A Short Segment of the R Domain of Cystic Fibrosis Transmembrane Conductance Regulator Contains Channel Stimulatory and Inhibitory Activities That Are Separable by Sequence Modification*

Received for publication, February 19, 2002, and in revised form, April 10, 2002

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The regulatory (R) domain of the cystic fibrosis transmembrane conductance regulator (CFTR) contains consensus phosphorylation sites for cAMP-dependent protein kinase (PKA) that are the basis for physiological regulation of the CFTR chloride channel. A short peptide segment in the R domain with a net negative charge of B9 (amino acids 817–838, NEG2) and predicted helical tendency is shown to play a critical role in CFTR chloride channel function. Deletion of NEG2 from CFTR completely eliminates the PKA dependence of channel activity. Exogenous NEG2 peptide interacts with CFTR to exert both stimulatory and inhibitory effects on the channel function. The NEG2 peptide with sequence scrambled to remove helical tendencies also inhibits channel function, but does not stimulate. Similar results are found for a NEG2 peptide whose helical structure is disrupted by a proline residue. When six of the negatively charged carboxylic acid residues are replaced by their cognate amides, reducing net negative charge to B3, but increasing helical propensity as assessed by circular dichroism, the peptide stimulates CFTR channel function, but does not inhibit. We speculate that the NEG2 region interacts with other cytosolic domains of CFTR to control opening and closing transitions of the chloride channel.

Defects in CFTR, a chloride channel located in the apical membrane of epithelial cells, are associated with the common genetic disease, cystic fibrosis (1–3). CFTR is a 1480-amino acid protein that is a member of the ATP binding cassette transporter family (4). The general structure of these membrane proteins includes two membrane spanning domains, each consisting of six transmembrane segments, and two nucleotide binding folds (NBF1 and NBF2). Most members of the ATP binding cassette family use the free energy of ATP hydrolysis to actively transport substrates across the membrane (5). However, unlike the other members of this family, CFTR contains a unique regulatory (R) domain, and encodes a cAMP-regulated chloride channel (6–8).

The R domain of CFTR contains several consensus PKA phosphorylation sites (9–11) that are the basis for physiological regulation of this chloride channel. CFTR channel opening requires phosphorylation of serine residues in the R domain, and ATP binding and hydrolysis at the nucleotide binding folds (7, 12, 13). Phosphorylation adds negative charges to the R domain, and introduces global conformational changes reflected by a reduction in the α-helical content of the R domain protein (14). Thus, electrostatic and/or allosteric changes mediated by phosphorylation are likely responsible for interactions between the R domain and other CFTR domains that regulate channel function (15, 16).

Rich et al. (17) showed that deletion of amino acids 708–835 from the R domain (ΔR-CFTR), which removes most of the PKA consensus sites, allows the CFTR chloride channel to open without phosphorylation. The open probability of AR-CFTR is one-third that of the wild type (wt) CFTR channel and does not increase upon PKA phosphorylation, although other biophysical properties of the channel (i.e. conductance and anion selectivity) are similar to wt-CFTR (18, 19). These data suggest that deletion of the R domain removes both inhibitory and stimulatory effects conferred by the R domain on CFTR chloride channel function. In support of this suggestion, addition of exogenous unphosphorylated R domain protein (amino acids 588–858) to wt-CFTR blocks the chloride channel (20, 21), and the block is relieved if the R domain becomes phosphorylated, indicating that the unphosphorylated R domain is inhibitory. Conversely, exogenous phosphorylated R domain protein (amino acids 588–855 or 645–834) stimulates the ΔR-CFTR channel, suggesting that the phosphorylated R domain is stimulatory (18, 19). Therefore, it appears that the phosphorylation state of the R domain determines whether it functions to stimulate or inhibit chloride channel activity.

In this work we identify a stretch of negatively charged amino acids at the carboxyl terminus of the R domain (817–838, NEG2), with a net charge of −9, which appears to be involved in both the stimulatory and inhibitory functions of the R domain on chloride channel activity. Furthermore, by modifying the sequence of this peptide, we were able to separate its stimulatory and inhibitory functions.
pCEP4 wt-CFTR with the mutant fragment between the BarZ717 and XhoI restriction sites. The ΔNEG1-CFTR cDNA has 27 bases deleted (amino acids 725–733). The ΔNEG2-CFTR cDNA has 66 bases deleted (amino acids 817–838). Deletions were confirmed by sequencing across the junction site.

Expression of CFTR in HEK 293 Cells—A human embryonic kidney cell line (293-EBNA HEK; Invitrogen) was used for transfection and expression of the CFTR proteins (18, 20–22). The HEK-293 cell line contains a pCMV-EBNA vector, which constitutively expresses the Epstein-Barr virus nuclear antigen-1 (EBNA-1) gene product and increases the transfection efficiency of Epstein-Barr virus-based vectors. Cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% l-glutamine. Geneticin (G418, 250 μg/ml) was added to the cell culture medium to maintain selection of the cells containing the pCMV-EBNA vector. LipofectAMINE reagent (Invitrogen) in Opti-MEM media (serum-free) was used to transfect the HEK-293 cells with pCEP4wt, pCEP4(ΔNEG1), or pCEP4(ΔNEG2). After 5 h, serum was added to the media (10% final serum concentration). Twenty-four hours after transfection, the transfection media was replaced with fresh media. The cells were harvested 2 days after transfection and microsomal membrane vesicles were prepared for single channel measurements in the lipid bilayer reconstitution system (18, 20–22).

In Vitro Phosphorylation of CFTR Proteins—CFTR proteins isolated in membrane vesicles were bound to protein G-agarose using a mouse monoclonal anti-human CFTR antibody (mAb 24–1, Genzyme). The protein G-agarose was washed and [32P]ATP (10 μCi) and protein kinase A (10 units/50 μl) was added. Samples were incubated at 30 °C for 60 min. At the end of the phosphorylation period, the reaction was stopped by extraction with 5% Triton X-100 in PBS. The [32P]phosphopeptide products were separated on SDS-PAGE under reducing conditions and autoradiographed. In most cases, the positions of the phosphopeptides on the autoradiographs were confirmed by excision of the gel segment and subsequent analysis of the peptides by MALDI-TOF mass spectrometry.

RESULTS
Subcloning of CFTR Gene—The wt-CFTR cDNA was cloned into an Epstein-Barr virus-based episomal eukaryotic expression vector, pCEP4 (Invitrogen, San Diego, CA), between the NcoI and XhoI restriction sites (18, 20–22). The ΔNEG1 and ΔNEG2 deletion mutants were created using the pALTER mutagenesis system and shuttled from pALTER into pCEP4 by substituting the corresponding fragment in

EXPERIMENTAL PROCEDURES

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Manager (version 1.51.00) data acquisition software. Spectra were obtained at 4 °C from 1-mm path length quartz cells. CD spectra were the average of eight scans collected at 0.1-nm intervals from 260 to 190 nm using standard instrument settings. Peptide concentrations ranged between 15 and 49 μM, at pH 6.7. Peptide stock solution concentrations were obtained by quantitative amino acid analysis performed by the Protein/Peptide Core Facility of the Massachusetts General Hospital.

Reconstitution of CFTR Channels in Lipid Bilayer Membranes—Electrophysiological analysis of single channel activity was performed as previously described (17, 21). Briefly, lipid bilayer membranes were formed across an aperture of ~200 μm diameter with a mixture of phosphatidylethanolamine:phosphatidylserine:cholesterol in a ratio of 5:5:1. The lipids were dissolved in decane at a concentration of 33 mg/ml. The recording solutions contained (in mM): cis (intracellular), 200 CsCl, 1 MgCl₂, 2 ATP, and 10 HEPES-Tris (pH 7.4); and trans (extracellular), 50 CsCl, 10 HEPES-Tris (pH 7.4). Vesicles (1–4 μl) containing wt-, ΔNEG1-, or ΔNEG2-CFTR were added to the cis solution. Unless otherwise noted, the PKA catalytic subunit was present at a concentration of 50 units/ml in the cis solution. Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments). The currents were sampled at 1–2.5 ms/point. Single channel data analyses were performed with pClamp and TIPS software.

RESULTS

Activity of CFTR Channels Lacking the NEG1 or NEG2 Sequence—Examination of the primary amino acid sequence of CFTR revealed two regions with high proportion of negatively charged residues in the R domain, amino acids 725–733 (NEG1) and amino acids 817–838 (NEG2) (Fig. 1A). The NEG2 sequence is highly conserved across species (Table I). To investigate the roles of NEG1 and NEG2 in CFTR function, these peptides as a function of TFE. Percent helicity was estimated from the band intensities using the maximum helical values of Chen et al. (32).

![Structure of wt NEG2 peptide and CD spectroscopy of NEG2 peptide analogues](http://www.jbc.org/)

**Fig. 3.** Structure of wt NEG2 peptide and CD spectroscopy of NEG2 peptide analogues. A, space filling model of the predicted α-helical structure of the NEG2 peptide. The negatively charged Glu and Asp residues are colored purple, the hydrophobic residues Leu, Ile, and Phe are orange, and Lys, light blue. The remaining residues are gray. B–E, circular dichroism spectra of NEG2i (pink), h-NEG2i (blue), s-NEG2 (light blue), p-NEG2 (red), and NEG2 (green) at 0, 33, 50, and 66% TFE in water at pH 6.7. F and G, mean residue molar ellipticity plots at 193 and 222 nm for the peptides as a function of TFE. Percent helicity was estimated from the band intensities using the maximum helical values of Chen et al. (32).
regions were deleted from CFTR. The resulting ΔNEG1- and ΔNEG2-CFTR proteins were transiently expressed in HEK 293 cells. Membrane vesicles containing CFTR proteins were isolated and subjected to SDS-PAGE. Like wt-CFTR, both ΔNEG1- and ΔNEG2-CFTR are present both in the core glycosylated (band B) and the fully glycosylated form (band C) (Fig. 1B). The PKA dependence of the ΔNEG1-CFTR channel is similar to wt-CFTR (Fig. 1C). No channel activity is observed in the absence of PKA, and the open probability ($P_o$) of the ΔNEG1-CFTR channel in the presence of PKA and ATP is similar to wt-CFTR. In contrast, the ΔNEG2-CFTR channel opens without PKA (Fig. 1C, right). The “constitutive” activity of the ΔNEG2-CFTR channel is unlikely to be due to the endogenous phosphorylation of the ΔNEG2-CFTR protein, since protein phosphatase 2A, which decreases activity of the wt-CFTR opened by PKA and ATP (18), has no effect on the ΔNEG2-CFTR channel ($n = 4$). Moreover, addition of PKA up to 200 units/ml, four times the concentration required to fully activate wt-CFTR (18), does not increase the open probability of the ΔNEG2-CFTR channel (Fig. 2). Although the conductance properties of ΔNEG2-CFTR are similar to those of wild type CFTR (25), its open probability is much less and cannot be increased with PKA (wt-CFTR $P_o = 0.254 \pm 0.024$, $n = 11$; ΔNEG2-CFTR, $P_o = 0.061 \pm 0.015$ without PKA and 0.053 $\pm 0.016$, with PKA, $n = 8$).

The failure of the ΔNEG2-CFTR channel to respond to PKA does not result from inability of the channel to be phosphorylated, an in vitro assay using [$\gamma$-32P]ATP shows comparable phosphorylation of wt-CFTR and ΔNEG2-CFTR (Fig. 1B). Densitometry readings for this gel were: wt-CFTR, Band C, 1063 units, Band B, 818 units; ΔNEG2-CFTR, Band C, 1000 units, Band B, 1145 units. However, since expression of ΔNEG2-CFTR in vesicles was slightly less than wt-CFTR, on Western blot, values for phosphorylated protein normalized to the amount of CFTR present were slightly higher for the ΔNEG2-CFTR. Densitometry units of radioactive phosphate per densitometry unit of ECL-labeled CFTR on Western blot for identical amounts of vesicles were as follows: for wt-CFTR, Band C, 1.3, Band B, 1.16; for ΔNEG2-CFTR, Band C, 1.58, Band B, 2.38. In a second experiment, the values were: wt-CFTR, Band C, 1.15, Band B, 0.99; for ΔNEG2-CFTR, Band C, 1.62, Band B, 2.08. Thus, ΔNEG2-CFTR was at least as well phosphorylated as the wt-CFTR under these conditions. The apparent molecular weight of the phosphorylated ΔNEG-2 CFTR protein is less than that of the wild type protein or the ΔNEG1-CFTR (apparent $M_r = 169,000$ for wild type-CFTR Band C versus 160,000 for the ΔNEG1-CFTR Band C versus $M_r = 155,000$ for the ΔNEG2-CFTR Band C). The apparent reduction in molecular weight with deleting these negatively charged sequences is greater than can be accounted for by the mass of the amino acids alone. This difference probably results from the deletion of substantial negative charges along with the sequence, since disproportion of acidic amino acids, especially when concentrated in a short sequence, is well known as a cause of anomalously high molecular weight determinations in SDS-PAGE (26–28).

The ΔNEG2-CFTR channel still contains all 10 consensus

### Table II

| Peptide | Sequence predicted helicity (consensus)* | Helicity by CD† | Channel stimulation‡ | Channel inhibition§ |
|---------|----------------------------------------|-----------------|----------------------|---------------------|
| NEG2    | GLEISEINE EDLKECFDDD ME                 | +               | ++                   | +                   |
| sNEG2   | LIKESEEDG ECLMIDEDEN EF                 | −               | −                    | +                   |
| pNEG2   | GLEISEINE EDLKECFDDD ME                 | −               | −                    | +                   |
| hNEG2   | GLEISEOING QNLKQSFEND ME                | +               | +++                  | −                   |

* H = α-helix; C = random coil; E = extended chain; ? = ambiguous states.
† Channel stimulation: + = no significant increase in $P_o$ at any concentration; ++ = significantly increased $P_o$ at concentration <1 μM; +++ = $P_o$ more than doubled at concentration < 1 μM.
‡ Channel inhibition: − = no significant decrease in $P_o$ at any concentration; + = $P_o$ significantly decreased at concentration > 11.2 μM; ++ = $P_o$ significantly decreased at concentration <2.2 μM.

![Fig. 4. Stimulation and inhibition of wt-CFTR channel by the exogenous NEG2 peptide.](image-url)

Diary plot (open probability versus time) of a wt-CFTR channel reconstituted into lipid bilayer membrane, illustrating the effect of the NEG2 peptide on channel activity. The cis-intracellular solution contained 2 mM ATP and 50 units PKA/ml. Arrows indicate the time when the peptides were added to the recording solution. The concentration of NEG2 is indicated above the plot. Representative single channel currents are shown at −80 mV, illustrating the stimulatory and inhibitory effects of the NEG2 peptide on wt-CFTR. This diary plot is from one of 12 experiments with the wt-CFTR channel. Stimulation was observed in 10 of 12, and inhibition, in 5 of 10 in which the channel survived to be tested at concentrations ≥4.4 μM. For stimulation, eight paired experiments were performed at 4.4 μM NEG2, for which baseline $P_o$ was 0.234 ± 0.023 and stimulated $P_o$ was 0.340 ± 0.032 ($p = 0.0097$). Statistically significant stimulation of channel activity was also observed in paired experiments for concentrations of 8.8 μM ($n = 6$, $p = 0.017$). Due to the sporadic nature of channel inhibition by the NEG2 peptide, statistically significant inhibitory effects were only observed at concentrations of 13.2 μM ($n = 9$, $P_o$ at baseline 18.8 ± 1.6%, and with 13.2 μM NEG 2, 4.5 ± 0.9%, $p < 0.0001$) and 26.4 μM ($n = 5$, $p < 0.0001$).
PKA phosphorylation sites. Riordan and co-workers (29, 30) showed that even wt-CFTR with all 10 consensus PKA sites mutated increases its $P_o$ in response to PKA, presumably by phosphorylation of a weak consensus sequence at Ser$^{735}$. Elimination of PKA responsiveness is therefore an unusual property of the ΔNEG2-CFTR channel. Since the ΔNEG2-CFTR channel exhibited reduced $P_o$, it is possible that removal of the NEG2 sequence also alters the stimulatory function of the R domain.

To test this possibility, the following sequence was used to assess the helical propensities of these peptides – increasing concentrations of trifluoroethanol (TFE, 0 - 66%) were used to assess the helical propensities of these peptides (Fig. 3, panels B - E). There was little or no helical tendency of the p-NEG2 or s-NEG2 peptides in the presence or absence of TFE as demonstrated by the lack of characteristic positive and negative α-helical bands at 193 and 222 nm, respectively (Fig. 3, panels F and G). Instead the large negative bands at 200 nm were used to assess the helical propensities of these peptides (31). The NEG2 and NEG2i peptides demonstrate relative little helicity in aqueous solution, however, with increasing TFE concentration, the helical content steadily increases as shown by the increase in the characteristic helical bands at 193 and 222 nm.

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of percent helicity (32). These plots clearly indicate that h-NEG2i is significantly more helical than NEG2 or NEG2i and that s-NEG2 and p-NEG2 almost completely lack helical structures. The different helical propensities between NEG2 and s-NEG2 were also confirmed by two-dimensional NOESY spectra. The different helical propensities between NEG2 and h-NEG2i is significantly more helical than NEG2 or NEG2i and the intermediate closed component, and (-a) is set at the nadir between the fast and intermediate closed times (arrow) and generate the closed-burst duration histograms (C). The solid lines represent the fit according to a double exponential equation: \( y = a_1 \exp(-a_2 t) + a_3 \exp(-a_4 t) \), where \( a_2 \) and \( a_4 \) are the fast and intermediate closed components, and \( a_3 \) is the long closed component. The best fit parameters are \( P_o = 0.511 \), \( t_o = 449 \) ms, \( P_c = 0.189 \), \( t_c = 2494 \) ms (control); \( P_o = 0.957 \), \( t_o = 105 \) ms, \( P_c = 0.043 \), \( t_c = 1652 \) ms (peptide-stimulated). Changes in \( P_o \) and \( t_o \) of the wt-CFTR channel in paired experiments, before and after addition of 4.4 \( \mu M \) wt-NEG2 peptide is shown in D.

Effect of Exogenous NEG2 and Its Congeners on CFTR Channel Activity—To test whether the NEG2 region is responsible for both stimulatory and inhibitory interactions between the R domain and other domains of CFTR, the wild type NEG2 peptide was added to the cis-intracellular side of a single CFTR channel captured in the planar lipid bilayer (Fig. 4). The diary plot of \( P_o \) as a function of time shows the activity of a single wt-CFTR channel during the course of the experiment (Fig. 4). After peptide addition (at arrows), there are periods of intense stimulation that last 4–8 min, often followed by return to the basal level of activity observed before peptide addition. In the example shown, activity reverted to baseline levels 4–8 min after addition of NEG2 peptide at concentrations 2.2 and 4.4 \( \mu M \), but at 8.8 \( \mu M \), stimulation persisted until a still higher concentration was added, at which time the channel closed. At higher concentrations, NEG2 produces an almost complete inhibition of the channel, where only a flickery 3 pS conductance is observed. During stimulation, the open probability more than doubled and more transitions were observed between the open and closed states (Fig. 4). In 10 of 12 experiments in which a peptide concentration \( \geq 0.44 \) \( \mu M \) was achieved, a stimulatory response was observed. For the two experiments for which it was not, one channel was inhibited upon initial peptide addition at a concentration of 4.4 \( \mu M \) and no stimulation was observed, and another channel did not display stimulation. Statistically significant increases in channel open probability are documented for NEG2 concentrations of 4.4 and 8.8 \( \mu M \), and increased activity, with \( p = 0.09 \), was observed at 2.2 \( \mu M \) NEG2. Profound inhibition, comparable with that shown in the example, was observed in five channels exposed to NEG2 peptide at concentrations \( \geq 4.4 \) \( \mu M \). However, in five other channels treated with NEG2 peptide at a concentration \( \geq 4.4 \) \( \mu M \), inhibition did not occur, even at concentrations up to 26.4 \( \mu M \). For some of these experiments, the channel may not have survived long enough in the bilayer following addition of peptide to be confident of lack of inhibition. Nevertheless, at the highest concentrations of peptide tested, 13.2 and 26.4 \( \mu M \), statistically significant inhibition was achieved when all channel recordings are considered.

Because inhibition did not occur with every experiment when
The R domain of CFTR contains two negatively charged regions, amino acids 725–733 (NEG1) and amino acids 817–838 (NEG2) which reside in close proximity to two PKA phosphorylation sites, Ser775 and Ser781, that are used in vivo (9). One amino acid substitution, noted in a patient with CF, is reported in the CF Mutation Consortium data base in the NEG1 region (E725K). Three mutations are reported in the NEG2 region (E822K, E826K, and D836Y), two of which were obtained from patients with cystic fibrosis (E822K and D836Y). Single channel studies of E822K and E826K indicate that both mutations result in reduced Po compared with wt-CFTR (34). Moreover, Cotten and Welsh (35) showed that N-ethylmaleimide modification of a cysteine residue in the NEG2 region (C832) produced irreversible stimulation of PKA-phosphorylated CFTR channel activity. The NEG2 region is highly conserved among species. Taken together, these data indicate the
importance of this portion of the R domain for the regulation of the CFTR channel. Our data demonstrate that the NE2G region of CFTR can both stimulate and inhibit chloride channel function. When this region is deleted from CFTR, the resultant channel opens without PKA, indicating loss of inhibitory function, but the P, never achieves that of wt-CFTR, and does not increase when phosphorylated with PKA, indicating a loss of stimulatory function. In support of a dual action for the NE2G sequence, addition of the NE2G sequence as a synthetic peptide to the intracellular side of the CFTR channel results in stimulation of channel openings at lower concentrations, but inhibition of channel activity at higher concentrations.

Stimulatory and inhibitory activities can be separated by sequence modifications. Inhibition alone is evident with peptides designed to retain the negative charge but disrupt the helical tendencies. Conversely, stimulation occurs with a peptide designed to retain and enhance the helical structure of the sequence, but to reduce the negative charge. Molecular modeling reveals an amphipathic feature of the NE2G sequence, i.e. the negatively charged residues mostly line up as a barber-pole stripe on the ɑ-helix. The surrounding residues are mostly hydrophobic. We speculate that the NE2G sequence, presented in different ways, could interact with CFTR at different sites to either stimulate or inhibit channel openings. Which function the NE2G sequence performs might be determined by how it is presented in the context of the intact molecule, which could be entrained, at least in part, by the phosphorylation state of the R domain. In this model, phosphorylated R domain favors presentation of the hydrophobic stripe of the helical conformation of NE2G to the stimulatory site, whereas the unphosphorylated R domain favors access of the negative charge in NE2G to an inhibitory site. Since the mechanism of increasing channel activity appears to be, both for NE2G and for h-NEG2i, mainly by increasing the number of channel openings, we speculate that the stimulatory activity might result from peptide binding increasing either binding or hydrolysis of ATP, probably at the first nucleotide-binding domain, which is often assigned the role of channel opening (36).

When the exogenous NE2G peptide is added to the intracellular side of the wt-CFTR channel, it could interact with either the stimulatory site or the inhibitory site, and it may compete with the endogenous sequence for access to these sites. The degree and duration of stimulation or inhibition by exogenous peptide will depend on the on and off rates at the stimulatory site and the inhibitory site, the effective concentration of the relevant structural form of the peptide, and competition from endogenous sequences (either the NE2G sequence itself, or sequences at other sites) at those two sites. When the channel is closed, as it is most of the time even in the phosphorylated state (since the P_i is only about 30%), the endogenous site for inhibition may often be occupied by the endogenous NE2G sequence, and thus binding of the exogenous peptide is favored at the stimulatory site. However, as stimulation increases, the inhibitory site may become more available, and eventually binding occurs at this site, and the channel is inhibited.

Naren and co-workers (37) reported that a sequence in the NH2-terminus of CFTR interacts with sequences in the proximal end of the R domain to increase CFTR channel activity. This work, taken together with the work reported here, is consistent with our earlier studies of segments of the R domain which inhibit channel function (21). Two segments, one containing amino acids 588–805 and the other, amino acids 672–855, proved inhibitory. The first segment may inhibit channel activity by interacting with the stimulatory NH2-terminal sequence in CFTR, preventing it from interacting with its target in the R domain, thereby preventing channel stimulation. The second inhibitory segment contains the NE2G sequence, and probably inhibits channel openings by presenting NE2G to its inhibitory site. It is intriguing that the binding portion of the NH2-terminus, which stimulates channel activity, appears to be a helical segment of the protein with one negatively charged face, and its activity is abrogated by reducing the negative charges in this segment (38). This observation reinforces the concept that negatively charged sequences may exert profound regulatory influence on channel activity.

A recent study by Baldursson et al. (39) found that deletion of amino acids 760–783 in the R domain resulted in CFTR channel activity in the absence of PKA phosphorylation; however, deletion of amino acids 784 to 835, which includes most of the NE2G sequence, did not allow the channel to open without phosphorylation. It may be that the two deletion mutants delete the NE2G sequence in whole or in part, ours (ΔNEG2(817–838)-CFTR) and that of Baldursson et al. (39) (Δ(784–835)-CFTR), assume different conformations compared with the native CFTR molecule and thus may behave differently in channel function. Baldursson’s (39) construct deletes 33 more amino acids (amino acids 783–816, including a putative PKC-phosphorylation site (Ser790)) than our ΔNE2G-CFTR construct. Since PKC phosphorylation has an essential role in PKA phosphorylation-dependent regulation of the CFTR channel (40), removal of this site may alter channel regulation. The potential role of PKC phosphorylation on the Δ(784–835)-CFTR and Δ(817–838)-CFTR remains to be studied. It is also possible that the R domain contains more than one inhibitory site, or both identified segments of R domain sequences (760–783 and 817–838) could act together to inhibit the unphosphorylated CFTR channel, and the lack of either one disrupts the inhibitory function of the R domain. King and Sorscher (41) also identify the COOH-terminal portion of the R domain (amino acids 723–836, which includes both the putative inhibitory sequences) as crucial in conferring PKA regulation on the channel.

Future studies to identify the sites of NE2G interaction within the CFTR molecule should provide new insights into the regulation of the CFTR channel. Particularly, understanding the stimulatory interaction of NE2G may facilitate the design of therapeutics to stimulate the CFTR opening to treat patients whose mutant forms of CFTR reach the cell surface.

REFERENCES
1. Quinton, P. M. (1986) Am. J. Physiol. 251, C649–C652
2. Welch, M. J., and Smith, A. E. (1993) Cell 73, 1251–1254
3. Zielenski, J., and Tsui, L. C. (1995) Annu. Rev. Genet. 29, 777–807
4. Riordan, J., Rommens, J., Kerem, B.-S., Nau, A., Rozmahel, R., Grzelczak, Z., Zielenkni, J., Lek, S., Flavioie, N., Chou, J.-J., Drumm, M., Iannuzzi, M., Collins, F., and Tsui, L.-C. (1989) Science 245, 1066–1073
5. Higges, C. F. (1992) Annu. Rev. Cell Biol. 6, 67–113
6. Bear, C. E., Li, C., Kastner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M., and Riordan, J. R. (1992) Cell 68, 809–818
7. Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welch, M. J. (1991) Cell 67, 775–784
8. Ma, J., and Davis, P. B. (1999) Clin. Chest Med. 19, 459–471
9. Rich, D. P., Berger, H. A., Cheng, S. H., Travis, S. M., Saxena, M. S., Smith, A. E., and Welch, M. J. (1993) J. Biol. Chem. 268, 20259–20267
10. Picciotto, M. R., Cohn, J. A., Bertuzzi, G., Greengard, P., and Nairn, A. C. (1992) J. Biol. Chem. 267, 12472–12475
11. Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welch, M. J., and Smith, A. E. (1991) Cell 68, 1027–1038
12. Talsukar, A. J., Chang, X.-B., Riordan, J. R., and Hanrahan, J. W. (1991) Nature 352, 628–631
13. Gunderson, K. L., and Kopito, R. R. (1995) Cell 83, 231–239
14. Dhallanty, A. M., and Riordan, J. R. (1994) Biochemistry 33, 4072–4079
15. Ma, J. (2000) Neuropsychoph. Sci. 15, 154–158
16. Wilkinson, D. J., Strong, T. J., Mansoura, M. K., Woud, D. L., Smith, S. S., Collins, F., and Dawson, D. C. (1997) Am. J. Physiol. 273, L127–L133
17. Rich, D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E., and Welch, M. J. (1991) Science 253, 205–207
18. Ma, J., Zhao, J., Drumm, M. L., Xie, J., and Davis, P. B. (1997) J. Biol. Chem. 272, 28133–28144
19. Winter, M. C., and Welch, M. J. (1997) Nature 389, 294–296
20. Ma, J., Tasch, J. E., Tao, T., Zhao, J., Xie, J., Drumm, M. L., and Davis, P. B. (1996) J. Biol. Chem. 271, 7351–7356
21. Tasch, J. E., Zerhusen, B., Zhao, J., Ma, J., and Davis, P. B. (1999) FEBS Lett. 445, 63–68
22. Xie, J., Drumm, M. L., Ma, J., and Davis, P. B. (1995) J. Biol. Chem. 270, 28084–28091
23. Geourjon, C., and Deleage, G. (1995) Comput. Appl. Biosci. 11, 681–684
24. Rost, B., and Sander, C. (1994) Proteins 19, 55–72
25. Tao, T., Xie, J., Drumm, M. L., Zhao, J., Davis, P. B., and Ma, J. (1996) Biophys. J. 70, 743–753
26. Iakoucheva, L. M., Kimsey, A. L., Masselon, C. D., Smith, R. D., Dunker, A. K., and Ackerman, E. J. (2001) Protein Sci. 10, 1353–1362
27. Armstrong, D. J., and Roman, A. (1993) Biochem. Biophys. Res. Commun. 192, 1380–1387
28. Graceffa, P., Jancso, A., and Mabuchi, K. (1992) Arch. Biochem. Biophys. 297, 46–51
29. Chang, X. B., Tabcharani, J. A., Hou, Y. X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W., and Riordan, J. R. (1993) J. Biol. Chem. 268, 11304–11311
30. Seibert, F. S., Tabcharani, J. A., Chang, X. B., Duhlanty, A. M., Mathews, C., Hanrahan, J. W., and Riordan, J. R. (1995) J. Biol. Chem. 270, 2158–2162
31. Perczel, A., Park, K., and Fasman, G. D. (1992) Proteins Struct. Funct. Genet. 15, 7–89
32. Chen, Y.-H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3350–3359
33. Rost, B., and Sander, C. (1994) Proteins 19, 55–72
34. Vanekerberghen, A., Wei, L., Jaspers, M., Cassiman, J.-J., Nilius, B., and Cuppens, H. (1998) Hum. Mol. Genet. 7, 1761–1769
35. Cotten, F. J., and Welsh, M. J. (1997) J. Biol. Chem. 272, 25617–25622
36. Carson, M. R., Travis, S. M., and Welsh, M. J. (1995) J. Biol. Chem. 270, 1711–1717
37. Naren, A. P., Cormet-Boyaka, E., Fu, J., Villain, M., Blalock, J. E., Quick, M. W., and Kirk, K. L. (1999) Science 286, 544–548
38. Fu, J., Ji, H.-L., Naren, A. P., and Kirk, K. L. (2001) J. Physiol. 536, 459–470
39. Baldursson, O., Ostedgaard, L. S., Rokhлина, T., Cotton, J. F., and Welsh, M. J. (2001) J. Biol. Chem. 276, 1904–1910
40. Jia, Y., Mathew, C. J., and Hanrahan, J. W. (1997) J. Biol. Chem. 272, 4978–4984
41. King, S. A., and Sorscher, E. J. (2000) Biochemistry 39, 9869–9875
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J. Biol. Chem. 2002, 277:23019-23027.
doi: 10.1074/jbc.M201661200 originally published online April 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201661200

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