Phosphorylation by cAMP-Dependent Protein Kinase Causes a Conformational Change in the R Domain of the Cystic Fibrosis Transmembrane Conductance Regulator†

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ABSTRACT: Individuals with cystic fibrosis have a defect in the CFTR protein, a chloride channel regulated by cAMP-dependent protein kinase (PKA). The majority of the phosphorylation sites of PKA are located in the R domain of CFTR. It has been postulated that this domain may act as a gate for the chloride channel. Of the many possible mechanisms whereby the R domain could gate the channel, including interdomain interactions, charge distribution, or conformational change, we investigated the possibility that phosphorylation leads to conformational changes in the R domain. To test this hypothesis, a protocol for purification of human R domain peptide synthesized in a bacterial expression system was developed. Purified R domain was phosphorylated by PKA, and CD spectra were obtained. As a result of phosphorylation by PKA, a significant spectral change, indicative of a reduction in the α-helical content, was found. CD spectra of the R domain of a shark homologue of CFTR indicated similar changes in conformation as a result of phosphorylation by PKA. In contrast, phosphorylation of the human R domain by PKC, which has only a small influence on CFTR channel activity, failed to elicit CD spectral changes, indicating no conformational change comparable to those induced by PKA phosphorylation. These observations provide the first structural characterization of the R domain and suggest that the gating of the CFTR chloride channel by PKA may involve a conformational change in the R domain.

Cystic fibrosis (CF),[1] the most common genetic disease in Caucasians, is characterized by severe respiratory and gastrointestinal dysfunction as a consequence of defective electrolyte and water transport (Tsui & Buchwald, 1991; Talamo et al., 1987). Knowledge of the CF gene product (Riordan et al., 1989) has allowed considerable advances in the understanding of the CF defect at the molecular level. In a number of cell types, expression of CFTR, the CF gene product, results in a cAMP-stimulated chloride conductance (Rich et al., 1990; Anderson et al., 1991; Kartner et al., 1991). Proof that CFTR is itself a PKA regulatable chloride channel came from the demonstration that the purified CFTR protein, in a planar lipid bilayer, mediated chloride conductance across the bilayer after addition of PKA and ATP (Bear et al., 1992). As expected, the key activity of the PKA on CFTR appears to be phosphorylation (Tabcharani et al., 1991).

Based on the predicted amino acid sequence for CFTR, a model was proposed of a transmembrane protein with three cytoplasmic domains, two involved in nucleotide binding and one in regulation by phosphorylation (Riordan et al., 1989). The latter, R domain, contains nine consensus sites for phosphorylation by PKA. Both the intact CFTR protein (Cheng et al., 1991; Gregory et al., 1990) and a recombinant polypeptide representing most of the R domain (Picciotto et al., 1992) have been shown to be phosphorylated by PKA at many of the predicted sites. Point mutation of these sites indicates that removal of an increasing number of PKA phosphorylation sites results in a decreased activation of chloride conductance (Gregory et al., 1990; Chang et al., 1993). Cells expressing a deletion mutant of CFTR, lacking the C-terminal half of the R domain and all but one PKA phosphorylation site, show a constitutive, cAMP-independent, chloride conductance (Rich et al., 1991). cAMP-stimulation causes a further increase in chloride conductance in these cells. Similar constitutive activity is observed when, as an alternative means of introducing negative charge, most of the serine residues which can be phosphorylated by PKA are converted to glutamic acid (X.-B. Chang and J. R. Riordan, unpublished observations). The R domain also contains consensus sites for phosphorylation by PKC (Riordan et al., 1989) and has been shown to be phosphorylated by PKC (Picciotto et al., 1992); however, the role of PKC phosphorylation in activation of CFTR is less clear than that of PKA (Tabcharani et al., 1991).

A simple model which could account for activation of the CFTR chloride conductance by phosphorylation of CFTR is that the R domain, in an unphosphorylated state, occludes the ion pore. Upon phosphorylation, the R domain might no longer prevent chloride from entering the channel. This could be accomplished by a number of potential mechanisms, including changes in charge distribution at the channel mouth, altered interdomain interactions in CFTR, or change in conformation of the R domain. To investigate the possibility of a conformational change in the R domain as a result of phosphorylation, the R domain sequence from human CFTR was expressed in a bacterial system and purified. CD spectroscopy was then used to compare the secondary structure characteristics of

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[1] Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; IPTG, isopropylthio-β-galactoside.

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the phosphorylated and unphosphorylated R domain polypeptide. To investigate the specificity of the conformational change, the conformation of human R domain phosphorylated with PKA or PKC was compared. To address the conservation of such a conformational change, purified R domain polypeptide from the shark homologue of CFTR was also phosphorylated with PKA.

MATERIALS AND METHODS

Protein Expression. DNA fragments encoding the human and shark (*Squalus axanthias*) R domains were obtained by PCR and cloned into plasmid expression vectors. PCR primers were engineered to contain the appropriate restriction enzyme recognition sites, to allow the ATG codon of aa 595 in the human CFTR (Riordan et al., 1989), or aa 596 of the shark analogue of CFTR (Marshall et al., 1991) to be utilized as the translational start site of the R domain, and to contain a mutation changing the first complete codon of exon 14 to a stop codon. The human CFTR cDNA fragment of exons 7–24 was employed as template (Riordan et al., 1989). The PCR product containing the human R domain sequences was digested with NheI (Gibco/BRL, Gibco Canada Ltd.) and ligated into the pET3A vector (Rosenburg et al., 1987). Transformation of DH5α (BRL) cells with the ligation mixture allowed isolation of the construct pNheShu2.6. Restriction enzyme analysis and double-stranded sequencing (Sequencase kit, United States Biochemical, Cleveland, OH) confirmed that pNheShu2.6 contains the R domain sequences in the correct orientation and free of PCR and cloning artifacts. pNheShu2.6 was then used to transform the host BL21(DE3) (Studier & Moffat, 1986). To generate the shark R domain DNA fragment, the template used was a cDNA clone including nucleotides 1346–5901 of the shark CFTR analogue (Grzelczak et al., 1990). The PCR product containing the shark R domain sequences was digested with *Hin*dIII (BRL) and ligated into the plasmid pFLAG (IBI Biosystems, Rochester, NY). The ligation mixture was used to transform DH5α cells. Restriction enzyme analysis and double-stranded sequencing confirmed that the shark R sequences were correctly represented in the construct pFsk6.

Expression of the R domain peptides was induced by the addition of 1 mM ITPG (BRL) to exponentially growing bacterial cultures. Translation of the correct peptide was confirmed by Western blotting. The amino acid content of each purified protein was determined (Tous et al., 1989).

Western Blotting. Samples were subjected to electrophoresis on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Nonspecific binding of antibody to the membrane was inhibited by incubation for 15 min in 3% BSA, 150 mM NaCl, and 10 mM Tris, pH 7.4. Membranes were then incubated with 1 μg/mL purified primary antibody in blocking solution for 1 h and then washed briefly in three changes of 10 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.4. The membranes were then incubated with the appropriate secondary antibody at 0.2 μg/mL in blocking buffer for 1 h and washed as above. Detection of the peptides in Western blotting experiments was accomplished with an alkaline phosphatase conjugated second antibody, either anti-mouse IgG or anti-rabbit IgG as appropriate, and development of color with the substrates nitro blue tetrazolium and 5-bromo-1-chloro-3-indolyl phosphate in 5 mM $MgCl_2$, 100 mM NaCl, and 100 mM Tris, pH 9.5 (Western blotting kit from Promega, Madison, WI). For the human R domain peptide, the primary antibody used for detection was a purified polyclonal, PAb6, raised against a synthetic peptide corresponding to aa 724–746 of CFTR. The crude serum was determined to detect the R domain on the basis of binding to immobilized antigen (synthetic peptide) and to two different R domain peptides expressed in bacteria; preimmune sera was used as a negative control. Interestingly, PAb6 does not detect full length CFTR well. Substantial decrease in the non-R-domain proteins detected with PAb6 was accomplished by preabsorption of the crude sera with bacterial extract from pET3A containing bacteria (Sambrook et al., 1989), ammonium sulfate fractionation, and chromatography on an FPLC protein A column (Pharmacia, Uppsala, Sweden). For the shark, the M1 monoclonal antibody supplied with the FLAG bacterial expression kit (IBI) was used, which is reactive against a four-peptide epitope (Asp-Tyr-Lys-Asp), engineered to be translated immediately before the R domain sequences.

Protein Purification. To purify the R domain polypeptides, cultures (1 L of bacterial media) were grown for 2 h after induction. The cells were harvested by centrifugation, subjected to one round of freeze/thawing, and then lysed with 0.3 mg/mL lysozyme (Boehringer Mannheim, Mannheim, Germany) in 10 mM Tris and 1 mM PMSF, pH 8.0. To reduce the viscosity of the lysate, $MgSO_4$ was added to 10 mM, and DNaseI (Boehringer Mannheim) to 1 μg/mL. The insoluble material was harvested by centrifugation, and then washed, to solubilize some of the bacterial membrane proteins, in a solution containing 40 mM KH_2PO_4, 20% glycerol, 12 mM $MgSO_4$, 0.5 mM PMSF, 6 mM dithioerthritol, and 1.2% octyl glucoside (Varadhachary & Maloney, 1990). After centrifugation of the octyl glucoside suspension, the R domain polypeptides were found in the pellet. This pellet was solubilized in 6 M guanidine HCl, 100 mM Tris, 50 mM dithioerthritol, and 1 mM PMSF, pH 7.5, at 4 °C overnight.

The protocol developed to purify the human peptide involved exchange of the solubilizing solution with 6 M urea and 50 mM bicine, pH 8.2. The guanidine hydrochloride solution provided more quantitative solubilization, while the urea solution was compatible with ion exchange chromatography. The protein was then loaded onto a Mono S (Pharmacia) cation exchange column. A linear NaCl gradient (0–0.3 M) was used to elute the protein. R domain containing fractions were pooled, diluted into 6 M urea and 50 mM Tris, pH 8.2, loaded onto a Mono Q (Pharmacia) anion exchange column, and eluted from the column with a linear NaCl gradient (0–0.3 M). R domain containing fractions were concentrated and then subjected to gel filtration on a Superose 12 (Pharmacia) column in 0.15 M NaCl, 6 M urea, and 50 mM $NaHPO_4$, pH 7.5. All column chromatography was performed on a Pharmacia FPLC system.

The protocol developed to purify the shark R domain is similar to that of the protocol for purification of the human polypeptide, except that the ion exchange steps were carried out at pH 7.2.

Purified R domain samples were renatured by step-wise dialysis out of 6 M urea, at 4 °C. Protein concentration was kept below 50 μg/mL during renaturation to prevent aggregation. The initial dialysis buffer contained 4 M urea, 50 mM Tris, 0.15 M NaCl, and 2 mM dithioerthritol at pH 7.5. At approximately 12-h intervals, the dialysis buffer was replaced with buffer containing half of the previous concentration of urea, while the concentration of Tris, NaCl, and dithioerthritol remained constant. This process was continued until the urea concentration was 60 mM.

Phosphorylation of the R Domain Peptides. Prior to phosphorylation, the samples in 60 mM urea were subjected to ultrafiltration in Centricon 10 units (Amicon) and then

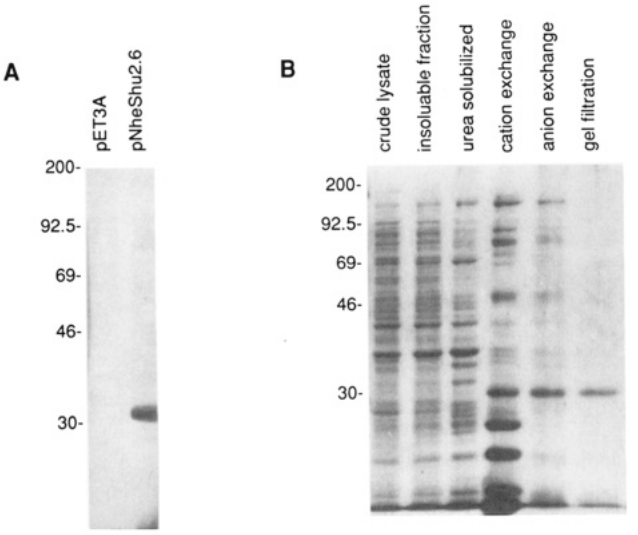

FIGURE 1: Expression and purification of human R domain. (A) Crude bacterial extracts from cells transformed with either the vector, pET3A, or pET3A containing the human R domain sequences, pNheShu2.6, were boiled in SDS–PAGE sample buffer and subjected to electrophoresis and Western blotting as described under Materials and Methods. (B) Samples from each stage of purification of the human R domain (see Materials and Methods for details) were subjected to SDS–PAGE on a 10% acrylamide gel and then stained with 0.2% Commassie brilliant blue, in 10% acetic acid/40% methanol. Molecular mass standards are indicated in kDa.

diluted into phosphorylation buffer, giving a final urea concentration of 6 mM. The R domain peptides were phosphorylated at a concentration of 100 μg/mL in 50 mM $KHPO_4$, pH 7.0, 10 mM $MgSO_4$, and 50 μM ATP with 5 μg/mL of the catalytic subunit of the PKA (Promega). PKC (Calbiochem) was used at 0.8 μg/mL, in 50 mM $KHPO_4$, pH 7.4, 1.5 mM $CaCl_2$, 10 mM Mg acetate, 0.05% 1,2-dioctanoyl-*sn*-glycerol (Sigma), and 50 μM ATP, on 200 μg/mL R domain at 30 °C for 75 min. These conditions were determined to provide maximal phosphorylation of the R domain polypeptides. In experiments where the polypeptides were radiolabeled, [γ-^{32}P]ATP (3000 Ci/mmol, 2 mCi/mL, Amersham) was included in the reaction mixture at a molar ratio of 1/4000 to nonradioactive ATP.

Circular Dichroism Spectroscopy. CD spectra were measured in a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd.) which was calibrated at regular intervals with a standard solution of d_{10}-camphorsulfonic acid. Each spectrum was the average of 10 scans. Spectra were measured in steps of 0.2 nm, with a path length of 0.1 mm, response time of 1 s, and scan speed of 50 nm/min, in the range 190–240 nm. The protein concentration in the CD experiments was 50 μg/mL. Each spectra was obtained on at least three different occasions, each with peptide from a separate preparation. Representative spectra are presented.

RESULTS

Human and shark R domain polypeptides were expressed in bacterial expression systems. A polypeptide, representing amino acids 595–831 of human CFTR, was detected in cells transformed with the construct pNheShu2.6, but not in those transformed with vector alone (Figure 1A). The R domain peptide was found to be expressed in the bacteria prior to the addition of the inducing agent. This suggests relaxed control of either the T7 gene 10 promotor which precedes the R domain or the β-gal promotor which controls the expression of the T7 RNA polymerase. The R domain detected migrates through the SDS–polyacrylamide gel with the apparent mobility of a 30 kDa protein, which is close to the predicted molecular mass of 26.8 kDa.

Table 1: Human R Domain Purification

	total protein (mg)[a]	percent R domain[b]	R domain yield (mg)[d]
crude lysate[c]	1280	0.5	6.4
insoluble material	625	1	6.2
urea	278	2	5.6
cation exchange	22.3	12	2.7
anion exchange	6.1	33	2.0
gel filtration	1.6	>95	1.6

[a] The starting material was a 1-L culture of bacteria. Protein concentration was determined by a modified Lowry assay. [b] Determined by scanning densitometry on a Sun Sparcstation with PDI Quantity One software. [c] Fractions were obtained as described in the text and are comprised of pools obtained following the step. [d] All values are approximate.

Figure 1B shows samples obtained from each of the steps of the purification of the human R domain peptide. The purification yields are summarized in Table 1. The human R domain peptide constitutes approximately 0.5% of the total bacterial protein following induction, a relatively low level of production for bacterial expression systems. The R domain was found in the water-insoluble fraction of the lysed bacterial suspension. To purify the human R domain, the insoluble material was washed in a solution containing octyl glucoside and then dissolved in a 6 M guanidine HCl solution and passed by Sephadex G-25 M column (Pharmacia) into 6 M urea and 50 mM bicine, pH 8.2. Chromatographic purification of the human R domain then proceeded with cation exchange, at pH 8.2, followed by anion exchange, also at pH 8.2, and finally gel filtration. Following gel filtration, the R domain samples were greater than 95% pure, as assessed by amino acid analysis and Commassie brilliant blue staining. From a bacterial culture grown in 1 L of medium, approximately 1.6 mg of R domain was obtained, giving an overall recovery of 25% of the R domain polypeptide.

Following purification of the R domain in 6 M urea, the peptide was renatured by gradual dialysis into a 100-fold lower concentration of urea. It was found that the critical factors required for renaturation were maintenance of the protein concentration below 50 μg/mL and a slow, gradual decrease in the urea concentration. Both of these factors were necessary to avoid aggregation of the renaturing peptide. The renatured protein was judged to be monomeric by its mobility on a gel filtration column (data not shown). Approximately 30% of the polypeptide initially subjected to dialysis was recovered in a stable, soluble form. The remaining 70% of the polypeptide precipitated from solution at approximately 0.25 M urea and was recovered by centrifugation.

For ease of detection, the shark R domain peptide was expressed in the FLAG bacterial expression system. The PCR product containing the shark R domain sequences (amino acids 595–841) was cloned into the pFLAG vector in a site preceded by sequences coding for the M1 epitope and a signal sequence for transport into the periplasm. The M1 epitope and signal sequences contribute an additional 27 residues at the N-terminus of the shark R domain peptide. Following induction, DH5α cells transformed with pFsk6 expressed the shark R domain peptide (Figure 2A); no expression was observed prior to induction. Uninduced cells containing the pFsk6 construct double significantly more slowly than cells containing the vector alone, and completely stop doubling when expression of the shark peptide is induced, implying that expression of the polypeptide is detrimental to cell viability.

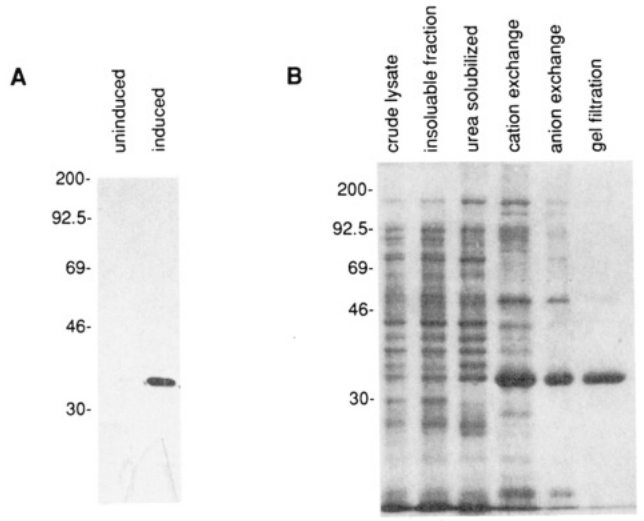

FIGURE 2: Expression and purification of shark R domain. (A) Crude bacterial extracts from DH5α cells transformed with pFsk6 (pFLAG containing the shark R domain sequences), before and after induction with ITPG, were boiled in SDS–PAGE sample buffer and subjected to electrophoresis and Western blotting as described under Materials and Methods. (B) Samples from each stage of purification of the shark R domain (see Materials and Methods for details) were subjected to SDS–PAGE on a 10% acrylamide gel and then stained with 0.2% Commassie brilliant blue, in 10% acetic acid/40% methanol. Molecular mass standards are indicated in kDa.

Table 2: Shark R Domain Purification

	total protein (mg)[a]	percent R domain[b]	R domain yield (μg)[d]
crude lysate[c]	500	<1	
insoluble material	306	<1	
urea	80	<1	
cation exchange	1.2	21	252
anion exchange	0.64	50	320
gel filtration	0.12	>95	120

[a] The starting material was a 1-L culture of bacteria. Protein concentration was determined by a modified Lowry assay. [b] Determined by scanning densitometry on a Sun Sparcstation with PDI Quantity One software. [c] Fractions were obtained as described in the text and are comprised of pools obtained following the step. [d] All values are approximate.

The shark peptide has an apparent molecular mass in SDS–PAGE of about 34 kDa, in agreement with the predicted mass of 31.2 kDa.

Table 2 and Figure 2B document the protocol for purification of the shark R domain. The shark peptide is expressed at less than 1% of the total bacterial protein. As with the human polypeptide, the shark R domain was found in the insoluble fraction of the bacterial lysate. Purification of the shark peptide proceeded essentially as with the human, except that the ion exchange chromatography was performed at pH 7.2 for the shark polypeptide. Purified shark R domain polypeptide is greater than 95% pure, as assessed by amino acid analysis and commassie brilliant blue staining. From the bacteria grown in 1 L of medium, 120 μg of shark peptide was obtained. The shark R domain was renatured, as described above, with a yield of about 50% soluble peptide from the peptide initially subjected to dialysis.

One objective of this study was to determine if phosphorylation of the R domain polypeptides caused any conformational changes which could be detected by CD spectroscopy. As it has been demonstrated that PKA activation of CFTR is associated with phosphorylation of multiple residues in the R domain (Cheng et al., 1991; Chang et al., 1993) and that

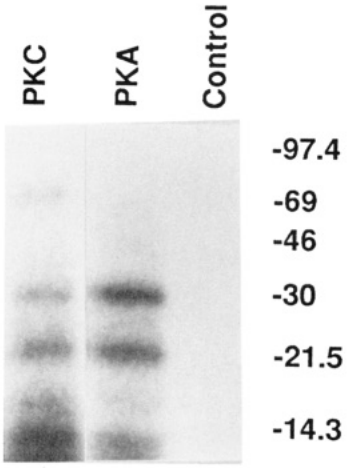

FIGURE 3: Phosphorylation of the human R domain by PKA and PKC. SDS–PAGE of phosphorylated human R domain: 50 ng of protein was phosphorylated as described under Materials and Methods and subjected to electrophoresis on 16% tricine buffered gels; the gel was dried and subjected to autoradiography. The control contains all components of the reactions except kinase. Autophosphorylated PKC can be seen with an apparent molecular mass of 80 kDa.

the *in vitro* phosphorylation of the R domain is similar to that of the *in vivo* phosphorylation of CFTR (Picciotto et al., 1992), the time of incubation of the polypeptides with the protein kinases and the concentration of ATP were adjusted to provide the maximum phosphorylation of the R domains.

Figure 3 depicts the results of phosphorylating the human R domain with PKA in the presence of trace amounts of [γ-^{32}P]ATP. The phosphorylated polypeptide migrates as a sharp band on the gel, suggesting that all of the molecules are uniformly phosphorylated. In agreement with Picciotto et al. (1992), the stoicheiometry of phosphorylation by PKA was 6 ± 3 molecules of phosphate per molecule of R domain and the phosphorylated polypeptide has an apparent molecular mass approximately 2 kDa greater than the unphosphorylated form (not shown). Also shown is the phosphorylation of the human R domain by PKC in the presence of trace amounts of [γ-^{32}P]ATP. As expected from previous studies (Picciotto et al., 1992), the labeling is less intense than with PKA, by a factor of about 2, and causes a small decrease in the mobility of the human R domain in SDS–PAGE. We observed that the phosphorylated samples of the R domain each contain small quantities of polypeptides of approximately 22 and 13 kDa, which arise during processing of the samples for gel electrophoresis. This conclusion is based on the following observations: although there are proteolytic fragments of the R domain detected in crude bacterial lysates, these fragments are separated from the R domain during purification; purified, renatured R domain migrates as a sharp peak in gel filtration experiments; incubation of either PKA, or PKC, phosphorylated, or unphosphorylated R domain at 30 or 37 °C, for various times up to 90 min prior to the addition of electrophoresis sample buffer, does not result in any increase in the fraction of R domain found in the fragments; within one experiment, the fragments are found in equal amounts in phosphorylated and unphosphorylated R domain samples; and, incubation for increasing time in electrophoresis sample buffer at 95 °C increases the fraction of R domain found in these fragments. The R domain may be cleaved by SDS in the sample buffer. It has been reported that SDS will cleave under these conditions at Asp-Pro sites (Rittenhouse & Marcus, 1984). There are no such sites in the R domain; however, there is a Glu-Pro site in the R domain at approximately the correct position to generated fragments of

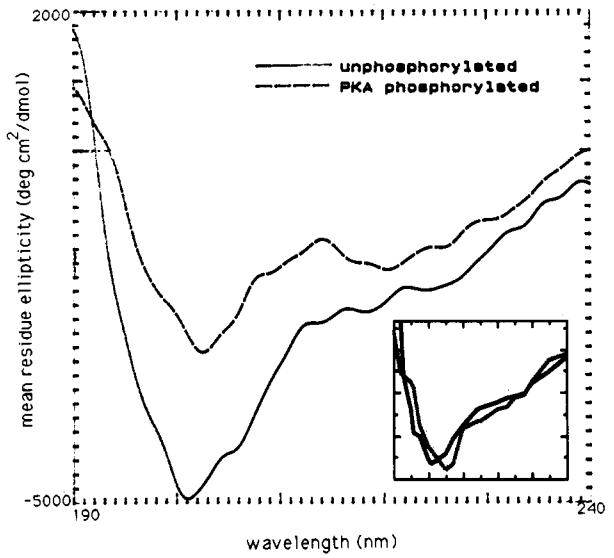

FIGURE 4: 4: CD spectra of human R domain in unphosphorylated and PKA phosphorylated forms. Spectra were obtained from samples containing 15 μg of protein, as described in the text. The spectrum of the unphosphorylated form of the peptide was determined in the phosphorylation buffer, 50 mM $KHPO_4$, pH 7.0, 10 mM $MgSO_4$ and 50 μM ATP. The spectrum of the buffer alone was subtracted from the R domain spectrum. The phosphorylated R domain sample was obtained by addition of PKA to the R domain in phosphorylation buffer and incubation at 30 °C for 15 min. To obtain a background spectrum, PKA alone was incubated in phosphorylation buffer. The spectrum for this sample was subtracted from that of the phosphorylated R domain. The inset compares the measured spectrum of the unphosphorylated R domain (solid line) with that predicted (dotted line) by the method described under Results.

the correct size. By Western blotting with an antibody which recognizes an epitope in the R domain between amino acid 590 and 698 (Kartner et al., 1992), the 13-kDa polypeptide has been identified as the N-terminal fragment of R domain. Western blotting with the antibody described under Materials and Methods, which recognizes an epitope among amino acids 724–746, revealed that the 22-kDa polypeptide contains the C-terminal fragment of the R domain. These fragments allow confirmation of utilization of different sites for phosphorylation by the two kinases, as the relative degree of phosphorylation of the two fragments in the PKA, compared to the PKC phosphorylated samples, differ.

Figure 4 contains the CD spectrum of PKA phosphorylated and unphosphorylated human R domain. Spectra of the R domain in 50 mM $KHPO_4$, pH 7.0, and the spectra of the R domain in 50 mM $KHPO_4$, pH 7.0, 10 mM $MgSO_4$, and 50 μM ATP minus the spectra of 10 mM $MgSO_4$ and 50 μM ATP were indistinguishable. ATP is a chiral molecule and has a CD spectra with a maximum magnitude of about 25% of that of the R domain between 190 and 200 nm; at higher wavelengths, the magnitude of the ATP spectra is approximately 10% of that of the R domain. The magnitudes of the R domain spectra shown in Figure 4 are relatively low, suggesting that the peptide was either present at a lower concentration than expected or that it contains a relatively low α-helical content and high β-sheet and random coil content. This latter suggestion is supported by the shape of the spectra and the deconvolution of the spectra, described below.

In general, each type of protein secondary structure has a characteristic and distinct CD spectrum (Johnson, 1988, 1990). The spectrum of most proteins is composed of a linear combination of the spectrum for each secondary structure type, in proportion to its content in the protein. The distinctive characteristics of the spectrum of the unphosphorylated human R domain include a local maximum around 215 nm, a maximum between 190 and 195 nm, which appears to be the superposition of two maxima at approximately 192 and 195 nm, and an asymmetric minimum at 207 nm. The apparent local maximum around 215 nm is characteristic of the superposition of two minima at 208 and 220 nm which characterize α-helical structure, as is the maximum around 192 nm. β-Sheet character in the polypeptide is indicated by the maximum at 195 nm. The asymmetric minimum at 207 nm indicates a spectral summation between a relatively large random coil component in the polypeptide and the α-helical component.

To deconvolve the CD spectra, standard curves for each secondary structure type derived from proteins of known secondary structure reported by Yang and coworkers (1986) were initially considered. Intuitively, it would seem that the correct approach would be to use standards derived from globular proteins. Further, previous reports indicate that these standard curves have been used successfully for secondary structure predictions of a number of globular proteins (Yang et al., 1986). However, in this study these standard curves gave unsatisfactory deconvolutions. The deconvolution of spectra of proteins of known structure were incorrect (e.g., DNaseI), spectra with very different appearance were assigned similar values for secondary structure elements, and the fit of the theoretical, calculated curves with the experimental curves was poor. It was suspected that the standard curve for β-sheet was grossly underestimated and at least part of the cause of the poor deconvolution. Therefore, the approach employed here for deconvolution used standard curves of Brahms and Brahms (1980) for pure secondary structures of homopolymeric peptides. The use of these curves as standards for deconvolution of CD spectra was found to provide good fit for proteins of know structure, in agreement with the reports of others [reviewed by Johnson (1988)]. The numerical method used to deconvolve the spectra was successive approximation: linear combinations of the secondary structure standards were compared to the experimentally determined spectra, and the fit was used to guide refinement in the linear combination of the standard spectra. The spectrum of the unphosphorylated R domain can be represented by a polypeptide of approximately 10% α-helix, 30% β-sheet, 10% β-turn, and 50% random coil. The inset to Figure 4 contains a superposition of the curve predicted by this method on the measured curve for the unphosphorylated R domain. The fit of the experimental and theoretical curves is within the variability observed experimentally. As indicated above, there is considerable variation in the standards employed in performing deconvolution to determine the secondary structure components. Therefore, the secondary structure composition values determined for the R domain are considered as only approximations.

The secondary structure composition of the human R domain was predicted with three different algorithms, each based on the most probable residue conformation in proteins of known three dimensional structure, and compared to that measured by CD spectroscopy, as shown in Table 3. It is notable that the three algorithms do not agree well with each other, particularly in the percentage of α-helix and random coil predicted to exist in the R domain. The Chou/Fasman (1978) method overestimated the helical content and underestimated the random coil character of the R domain, compared to that observed by CD spectroscopy. However, this method most accurately predicted the β-sheet content of the human R domain. The methods of Gascuel and Golmard (1988) and Garnier et al. (1978) agree better with each other,

Table 3: Secondary Structure of the Human R Domain: Predicted and Measured[a]

	Chou/ Fasman	Gascuel/ Golmard	Garnier et al.	measured by CD
α-helix	44%	24%	13%	10%
β-sheet	31%	18%	14%	30%
β-turn	5%		27%	10%
random coil	20%	58%	46%	50%

[a] The secondary structure of the human R domain was predicted using the algorithmis of Chou and Fasman (1978), Gascuel and Golmard (1988), and Garnier et al. (1978).

and with the measurements made by CD spectroscopy, in the prediction of α-helix and random coil content of the R domain. Both these methods underestimate the β-sheet content of the R domain.

There is a significant difference in the spectrum of the PKA phosphorylated R domain from that of the unphosphorylated form of the R domain, suggesting that phosphorylation of the R domain causes significant conformational change in the peptide. Relative to the unphosphorylated form of the R domain, the CD spectra of the phosphorylated form of the molecule has a decreased maximum at 192 nm (but not at 195 nm), and the minimum at 207 nm has decreased in magnitude and become more symmetric. By deconvolution with standard curves, these spectral changes are consistent with a decrease in α-helical content (to about 5% of the protein) and increase in random coil character of the phosphorylated R domain. When a 10-fold molar excess of PKA inhibitor (synthetic rabbit sequence, a 20 amino acid peptide containing the sequence Arg-Arg-Asn-Ala as a substrate analogue, Sigma), relative to the PKA, was included in the phosphorylation reaction, the R domain remained unphosphorylated, and the CD spectra was very similar to that of the R domain unexposed to PKA (data not shown). In each case where the R domain was incubated with PKA, either in the presence or absence of inhibitor, the final spectra was obtained by subtracting the PKA and buffer from the R domain spectra. In presence of the inhibitor, the final spectra contained contributions from both the R domain and the inhibitor and was the same before and after PKA addition. However, without the inhibitor, the final spectra contained only the contribution of the R domain and demonstrated the change illustrated in Figure 4 following incubation with PKA. These observations suggest that the method of subtraction of the PKA and buffer spectra from the R domain spectra is valid and that the conformational change observed in the R domain following incubation with PKA is a specific result of phosphorylation of the R domain.

Figure 5 contains the CD spectra of PKC phosphorylated and unphosphorylated human R domain. The spectra of the two forms of R domain show very little difference. If there is any conformational change in the R domain as a result of PKC phosphorylation, it is of the type that could not be detected by CD spectroscopy, such as minor spatial reorganization of secondary structure elements. The phosphorylation buffers alone were without significant effect on the R domain spectra, as indicated by the similarity in the spectra of the unphosphorylated polypeptides in Figures 4 and 5. There is an apparent correlation between the relative capacities of the two kinases to activate the CFTR chloride channel and their influence on the secondary structure elements of the R domain conformation.

To further assess the biological relevance of the observed conformational change in the R domain, R domain from the shark analogue was also expressed and purified. The R domains from the two species are the most divergent part of

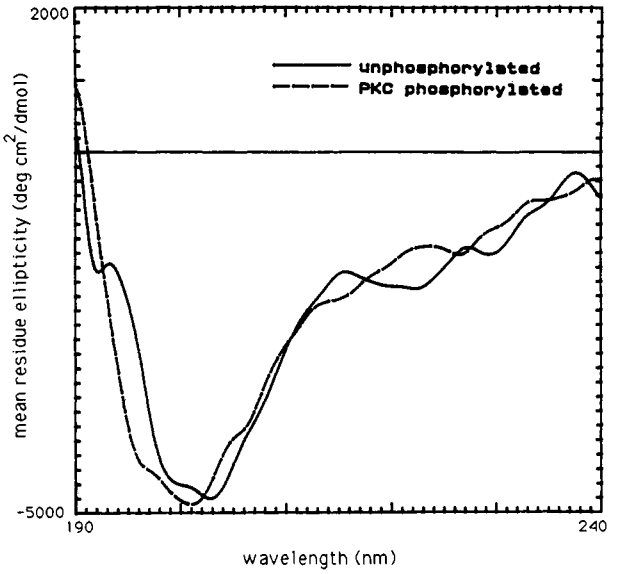

FIGURE 5: CD spectra of PKC phosphorylated and unphosphorylated forms of the human R domain. CD spectra of phosphorylated and unphosphorylated forms of human R domain. The spectrum of the polypeptide (10 μg) was determined in PKC phosphorylation buffer: 50 mM $KHPO_4$, pH 7.4, 1.5 mM $CaCl_2$, 10 mM Mg acetate, 0.05% 1,2-dioctanoyl-*sn*-glycerol, and 50 μM ATP. The spectrum of the buffer alone was subtracted from the R domain spectrum. The spectrum of PKC, at the appropriate concentration, in PKC phosphorylation buffer was subtracted from the spectra of the phosphorylated R domain.

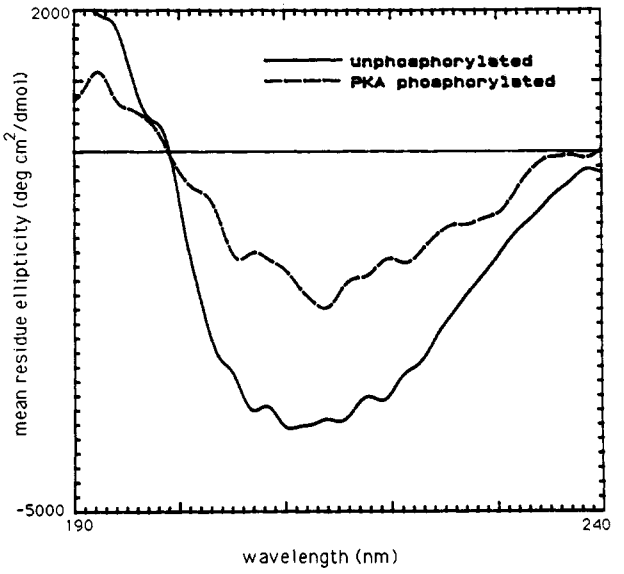

FIGURE 6: CD spectra of the PKA phosphorylated and unphosphorylated forms of the shark R domain. Shark R domain was phosphorylated and CD spectra for phosphorylated and unphosphorylated forms (10 μg) were obtained as described for the human R domain in the legend to Figure 4.

the CFTR molecules in terms of primary structure. Overall, the human and shark CFTR analogues are 72% identical at the amino acid level, but the R domains of the two species show only 57% identity (Marshall et al., 1991; Grzelczak et al., 1990). Both human and shark R domains contain nine consensus sites for phosphorylation by PKA, although two of these sites do not appear equivalent in an alignment of the two sequences. CD spectra of PKA phosphorylated and unphosphorylated forms of the shark R domain were also obtained and are shown in Figure 6. The shark and human R domains show similarities in their secondary structure in that both have a low α-helical content, approximately one-third β-sheet,

and greater than 50% of the molecule is β-turn and random coil. Emphasis cannot be placed on the absolute values of the secondary structural elements of the shark peptide as approximately 10% of the polypeptide is the FLAG epitope and periplasmic leader sequence. Strikingly, the phosphorylated form of the shark R domain also shows considerable conformational change from the unphosphorylated form. Like the human, a decrease in helical and increase in random coil content are observed when the shark R domain is phosphorylated by PKA. Since both PKA phosphorylation and the consequent conformational change are more highly conserved than the primary sequence of the domain, it is reasonable to conclude that the former two features are functionally important in the molecule.

DISCUSSION

This study describes the expression and purification of R domain polypeptides from both human and shark analogues of CFTR. The primary assumption on which the significance of the study rests is that the recombinant R domain has been renatured to a form that closely resembles that of the R domain contained in endogenous CFTR. Although the final proof of this must await structural analysis of CFTR, the reasons that follow suggest the validity of the approach. The choice of the R domain as a separate functional domain is based on alignment of CFTR with other members of a broad class of membrane proteins. The unit structure of these proteins consists of six transmembrane helices and a nucleotide binding domain (Riordan et al., 1989). Of the many members of this family, only CFTR contains a highly charged, cytoplasmic domain, which contains many consensus sites for phosphorylation by protein kinases. This suggests that, in evolutionary terms, the R domain may be a relatively recent addition to an ancestral precursor to CFTR, and thus a separate functional unit.

The literature is replete with examples of proteins and protein domains, expressed in recombinant bacterial systems, which maintain structural and functional integrity. For example, streptokinase, when expressed in *Escherichia coli*, maintains its ability to activate fibrinolysis (Estrada et al., 1992). Crystals of indistinguishable structure from that of the protein obtained from nonrecombinant sources have been obtained for the recombinant ribosomal protein, S5, and a human muscle fatty-acid binding protein (Ramakrishnan & White, 1992; Zanotti et al., 1992). Renaturation from strong chaototropic agents has been observed to yield biologically active bovine growth hormone, urokinase, and various other proteins (George et al., 1985; Winkler et al., 1985; Rinas et al., 1992). Examples of isolated domains which maintain functional integrity are the DNA binding domain of the yeast transcriptional activator, GAL(4) (Kraulis et al., 1992), the phosphorecognition domain of v-src (Waksman et al., 1992), and the cytoplasmic domain of the erythrocyte band 3 (Wang et al., 1992). Therefore, it is reasonable to expect the R domain, when expressed in isolation, to form a functional unit and to have properties similar to those of the native domain.

A significant change in the secondary structural characteristics of both human and shark R domains was observed as a result of phosphorylation of the peptides by PKA. These observations were made under conditions of the maximum possible phosphorylation of the peptides by the PKA. Cheng and co-workers (1991) have shown that, *in vivo*, amino acids 660, 737, 795, and 813 in CFTR are phosphorylated as a result of cAMP-stimulation. *In vitro*, PKA phosphorylates these residues in CFTR, as well as amino acids 700, 712, and 768. In an isolated R domain peptide, slightly smaller than the one used here, amino acids 660, 700, 737, 813, and probably both of 768 and 795 were found to be phosphorylated by PKA (Picciotto et al., 1992). Preliminary data indicate that at least six of the available PKA sites were phosphorylated under the conditions used here. Thus, as found by Picciotto and co-workers, the phosphorylation pattern of the isolated R domain is similar to that of the intact CFTR and also similar to that obtained *in vivo*. Having demonstrated an effect on R domain conformation with maximal phosphorylation, it is now possible to pursue investigation of the contributions of phosphorylation of the individual sites to this effect.

Phosphorylation of CFTR by PKA has been shown to activate chloride conductance. Stimulation of CFTR by PKC has also been shown to produce a chloride current in membrane patches, although the magnitude of this current is much less than that elicited by PKA (Tabcharani et al., 1991; Berger et al., 1993). Therefore, it might be expected that if conformational change in the R domain as a result of PKA phosphorylation was involved in activation, then PKC phosphorylation might also cause a similar conformational change in the R domain. However, most of the PKA consensus sites for phosphorylation (9 of 10) are in the R domain, while many other consensus sites for PKC phosphorylation exist in CFTR outside the R domain. Thus, the observations reported here may indicate that the PKC sites may also participate in mediation of the large potentiation of PKA activation which PKC causes (Tabcharani et al., 1991; Chang et al., 1993).

The CD spectra of the PKA phosphorylated R domains were significantly different from that of the unphosphorylated peptides. The change in secondary structure indicated by the spectra was a decrease in, but not complete removal of, α-helical content. The unphosphorylated form of the R domain was shown to be approximately 10% α-helical; therefore, approximately 24 amino acids in the polypeptide will assume an α-helical conformation. As a helix should contain at least six amino acids, there are likely to be, depending on the length, one to four α-helices in the R domain. The increase in random coil nature of the phosphorylated R domain suggests that one or two α-helices may unwind to a less ordered state as a result of phosphorylation.

How might this change in R domain conformation result in gating of the chloride conductance in CFTR? The shaker, or *Drosophila* potassium channel, provides an example of an ion channel which is gated by the plugging and unplugging of the ion pore with a cytoplasmic domain (Hoshi et al., 1990). In analogy to the shaker model, it has been speculated that phosphorylation of the R domain may cause sufficient electrostatic repulsion to displace the R domain from the mouth of the channel (Fuller & Benos, 1992). Another mechanism, suggested by the results presented here, is that phosphorylation of the R domain causes a conformational change which allows the domain to slide out of the channel. Conformational change could function in place of, or along with, the electrostatic repulsion model.

The data presented here provide the first evidence that an important component in the function of CFTR is a conformational change in the R domain as a result of phosphorylation by PKA.

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