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Mutation Of Potential Phosphorylation Sites In The Recombinant R Domain Of The Cystic Fibrosis Transmembrane Conductance Regulator Has Significant Effects On Domain Conformation

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Mutation of potential cAMP dependent protein kinase sites in the R domain of the cystic fibrosis transmembrane conductance regulator has significant effects on protein function. Mutation of the potential phosphorylation sites from serine to alanine, to abolish the site, reduced sensitivity to activation, or to glutamic acid, to mimic phosphorylation, caused some constitutive activity. To explore the structural effects of these mutations, recombinant R domain peptides were studied: the wild type, a mutant with nine serine residues changed to alanine, and a mutant with eight serine residues changed to glutamic acid. As assessed by C.D. spectroscopy, the mutants have substantially different secondary structure than the wild type, in agreement with the predictive algorithm of Gascuel and Golmard. The results show that mutagenesis of residues alters the polypeptide structurally as well as preventing it from serving as a phosphorylation substrate. Hence, the functional consequences of the mutations may not be entirely due to effects on phosphorylation.

Protein phosphorylation and dephosphorylation are ubiquitous mechanisms for controlling a wide variety of cellular functions (1). CFTR, the protein which is dysfunctional in individuals with the genetic disease, cystic fibrosis (2), is a chloride channel, which is activated when it is phosphorylated by the cAMP dependent protein kinase (PKA) (3,4,5). Nine of the ten dibasic consensus sites for PKA phosphorylation (6) in CFTR are in the R domain of the protein (2). Many of the predicted sites are utilized, with varying stoichiometry (7,8). It has been demonstrated that a recombinant peptide representing the R domain undergoes a conformational change with phosphorylation by PKA (9). Mutation of nine of the PKA phosphorylatable residues in CFTR to alanine produced a chloride channel with a PKA stimulatable chloride channel activity of approximately 40% of the wild type level (10). Substitution of acidic residues to mimic the negative charge of the phosphorylated serines in CFTR produced a molecule with constitutive chloride conductance (11). Deletion of most of the R domain from CFTR results in a channel which constitutively conducts chloride (12). To further investigate the properties of the mutant and

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Abbreviations: CFTR-cystic fibrosis transmembrane conductance regulator, PKA- cAMP dependent protein kinase, C.D.- circular dichroism.

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phosphorylated R domains, recombinant R domain peptides with either PKA phosphorylatable serines mutated to alanine or to glutamic acid were studied.

In general, point mutations do not change protein structure substantially, unless the mutation completely disrupts folding (13). It has been observed that many protein kinase phosphorylation sites occur in what are predicted to be β turn secondary structures (14). These predictions were made with the database compiled by Chou and Fasman, indexing the propensity of each residue to be found in each secondary structure type. It is not surprising that cAMP dependent protein kinase phosphorylation sites are predicted to be in β turns, as the residues of the consensus sequence have moderately high β turn propensity (15). The mutations of serine to alanine and serine to glutamic acid, which are commonly made in investigating the significance of kinase phosphorylation sites, replace a residue with high β turn potential with a residues of high (alanine) or moderate (glutamic) α helical potential (16). This suggests that the phosphorylation site mutations in the R domain may effect the secondary structure of the peptide.

Recombinant peptides of the wild type and mutant R domains have been expressed and purified. Their secondary structure characteristics were investigated by C.D. spectroscopy and compared to secondary structure predictions.

Materials and Methods

Expression and Purification of Proteins: Using mutant CFTR templates, mutant R domain sequences (representing amino acids 595 to 831 of CFTR) were inserted into pET3A (17), as described for the wild type (9). 9SA R domain, in which nine consensus PKA phosphorylatable serine or threonine residues were mutated to alanine, and 8SE R domain, in which eight consensus PKA phosphorylatable serines were mutated to glutamic acid were expressed. The locations of the mutations are indicated in Table 1. Expression plasmid sequences were confirmed by restriction enzyme analysis and double stranded sequencing. All proteins were expressed in the BL21(DE3)plysS host (18). Cultures were grown at 37°C to an optical density of 0.8 at 600 nm and protein expression was induced with 1 mM isopropylthio-β-galactoside and grown for a further 24 hours. Expression of the correct peptide was verified by Western blotting with five antibodies to different epitopes in the R domain, all of which recognized the mutants as well as the wild type (not shown). The 9SA peptide was purified in exactly the same manner as the wild type R domain (9). Purification of the 8SE peptide differed from the wild type only in the pH and buffer used during cation exchange chromatography. Cation exchange of the wild type and 9SA

Table 1: Predicted secondary structure of the PKA phosphorylatable residues in the R domain. The amino acid type, (S-serine, T-threonine, E-glutamic acid, A-alanine) in the wild type, 9SA and 8SE R domains is indicated. Residue numbering scheme is that of full length CFTR (2). Secondary structure prediction was performed with the algorithms of Garnier et al and Gascuel and Golmard. The predicted secondary structure (t-beta turn, c-random coil, b-beta sheet, h-alpha helix) is indicated for the four residues which constitute the consensus site for PKA phosphorylation.

residue no.	amino acid type			Garnier et al prediction			Gascuel and Golmard prediction		
	WILD TYPE	8SE	9SA	WILD TYPE	8SE	9SA	WILD TYPE	8SE	9SA
660	S	E	A	t	c	c	c	h	h
686	S	E	A	t	c	t/c	c	c	c/h
700	S	E	A	t	t	t	c	c	h
712	S	E	A	t/b	b	b	h	h	h
737	S	E	A	t	h	h	h	h	h
768	S	E	A	t	t	t	h	h	h
788	T	T	A	t/c	t/c	c	c	c	h
795	S	E	A	t	c/b	b	b	h	h
813	S	E	A	c	c	c	c	h/c	h

peptides was performed at pH8.2 in 50 mM bicine, while 50 mM MES pH6.0 was used for the 8SE peptide. Purity of the peptides was assessed by silver staining (kit from Sigma). Renaturation of peptides was carried out essentially as described (9). Samples equilibrated in 60 mM urea were dialyzed overnight at 4°C against 20 mM $KHPO_4$, pH 7.5 prior to C.D. spectroscopy.

Secondary Structure Prediction: The two algorithms used for secondary structure prediction are those that agreed with the observed C.D. spectra of the wild type peptide (9) and were performed on a Compac Deskpro 386s personal computer with the software package PCgene (version 6.01, Intelligenetics, Inc.). The algorithms of Garner et al (19) and Gascuel and Golmard (20) are based simply on the statistical likelihood of a given residue being in a given conformation.

C.D. Spectroscopy: C.D. Spectroscopy was performed as described (9). A modified version of the method of Yang et al (21) was employed to deconvolve the experimental spectra. Also, spectra of peptides of single secondary structure type (22) were combined in proportion of the predictions of the secondary structure algorithms and compared to the experimentally determined spectra.

Results

Three forms of R domain peptide were expressed in a bacterial expression system: the wild type sequence, 9SA and 8SE. Figure 1 indicates that each of the peptides is found in the water insoluble fraction of bacterial lysates. The wild type and 9SA peptide migrate in SDS-PAGE with apparent molecular mass of 30 kDal, while the 8SE mutant migrates with apparent molecular mass of 33kDal, even though the predicted mass is only 0.4 kDal greater than the wild type. Phosphorylated wild type R domain shows a similar decrease in mobility, suggesting the mobility

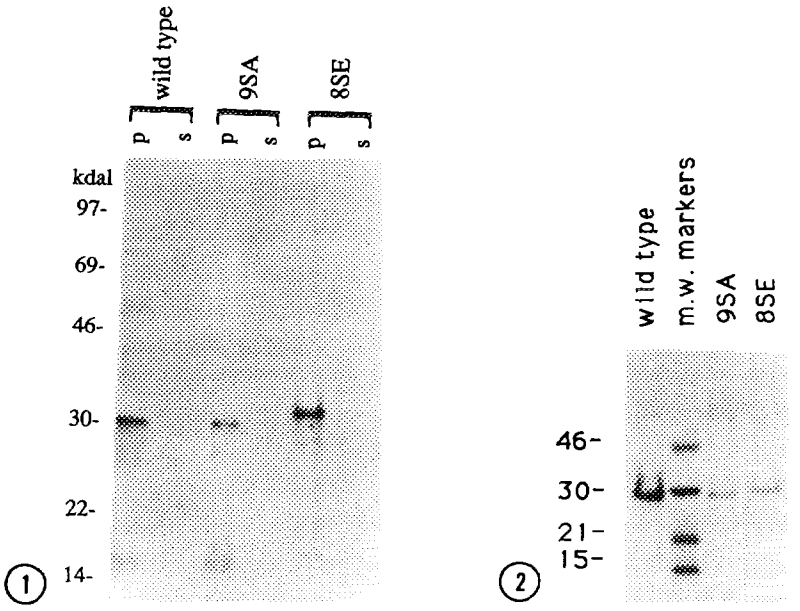

Figure 1. Expression of R domain peptides in bacteria. Bacterial hosts were induced to express the R domain peptides as described in the text. Water soluble (S) and insoluble (P) fractions of lysed samples were subjected to SDS-PAGE on 10% tricine buffered gels, transferred to nitrocellulose filters and subjected to Western blotting with the monoclonal antibody L11E8 (34) as described (9).

Figure 2. Purification of R domain peptides. Peptides were purified from bacterial expression systems as described in the text. The protein purified from one ml of initial bacterial culture was subjected to SDS-PAGE on a 10% tricine buffered gel. The gel was stained with commassie brilliant blue, as described (9).

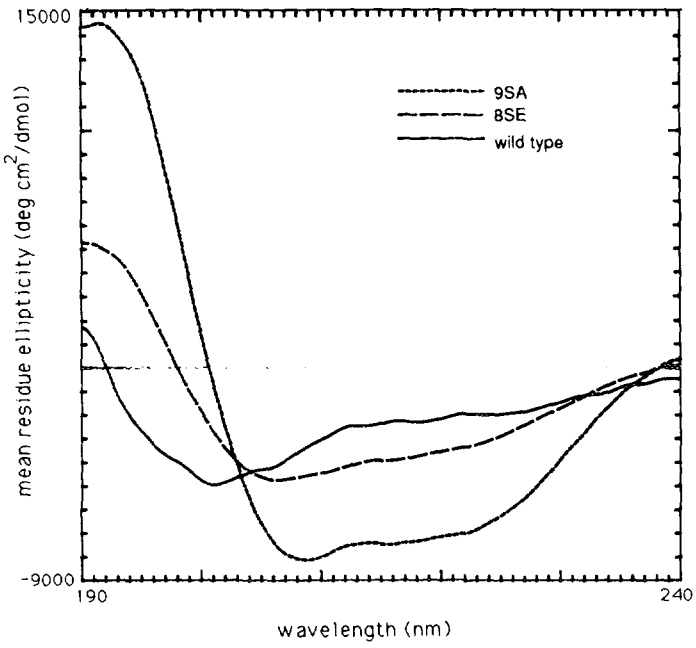

Figure 3. CD spectra of R domain peptides. CD spectra of the wild type (solid line), and mutants, 9SA (dotted line) and 8SE (dashed line) were obtained as described in the text.

of the peptide in SDS-PAGE is effected by the net charge, as well as molecular mass (7,9). It was previously reported that the wild type R domain was expressed at about 1% of the total bacterial protein (9). Following optimization of expression, 9SA and 8SE peptides were found at approximately 0.25 and 0.5% of the total bacterial protein.

Each peptide was purified from the insoluble fraction of the bacteria after solubilization in 6M guanidine HCl and then 6M urea, by ion exchange and gel filtration. Figure 2 shows purified R domain peptides. The wild type peptide has been reported previously to be recovered at 1 to 2 mg of wild type R domain from one litre of bacterial culture. For the mutants, the yield was similar: 350 to 500 ug of peptide per litre of culture. Following purification, the peptides were renatured by gradual dialysis out of 6M urea. For the wild type, this process was about 25% efficient (9), as it was for 9SA. The 8SE mutant renatured at nearly 100% efficiency. As predicted, the pI of the wild type and 9SA was approximately 8.3; while the pI of 8SE was approximately 4.5 (data not shown), as determined by IEF gel electrophoresis (kit from Novex).

Figure 3 contains the CD spectra of the renatured R domain peptides. Each peptide has a spectra indicative of a high degree of secondary structure. Maxima around 190 to 195 nm and broad minima (which is the superposition of two minima) between 200 and 230 nm indicate the presence of α helical and β sheet structures. The spectra of each of the wild type, 8SE and 9SA R domains are clearly different. The spectra of the 9SA peptide has significantly more helical contribution than the others, while the spectra of the 8SE peptide appears to have more β sheet character than the others. It should be noted that the spectra of 8SE does not resemble the spectra of phosphorylated wild type R domain (9).

Table 2: Secondary structure composition of the R domain peptides. The secondary structure of the wild type and mutant R domain peptides was predicted with the algorithms of Garnier et al and Gascuel and Golmard and compared to the values obtained from deconvolutions of the experimental spectra by a modified version of the method of Yang and coworkers. rms difference -calculated as : the square root of [the sum of (the square of the difference between the deconvolved value for each secondary structure type and the value for that secondary structure type predicted by the algorithm)].

		alpha helix	beta sheet	beta turn	random coil	rms difference
wild type	measured	9%	40%	7%	44%	
	Garnier et al	13%	14%	27%	46%	33
	Gascuel/Golmard	24%	18%		58%	28
8SE	measured	16%	48%	0%	36%	
	Garnier et al	15%	13%	21%	50%	43
	Gascuel/Golmard	33%	12%		56%	45
9SA	measured	45%	23%	0%	32%	
	Garnier	14%	18%	22%	46%	41
	Gascuel/Golmard	41%	11%		48%	20

To deconvolve the spectra and compare the secondary structure composition of the peptides to the predictive algorithms, two approaches were used. The method of Yang and coworkers (21) had previously been found to be inaccurate at deconvolving CD spectra (9). However, a modification of this method produced results for the wild type R domain which agreed favorably with those obtained by successive approximation (9). The modification removed the constraint of having the secondary structure components in the deconvolution sum to 100%. Table 2 contains the deconvolution values for each of the R domain peptides compared to the predictions. The algorithm of Gascuel and Golmard agrees with the deconvolution of the experimental spectra for the wild type and 9SA more closely than the algorithm of Garnier et al. Neither of the two algorithms agrees well with the deconvolution of the 8SE spectra, possibly because of the weakness of the deconvolution method for detecting β sheet (23).

As an alternative means of comparing the experimental spectra and the predictive algorithms, theoretical spectra were prepared. Figure 4 shows two sets of theoretical CD spectra generated by combining the curves for homopolymeric peptides of a single secondary structure

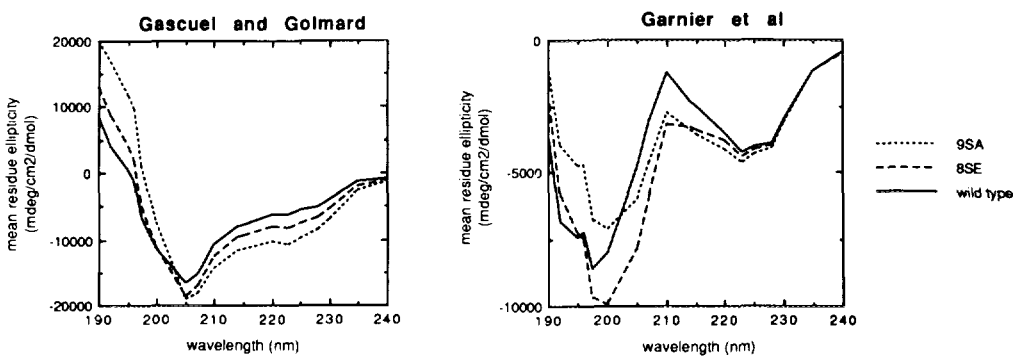

Figure 4. Spectra predicted for R domain peptides by secondary structure prediction algorithms. Spectra for homopolymeric peptides of single secondary structure type were combined in the proportions predicted by the algorithms of Gascuel and Golmard or Garnier and coworkers.

type (22) in proportion to the predictions of the algorithms (values in Table 2). Clearly, the spectra predicted by Gascuel and Golmard are reflective of the experimental spectra (Figure 3), while those predicted by Garnier and coworkers are not.

Discussion

The experiments described here clearly demonstrate that the mutation of the phosphorylatable serines in the R domain to either alanine or glutamic acid cause significant conformational change in the domain. What are the implications of these and other studies on the conformation of recombinant R domain to the observed biological activity of wild type, 9SA and 8SE CFTR molecules? One trivial interpretation of the data is that the conformation of the recombinant R domains is different than the R domains of intact CFTR. However, this is unlikely, as there are examples of recombinant domains from proteins which have the same function in isolation as they do in the intact protein (24,25,26). Further, there are numerous examples of recombinant proteins, renatured from inclusion bodies, which have native biological function (for example, 27,28,29). In a direct comparison of the structure of recombinant peptides and the natural domains of fibronectin, it was found that the recombinant peptides have very similar structures to their counterparts obtained from proteolysis of the intact protein (30). Therefore, the investigation of structural and functional characteristics of recombinant domains is a feasible approach to determining their properties in the intact protein.

9SA and 8SE CFTR molecules have altered function as follows: diminished function with abolition of the phosphorylation sites and constitutive activity with mimicry of the phosphorylated sites (8,10,11). These observations might seem contradictory to the observation of significant change in the secondary structure of 9SA and 8SE R domains. One simple interpretation of these observations is that the overall structure of the R domain is not important, its ability to be phosphorylated is. If the R domain is considered to be two discrete domains (31), RD2, the domain which contains the majority of the phosphorylation sites (residues 679-798), is very poorly conserved, maintaining only 23% identity in the amino acid sequence among ten species of CFTR. The only parts of RD2 that are conserved are the PKA phosphorylation sites. Therefore, it is possible that the secondary structure of RD2 is of secondary importance to the ability of the domain to be phosphorylated. It was anticipated when the experiments described here were initiated that the 8SE R domain peptide might have a conformation similar to the phosphorylated wild type R domain peptide (9). However, the two do not have the similar conformations, supporting the contention that the conformation of RD2 is not the determining factor in CFTR channel activation.

This interpretation might suggest that the previously observed conformational change of a recombinant R domain with phosphorylation (9) was not a biologically significant event. However, this seems unlikely for a number of reasons. 1) Site directed mutagenesis and post-translational modification are not comparable. 2) The above discussion concluded that the significant property of the R domain is its ability to be phosphorylated. Therefore, changes in conformation resulting from phosphorylation would be inherently significant. 3) Preliminary experiments indicate that the 9SA and 8SE R domains still exhibit moderate conformational change, in an analogous way to the wild type, on phosphorylation.

The mutation of serine to alanine is a common approach to studying the role of phosphorylation in protein function. For example, this approach was used with the Na^+,K^+-ATPase (32) and the voltage sensitive Na^+ channel (33). A conformational change is predicted to result from replacing a residue with moderately high turn potential with one of high helical potential. A better substitution for the serine might be asparagine, which is small, hydrophilic, and uncharged. Asparagine also has a moderate turn potential and low helical potential.

To conclude, care should be taken in the interpretation of data concerning the mutation of phosphorylation sites, as the potential exists for conformational change with mutation. The secondary structure prediction algorithm of Gascuel and Golmard may serve as a guide to indicate conformational changes.

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