DNA or RNA was added to the reticulocyte lysate system, and when $^{35}$S-methionine was added to this mix and incubated exactly as for the R domain translation, no translated protein bands were detected on autoradiography (Fig. 1A, lane 1). This mix does, however, contain all of the endogenous reticulocyte lysate proteins. The second control translation mix was obtained by expressing the six proteins of the brome mosaic virus, one of which migrates on electrophoresis at about 35 kDa, where the R domain protein migrates. This control provides not only the ribosomes and proteins of the reticulocyte but also complete and possibly nascent proteins of about the same molecular weight as the R domain translation product.

Expression of CFTR in HEK 293 Cells—The wild type CFTR cDNA (12) was subcloned into the eukaryotic expression vector pCEP4 (Invitrogen) between the NheI and XhoI restriction sites. A human embryonic kidney cell line (HEK 293-EBNA, Invitrogen) was used for the transfection and expression of CFTR protein. The cell line contains a mosaic virus, one of which migrates on electrophoresis at about 35 kDa, and the R domain translation product, no translated protein bands were detected on autoradiography (Fig. 1A, lane 1).

Isolation of Microsomal Membrane Vesicles from HEK 293 Cells—Microsomal vesicles were isolated from HEK 293 cells expressing the wild type CFTR protein (13). Twelve 75-cm² flasks of 293 HEK cells transfected with pCEP4(CFTR) vectors were harvested. The cell pellet was resuspended in ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.2, 1 mM EDTA, 5 μM diisopropyl fluorophosphate, 10 μM pepstatin A, 10 μM aprotinin, and 10 mM benzamidine) prior to lysis by 10 strokes in a tight fitting Dounce homogenizer, followed by 15 strokes after addition of an equal volume of sucrose buffer (500 mM sucrose, 10 mM HEPES, pH 7.2). Microsomes were collected by centrifugation of postnuclear supernatant (600 × g, 15 min) at 100,000 × g for 45 min and resuspended with 1.0 ml of prephosphorylation buffer (250 mM sucrose, 10 mM HEPES, pH 7.2, 5 mM Mg-ATP, and 100 units/ml PKA catalytic subunit). Microsomal vesicles were stored at a protein concentration of 2–6 mg/ml at −75°C until use. Usually, 1–3 μl of microsomal membrane vesicle solution (1-ml volume) for the incorporation of CFTR channels.

Isolation of Microsomal Membrane Vesicles from T84 Cells—T84 cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Microsomal membrane vesicles from T84 cells were isolated following the procedure of Tilly et al. (14). Briefly, cells were homogenized in a buffer containing 250 mM sucrose, 2 mM EDTA, 10 mM HEPES-Tris (pH 7.4), plus protease inhibitors. The resulting plasma membrane was pelleted by centrifugation at 45,000 × g and resuspended in 150 mM KCI, 10 mM HEPES-Tris (pH 7.2) at a protein concentration of 1–3 mg/ml. The membrane vesicles were stored at −75°C until use.

Reconstitution of CFTR Channels—Lipid bilayer membranes were formed across an aperture of ~200 μm diameter with a lipid mixture of phosphatidyl ethanolamine and phosphatidyl serine cholesterol in a ratio of 6:6:1; the lipids were dissolved in decane at a concentration of 40 mg/ml (13). The recording solutions contained: cis (intracellular), 200 mM KCI, 2 mM ATP, 1 mM MgCl₂, and 10 mM HEPES-Tris (pH 7.4); trans (extracellular), 50 mM KCI, 10 mM HEPES-Tris (pH 7.4). Micromolar solutions (1–3 μl) containing wild type CFTR proteins were added to the cis solution. 50 units/ml PKA catalytic subunit (Promega) were always present in the cis solution. The experimental conditions selected only CFTR channels that were oriented in the bilayer membrane in the cis intracellular trans extracellular manner.

To maintain stability of the bilayer membrane and the CFTR channel activities, designed pulse protocols were used to measure currents through the single CFTR channels. The bilayer membrane was kept at a holding potential of 0 mV and pulsed to different test potentials of either 2- or 5-s durations. The interval between consecutive episodes was 10 s. Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments). Data acquisition and pulse generation are performed with a 466 computer and 1200 Digidata A/D converter (Axon Instruments). The currents were sampled at 1–2.5 ms/poin and filtered at 60 Hz through an 8-pole Bessel filter. Single channel data analyses were performed with pClamp, TIPS, and custom softwares.

**RESULTS**

Expression of the RDP—When no source of DNA or mRNA was included in the reticulocyte lysate translation mix, no $^{35}$S-methionine-labeled translation products were detected by SDS-PAGE and autoradiography (Fig. 1A, lane 1). However, when a Bluescript plasmid containing the gene encoding RDP, which contained the entire exon 13 plus 85 base pairs of exon 14 or 28 amino acids was included, a translation product was detected at molecular mass of ~35 kDa (Fig. 1A, lane 2). The R domain contains a second ATG codon in frame at a site nine or 14 or 28 amino acids was included, a translation product was detected at molecular mass of ~35 kDa. The R domain contains a second ATG codon in frame at a site nine amino acids downstream from the initial start site, and this second start site probably accounts for the doublet appearance of the protein. The apparent molecular mass of this protein is slightly higher than the calculated molecular mass (32 kDa) for reasons that are not entirely clear. However, this protein appears to be the authentic R domain protein because (i) sequenc-
body and phosphorylated with protein kinase A (Fig. 1A, lane 4). The concentration of RDP in the translation mix varied from 30 to 70 ng/ml, based on the incorporation of [35S]methionine (n = 3).

Expression of CFTR and Characterization of Channel Activity—CFTR was expressed by transfecting the CFTR cDNA under cytomegalovirus promoter control into the HEK 293 cells (13). After expression of the CFTR protein, microsomal membrane vesicles were isolated from the HEK 293 cells. The Western blot shows that these vesicles contain abundant CFTR protein (Fig. 1B). Alternatively, vesicles prepared from T84 cells were used for bilayer fusion. To measure the CFTR channel activities, 2 mM Mg-ATP and 50 units/ml catalytic subunit of PKA were always present in the cis intracellular solution. If PKA and ATP were not present in the cis solution, no CFTR-like channels were detected in 21 bilayer fusions with HEK293 vesicles and more than 100 fusions with T84 vesicles.

In a KCl concentration gradient of 200 mM (cis)/50 mM (trans), a single CFTR channel had a linear conductance of 8.2 ± 0.6 pS, with a reversal potential of +22 ± 1.4 mV. At −80 mV, the channel had an average open probability of 0.318 ± 0.028, with mean open lifetimes of τo1 = 23.6 ms and τo2 = 110 ms (n = 76) (13). Values were the same for channels from T84 vesicles, except for a small difference in the relative occurrence of τo1 and τo2 (15). An inhibitor of the CFTR channel, diphenylcarboxylate (3 mM), when added to the trans solution, completely blocked channel activity. 4,4'-Diisothiocyanate 2,2'-stilbene disulfonate-sensitive Cl− channel detected in the T84 cells (3.2 pS, distinct from the CFTR channel) (15, 17) (Fig. 4A, n = 6) nor on a 98-pS cation channel endogenous to the T84 cells (Fig. 4B, n = 5). The results indicate that RDP binds specifically to the intracellular portion of the CFTR protein, and the results are the same whether native CFTR or heterologously expressed CFTR is studied.

Role of Phosphorylation of RDP in Channel Block—Since these initial experiments were conducted with active PKA plus ATP present on the intracellular side of the channel and we have shown that the R domain protein is susceptible to phosphorylation by PKA (Fig. 1A, lane 4), it is likely that the RDP, though not phosphorylated when it is added to the bilayer system, undergoes phosphorylation during the course of the experiment. To test the role of phosphorylation in mediating the effect of RDP block of the CFTR channel, two sets of experiments were performed. First, to prevent phosphorylation of RDP, a specific peptide inhibitor for PKA, protein kinase

Phosphorylation-dependent Block of CFTR Chloride Channel

Fig. 2. Transient inhibition of CFTR chloride conductance by R domain protein. A single CFTR Cl− channel was incorporated into the bilayer membrane from microsomal membrane vesicles isolated from HEK 293 cells. Channel open probabilities (Po) were calculated as the fractional time occupied by the open state during the 5-s test pulse to −80 mV. The calculated Po during consecutive test pulses was plotted as a function of time ("diary plot"), as shown in the upper panel. RDP exhibited a biphasic effect on the CFTR channel, promoting channel closure for ~12 min, followed by spontaneous recovery. Representative single channel traces were shown for the control channel (A), 3 min after the addition of RDP (−15 pm final concentration) (B), and 16 min after the addition of RDP (C). Marks to the left of the single channel traces show base-line (zero current, closed channel) levels. Data are representative of seven separate experiments.
inhibitor (PKI), was added to the recording solution before the addition of RDP (Fig. 5). The inclusion of PKI did not cause rundown of CFTR channels in the bilayer either for heterologously expressed channels in HEK293 cells or for endogenous T84 channels, suggesting that the CFTR channels, once phosphorylated by PKA, did not undergo dephosphorylation in the bilayer membrane over the time course of the experiment (10–45 min, n = 2, T84 cells; n = 11, transfected HEK293 cells). In other words, phosphatases were not active in the vesicles fused with the bilayer membrane. These results are similar to those reported by Anderson et al. (5) in patch clamp studies of CFTR. Under these conditions, in which RDP cannot be phosphorylated, addition of RDP resulted in an irreversible block of CFTR channels (Fig. 5). In addition, the RDP was phosphorylated in a test tube before addition to the recording solution. The phosphorylated RDP had no significant effect on the channel activities over a period of 12 min (Fig. 6, n = 3).

The results indicate that phosphorylation plays a key role in mediating the binding of RDP to the CFTR molecule; only the unphosphorylated form of RDP is capable of blocking the CFTR channel.

**DISCUSSION**

The large intracellular R domain is a special feature of the CFTR molecule, alone among members of the ABC family. In the presence of adequate concentrations of intracellular ATP,
phosphorylation of any one of four main phosphorylation sites (18, 19) is sufficient for full opening of the channel, and indeed, small increases in channel open probability can be detected in response to PKA phosphorylation even in native CFTR molecules with all consensus phosphorylation sites converted to alanines (20). However, CFTRΔR-S660A, a molecule with amino acids 708–835 deleted (to render it constitutively open) with the remaining consensus PKA site mutated, is insensitive to activation by PKA (9), suggesting that activation by phosphorylation takes place in the R domain. Our results show that the exogenous RDP interacts specifically on the intracellular side of the CFTR channel. Inhibition of channel activity is

**Fig. 5. Preventing phosphorylation of RDP causes sustained CFTR channel block.** PKI was added to the intracellular side of a single CFTR channel to a final concentration of 10 μM, approximately 5 times the concentration necessary to prevent phosphorylation of RDP by PKA determined in separate assays. Channel open probabilities (Po) were calculated as the fractional time occupied by the open state during the 5-s test pulse to -80 mV. The diary plot (upper panel; see Fig. 2 legend) shows that addition of PKI did not affect Po of the control channel, but it prevented the recovery of channel activity closed by RDP. In the presence of PKI, the blocking effect of RDP became essentially irreversible. The channel remained closed during the whole period of the experiment. Selected single channel traces are shown for the control condition (A), 5 min after addition of PKI (B), and 13 min after addition of RDP (C). Base line (zero current, closed channel) is indicated for each line by marks on the left. This experiment is representative of four others.

**Fig. 6. Prephosphorylation of R domain protein eliminates its inhibitory activity.** The RDP was prephosphorylated in a test tube with 100 units/ml catalytic subunit of PKA and 5 mM Mg-ATP before addition to the CFTR channel, which was captured in a vesicle from HEK293 cells. Under control conditions, the bilayer contained at least three active CFTR channels (A), which remain stable over a period of 12 min. Panel B illustrates channel activity following addition of the phosphorylated RDP. No inhibition was seen over a period of 12 min. Subsequent addition of PKI and unphosphorylated RDP resulted in progressive inhibition of CFTR channel activity. The traces shown in C were taken 12 min after addition of PKI and unphosphorylated RDP. The residual channel activity remaining could be completely blocked by adding 3 mM diphenylcarboxylate to the trans solution (data not shown). Base line (zero current, closed channels) is shown by a dashed line. This experiment is representative of three others.
abolished by phosphorylation of RDP.

This inhibitory activity is specific for the R domain, since an anonymous protein of comparable molecular weight translated under the same conditions fails to block the channel, and inhibition appears to be specific for CFTR, since the small linear chloride channel is not blocked, nor is a large cation channel identified in the T84 vesicle preparations. The inhibition depends on the phosphorylation state of the R domain protein, however. Prephosphorylated R domain protein fails to block the channel, and when R domain protein is added under conditions where phosphorylation has been inhibited, the channel is closed indefinitely. However, when unphosphorylated R domain protein is added to the bilayer system in the presence of PKA and ATP, there is an initial block, followed by slow reversal as (presumably) the R domain protein undergoes phosphorylation during the course of the experiment. A second round of block and reversal can be demonstrated by adding fresh, unphosphorylated R domain protein. These experiments demonstrate that block by the R domain protein is not due to destruction of the CFTR channel, since the channel recovers with time (and phosphorylation of the R domain protein), and recovery of the channel is not accompanied by insensitivity to R domain block, since addition of fresh R domain protein restores the block.

These data are consistent with the hypothesis that the R domain protein binds reversibly to the CFTR channel in its open state, probably in the region of the pore. In the conditions in which PKA and ATP are still available on the intracellular side of the CFTR molecule, unbound R domain protein is gradually phosphorylated. As the R domain protein disengages from CFTR, there is, over time, less and less R domain protein remaining unphosphorylated to interact with CFTR. Eventually, the concentration of unphosphorylated R domain protein falls below that required to bind to and block the channel. When the R domain protein is prephosphorylated, there is no interaction. When the R domain protein is protected from phosphorylation by inclusion of PKI on the intracellular side of CFTR, there is always sufficient unphosphorylated R domain protein to interact with the channel. Since phosphorylation at even a single site in CFTR in the native molecule is sufficient to open the CFTR channel (9, 18, 19), we speculate that binding of the R domain to the pore requires an unphosphorylated R domain protein molecule. An alternative hypothesis is that the exogenous R domain binds to a site in the closed state of the CFTR molecule, exerting steric hindrance to channel opening. In this case, one would expect that the phosphorylated R domain protein would be a better blocker than the unphosphorylated protein, because in the native molecule, stabilization of the open state must occur when the R domain is phosphorylated. Therefore, our data are less consistent with this hypothesis.

The reconstitution system presented here demonstrates specific interaction between the R domain and CFTR, which has functional consequences, and should be useful for further investigation of the specific amino acid residues involved in the binding sites on both RDP and CFTR itself.

REFERENCES

1. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
2. Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Science 245, 1066–1073
3. Tabcharani, J. A., Chang, X.-B., Riordan, J. R., and Hanrahan, J. W. (1991) Nature 352, 628–631
4. Bear, C. E., Li, C., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjesingh, M., and Riordan, J. R. (1992) Cell 68, 809–818
5. Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) Cell 67, 775–784
6. Quinton, P. M., and Reddy, M. M. (1992) Nature 360, 79–81
7. Duhlanty, A. M., and Riordan, J. R. (1994) Biochemistry 33, 4072–4079
8. Rich, D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E., and Welsh, M. J. (1991) Science 253, 205–207
9. Rich, D. P., Berger, H. A., Cheng, S. H., Travis, S. M., Saxena, M., Smith, A. E., and Welsh, M. J. (1993) J. Biol. Chem. 268, 20259–20267
10. Armstrong, C. M., Bezanilla, F., and Rqkas, E. (1973) J. Gen. Physiol. 62, 375–391
11. Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990) Science 250, 568–571
12. Drumm, M. L., Pope, H. A., Cliff, W. H., Rommens, J. M., Marvin, S. A., Tsui, L.-C., Collins, F. S., Frizzel, R. A., and Wilson, J. M. (1990) Cell 62, 1227–1233
13. Xie, J., Drumm, M. L., Ma, J., and Davis, P. B. (1995) J. Biol. Chem. 270, 28084–28091
14. Tilly, B. C., Winter, M. C., Ostedgaard, L. S., O’Riordan, C., Smith, A. E., and Welsh, M. J. (1992) J. Biol. Chem. 267, 9470–9473
15. Tao, T., Xie, J., Drumm, M. L., Zhao, J., Davis, P. B., and Ma, J. (1996) Biophys. J. 70, 743–753
16. Gadsby, D. C., and Nairn, A. C. (1994) Trends Biochem. Sci. 19, 513–518
17. Gabriel, S. E., Price, E. M., Boucher, R. C., and Stutts, M. J. (1992) Am. J. Physiol. 263, C708–C713
18. Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welsh, M. J., and Smith, A. E. (1991) Cell 66, 1027–1036
19. Picciotto, M. R., Cohn, A. J., Bertuzzi, G., Greengard, P., and Nairn, A. C. (1992) J. Biol. Chem. 267, 12742–12752
20. Chang, X.-B., Tabcharani, J. A., Hous, Y. Y., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W., and Riordan, J. R. (1993) J. Biol. Chem. 268, 11304–11311
