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Effects of domain interactions of function and regulation of the cystic fibrosis transmembrane conductance regulator.

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EFFECTS OF DOMAIN INTERACTIONS ON FUNCTION AND REGULATION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

by

SCOTT A. KING

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title Effects of Domain Interactions on Function and Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator

Cystic fibrosis (CF) is caused by the aberrant function of the cystic fibrosis transmembrane conductance regulator (CFTR). Understanding intramolecular interactions within CFTR may illuminate functional processes disrupted in the disease. In this dissertation, domain binding interactions within CFTR were tested for their influence on protein function and regulation. Functional and physical interactions were observed between proximal (M837X) and distal (Δ1-836) halves of CFTR. Unlike wild-type CFTR, coexpression of M837X with Δ1-836 conferred halide permeability in cells without stimulation of cyclic AMP dependent protein kinase A (PKA). Interestingly, the physical binding between M837X and Δ1-836 led to an M837X protein that was detected in a phosphorylation dependent, reduced mobility form. Kinase inhibitors diminished both the high basal activity and the shifted M837X protein, indicating that endogenous PKA activity was responsible for both events. The distal portion of the regulatory domain (R-domain) was necessary for the PKA dependent activity and the mobility shift of M837X. Soluble R-domain also co-immunoprecipitated with Δ1-836 in a fashion that led to a phosphorylation dependent conformational change in the R-domain protein. Further analysis

of regulatory subdomains isolated a region (amino acids 740-813) necessary for the change due to phosphorylation. Another cytosolic domain, the first nucleotide binding domain (NBD1), was also found to coimmunoprecipitate with Δ1-836. Because intramolecular binding is often an important prerequisite for protein biosynthesis in the endoplasmic reticulum (ER), we tested the ability of the common ΔF508 mutation to disrupt intramolecular binding within CFTR. The ΔF508 mutation eliminated the enhanced halide permeability detected upon coexpression of M837X with Δ1-836 but did not disrupt physical interactions of NBD1 with distal domains. Therefore, while the ΔF508 mutation can still be recognized in cells when CFTR domains self-assemble, a defect in domain binding cannot be implicated as underlying the ΔF508 processing abnormality. These studies provide new information concerning the ways CFTR domains bind each other and indicate the importance of domain:domain interactions in CFTR phosphorylation, regulation, and function.

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LIST OF ABBREVIATIONS

4PBA	sodium 4-phenylbutyrate
ABC	ATP-binding cassette
ATP	adenosine triphosphate
ß-gal	ß-galactosidase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
C-terminal	carboxy terminal
CBAVD	congenital bilateral absence of the vas deferens
CD	circular dichroism
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGK	cGMP dependent protein kinase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DOC	deoxycholic acid
EnaC	epithelial sodium channel
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FBS	fetal bovine serum
HisM	prokaryotic histidine permease hydrophobic domain

LIST OF ABBREVIATIONS (Continued)

HisP	prokaryotic histidine permease NBD
HisQ	prokaryotic histidine permease hydrophobic domain
I/V	current/voltage
IBMX	3-isobutyl 1-methyl xanthine
kD	kiloDalton
MalF	prokaryotic maltose permease hydrophobic domain
MalK	prokaryotic maltose permease NBD
MDR	multi-drug-resistant
MOI	multiplicity of infection
NBD	nucleotide binding domain
NBT	4-nitro blue tetrazolium chloride
ORCC	outwardly rectifying chloride channel
PBS	phosphate-buffered saline
P-gly	P-glycoprotein
PKA	cyclic AMP dependent protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PVDF	poly(vinylidene difluoride)
R-domain	regulatory domain
RD1	amino acids 587-672
RD2	amino acids 679-798

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPQ	6-methoxy-N-(3-sulfopropyl)quinolonium
STE6	yeast a-factor mating peptide transporter
TMD	transmembrane domain
vTF7.3	vaccinia virus encoding the T7 polymerase

INTRODUCTION

Cystic Fibrosis and the Cystic Fibrosis Transmembrane Conductance Regulator.

Cystic Fibrosis. CF is an autosomal recessive disease with an incidence of approximately 1 in 3,000 live births in the Caucasian population, 1 in 10,000 in the Hispanic population, and low incidence in Asian and African populations (NIH, 1999). The prognosis of patients with CF has improved over the past 50 years from death in early childhood to a life expectancy of greater than 31 years (Shwachman *et al.*, 1949; Cystic Fibrosis Foundation, 1998). Clinically, the disease is characterized by chronic obstructive lung disease with recurrent infections, intestinal obstruction, loss of pancreatic function, high salt in the sweat (a diagnostic indicator), and male infertility (Quinton, 1999). This diversity of symptoms complicated early endeavors to isolate the source of the disease.

Historically, references to CF can be traced back to the 17th century, when "bewitched" children with salty skin were known to have poor growth and early demise. Formal classification of CF as a clinical disease was not made until the 1930s. The variety of symptoms inspired several early names, including "cystic fibromatosis with bronchiectasis," suggested by Fanconi to define the lung disease; "cystic fibrosis of the pancreas," by Anderson, relating to the pancreatic pathology; and "mucoviscidosis," termed by Farber to describe the thick mucous secretions (see reviews: Welsh *et al.*, 1995; Ramsey, 1998; Quinton, 1999). The range of names reflected the difficulty of defining a

global model of pathogenesis that would account for myriad symptoms. With the advent of electrophysiology and advanced biochemical techniques, a defect common to the involved organs was defined. Altered transport of sodium and chloride was discovered in the respiratory epithelium and sweat glands of CF patients (Knowles *et al.*, 1981; Quinton and Bijman, 1983). These results and others that followed indicated that the source of the disease was an inability to regulate ion transport across the epithelial membrane of organs necessary for proper function (Patton *et al*, 1982; Sorscher and Breslow, 1982; Widdicombe, 1986).

Discovery of the gene aberrant in CF. The discovery of the gene responsible for CF occurred in the late 1980s. Positional cloning techniques were utilized to isolate DNA sequences associated with CF. The search for genetic markers resulted in the discovery of MET and D7S8 on chromosome 7 residing close to the prospective gene (Zengerling *et al*, 1987; Poustka *et al*, 1988). Further isolation using chromosome walking and jumping identified a gene at chromosome 7q31.2 mutated in the CF population (Kerem *et al*, 1989; Riordan *et al*, 1989; Rommens *et al*, 1989). The gene encompasses 230 kb encoding 27 exons and an mRNA of approximately 6.5 kb.

CFTR. The protein product, CFTR, contains 1480 amino acids and has a molecular weight of 140-170 kD (Riordan *et al*, 1989; Harris, 1992). Sequence analysis of the protein encoded by CFTR DNA identified two transmembrane domains and two regions predicted to bind adenosine triphosphate (ATP). These nucleotide binding domains (NBDs) of CFTR consist of sequences common to the ATP-binding cassette

(ABC) gene family, including Walker A and Walker B sequences, and an LSGGQ motif (Riordan *et al.*, 1989). The topology of CFTR (Fig. 1) was predicted to consist of five large domains, four typical of ABC proteins, including the first transmembrane domain (TMD1), the first nucleotide-binding domain (NBD1, initially designated as amino acids 433 to 586, also see below), TMD2, and NBD2 (residues 1214 to 1386). One domain unique to CFTR, the R-domain (amino acids 590 to 830), is located in the middle of the protein and divides the two TMD-NBD motifs. The R-domain is unlike other linker regions in the ABC family due to the abundance of charged residues. (Almost 1/3 of the R-domain is composed of charged amino acids.) The R-domain also contains several PKA phosphorylation sites and at least two sites for protein kinase C (PKC) dependent phosphorylation. The topology predicted following the discovery of CFTR has proven to be quite accurate, although the boundaries of NBD1 have recently been questioned and may extend beyond original predictions by 50-100 amino acids (Annereau *et al.*, 1997; Bianchet *et al.*, 1997; Hoedemaeker *et al.*, 1998).

CFTR is a membrane-associated glycoprotein detected as multiple bands on Western blots (Gregory *et al.*, 1990). Two N-linked glycosylation consensus sequences (NXS/T) reside on the loop between transmembrane helices M7 and M8, resulting in the glycosylation of asparagines 894 and 900. Glycosylation of these sites confirms the predicted position of this loop in an extracellular location. Antibody studies have demonstrated that the loop between M1 and M2 is also extracellular, indicating that the N-terminal tail is intracellular (Denning *et al.*, 1992b). R-domain and C-terminal tail antibodies also mapped these regions to the cytoplasmic side of the membrane. Additional studies in which glycosylation sites were inserted into regions predicted to be cytoplas-

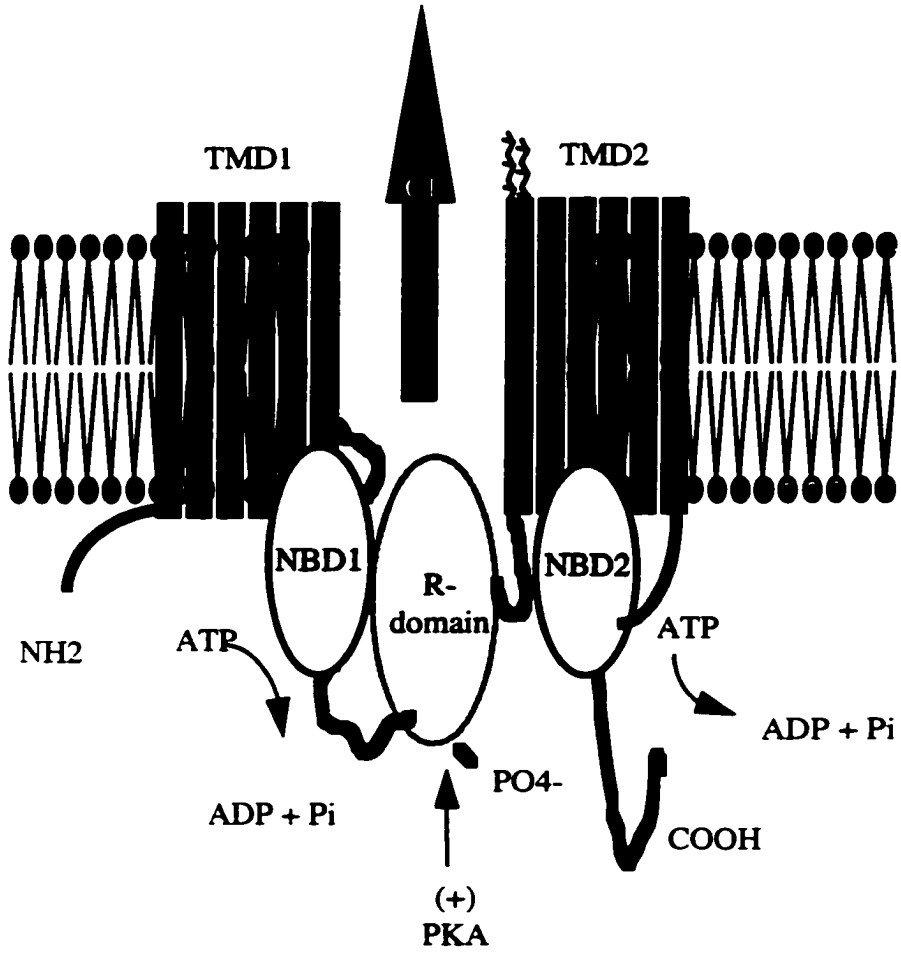


FIG. 1. CFTR model. CFTR is composed of two TMDs (TMD1 and TMD2), two NBDs (NBD1 and NBD2), and an R-domain with several PKA sites. Glycosylation sites are shown within TMD2 (≋).

mic or extracellular also support the original models of CFTR topology (Chang *et al.*, 1994).

Specific antibodies produced against CFTR have been useful in screening tissues for the protein. CFTR immunohistochemistry reinforced the conclusions of earlier tissue electrophysiology reports that CFTR is localized to the epithelial membranes of respiratory and gastrointestinal tissues, sweat glands and the pancreas (Cohn *et al.*, 1991; Crawford *et al.*, 1991; Marino *et al.*, 1991). CFTR has also been identified in the heart and kidneys, although these organs do not appear to be affected in the disease and the function of the protein in these tissues is unknown (Levesque *et al.*, 1992; Horowitz *et al.*, 1993; Morales *et al.*, 1996).

ABC proteins. CFTR belongs to a large family of proteins that transport a variety of molecules (such as short peptides and monosaccharides) across cell membranes (Schneider and Hunke, 1998). Proteins in the ABC family are defined by structural features, including TMDs and NBDs. Other examples of this gene family include STE6, the yeast α-factor mating peptide transporter; prokaryotic carbohydrate and amino acid permeases such as prokaryotic maltose and prokaryotic histidine permease (MalK, HisP); and P-glycoprotein (P-gly), the multi-drug-resistant gene product found in certain cancers. ABC transporters also carry out many types of functions, including active transport (P-gly and MalK), regulation of other channels (SUR and CFTR), and passive ion transport (CFTR) (Schneider and Hunke, 1998).

Interestingly, the sequence homology across the ABC family is minimal, except within the NBDs, where the primary sequence is fairly conserved (Welsh *et al.*, 1998).

The functional significance of NBD homology was demonstrated by replacement of an NBD from STE6 with CFTR NBD1 (Teem *et al.*, 1993). The resulting STE6-CFTR chimeric protein was able to transport α-factor, indicating that CFTR NBD1 could substitute for an STE6 NBD and provide STE6 the energy necessary for mating factor transport. While the NBDs share homology in both sequence and function, the TMDs have little sequence homology and are believed to provide the specificity of substrate and function to these proteins (Welsh *et al.*, 1998).

CFTR function. Expression of CFTR in Xenopus oocytes (Bear *et al.*, 1991; Drumm *et al.*, 1991) or nonepithelial cells (Rommens *et al.*, 1991) without endogenous CFTR led to the appearance of cAMP-regulated chloride channel activity with the same conductance properties as in native epithelial cells that express CFTR endogenously. Direct evidence that CFTR itself conducted chloride resulted from experiments in which CFTR was expressed in Sf9 cells, purified, and inserted into planar lipid bilayers (Bear *et al.*, 1992). Low conductance, cAMP-activated chloride channel activity was detected in the bilayers, demonstrating that CFTR did not require additional proteins to conduct chloride.

Functions in addition to chloride transport have also been attributed to the protein. CFTR was shown to regulate the activity of other channels in the cell membrane, suggesting that the protein helps coordinate the movement of salts and fluids (Al-Awqati, 1995; Kunzelmann, 1999). For example, early patch-clamp studies in CF epithelial cells indicated abnormalities in chloride conductance of an outwardly rectifying chloride channel (ORCC) of approximately 30-50 pS (Halm *et al.*, 1988; Welsh *et al.*, 1989). This

channel was stimulated by both PKA and PKC catalytic subunits and was found to be absent in CF cells (Hwang *et al.*, 1989). When the CF gene was identified, it was surprising that the encoded Cl^- channel did not have large conductance and outwardly rectifying properties but instead produced a low conductance, linear current/voltage (I/V) channel. It was subsequently established that these two currents result from two different channel proteins and that the lower conductance CFTR positively regulates the larger ORCC (Egan *et al.*, 1992). In addition to epithelial chloride currents, regulation of an epithelial sodium channel (ENaC) by CFTR has also been demonstrated (Stutts *et al.*, 1995; Ismailov *et al.*, 1996). In contrast to ORCC, ENaC is negatively regulated by CFTR, so that in the absence of CFTR, ENaC activity is enhanced. Recently, domains of CFTR contributing to ENaC regulation have been isolated. Intact NBD1 was found to be important for ENaC down regulation, while C-terminal domains were not required (Schreiber *et al.*, 1999). This study demonstrates the importance of specific CFTR domains in the regulation of other channels.

Other transport functions have also been described for CFTR, including transmembrane movement of ATP (Schwiebert *et al.*, 1995). Under hypotonic conditions, non-CF cells were shown to release ATP into extracellular fluid, while cells lacking CFTR were unable to respond in this way (Taylor *et al.*, 1998).

Relationship of CFTR to disease pathogenesis. The relationship between aberrant CFTR molecular function and the pathogenesis in different organs is not yet fully understood. Initial symptoms observed in newborns (*i.e.*, meconium ileus and failure to thrive)

may be attributed to dysfunction of the pancreas, intestine, or both (Wilschanski and Durie, 1998). The loss of CFTR in the pancreatic proximal duct results in protein-rich dehydrated secretions that obstruct the ducts, lead to acinar cell destruction and contribute to intestinal blockage (Durie, 1989; Nousia-Arvanitakis, 1999). Nearly 85% of CF patients ultimately become pancreatic insufficient. Similarly, congenital bilateral absence of the vas deferens (CBAVD) occurs in the majority of male patients and is believed to result from obstruction and atrophy of the ducts (Gaillard *et al.*, 1997). The pathogenic basis of these changes may also result from inadequate hydration of luminal content due to diminished secretion of fluid and electrolytes.

While intestinal complications were at one time the most common cause of mortality in CF, respiratory disease is now the leading cause of death in this population (Welsh *et al.*, 1995). Several theories have been proposed relating defective Cl^- channel function to the symptoms of CF lung disease. Traditionally, the lung disease is thought to result from thick secretions and the resulting inability of ciliary movement to clear the airways of infectious agents (Wine, 1999). Strong supporting evidence indicates that dilated airway acinar and ductal lumens in CF patients can be demonstrated before chronic infection is observed (Sturgess, 1982). New findings concerning the lung disease, however, suggest that predisposition to infection is not strictly caused by lack of mucous clearance but may also result from elevated ion content in the surface fluid lining the airways (Travis *et al.*, 1999). Defensins, antibacterial peptides that help protect the lungs from numerous pathogens, are inactivated by high salt concentrations and unable to abate bacterial growth *in vitro* (Goldman *et al.*, 1997). The airway surface liquid salt concentra-

tion in humans is an area of considerable debate. It has been suggested that elevated salt in CF airway fluid inactivates defensins and increases the susceptibility to bacterial infections. Other reports, however, have indicated that the salt concentration in the fluid lining the CF respiratory tract is unchanged (Knowles *et al.*, 1997).

Biogenesis, conduction, and regulation of CFTR

Biogenesis. The biogenesis of CFTR is typical for a glycosylated plasma membrane protein (Jilling and Kirk, 1997). Translation of CFTR occurs at the ER membrane, where CFTR is core glycosylated and folds to a tertiary conformation with the aid of protein chaperones. Core glycosylated, immature CFTR can be detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as an approximately 140-kD protein (Cheng *et al.*, 1990; Gregory *et al.*, 1990). From the ER, proteins are transported to the Golgi, where carbohydrate side chains are further modified, resulting in a protein of 150-170-kD (Fig. 2). Mature CFTR is packaged into vesicles in the trans-Golgi network for trafficking to the plasma membrane. While no discrete plasma membrane (or apical membrane) targeting signals have yet been demonstrated for CFTR, removal of a region immediately distal to NBD2 may impair retention of CFTR at the plasma membrane. This region, in essence, may be considered as a plasma membrane targeting domain (Haardt *et al.*, 1999).

Approximately 70% of wild-type CFTR is not processed beyond the ER but instead is rapidly degraded with a half-life of approximately 30 minutes (Lukacs *et al.*, 1994). Inhibition of the 26S proteasome by lactacystin or the peptide aldehyde, ALLN, stabilizes the half-life of immature CFTR and increases the amount of the ubiquitinated

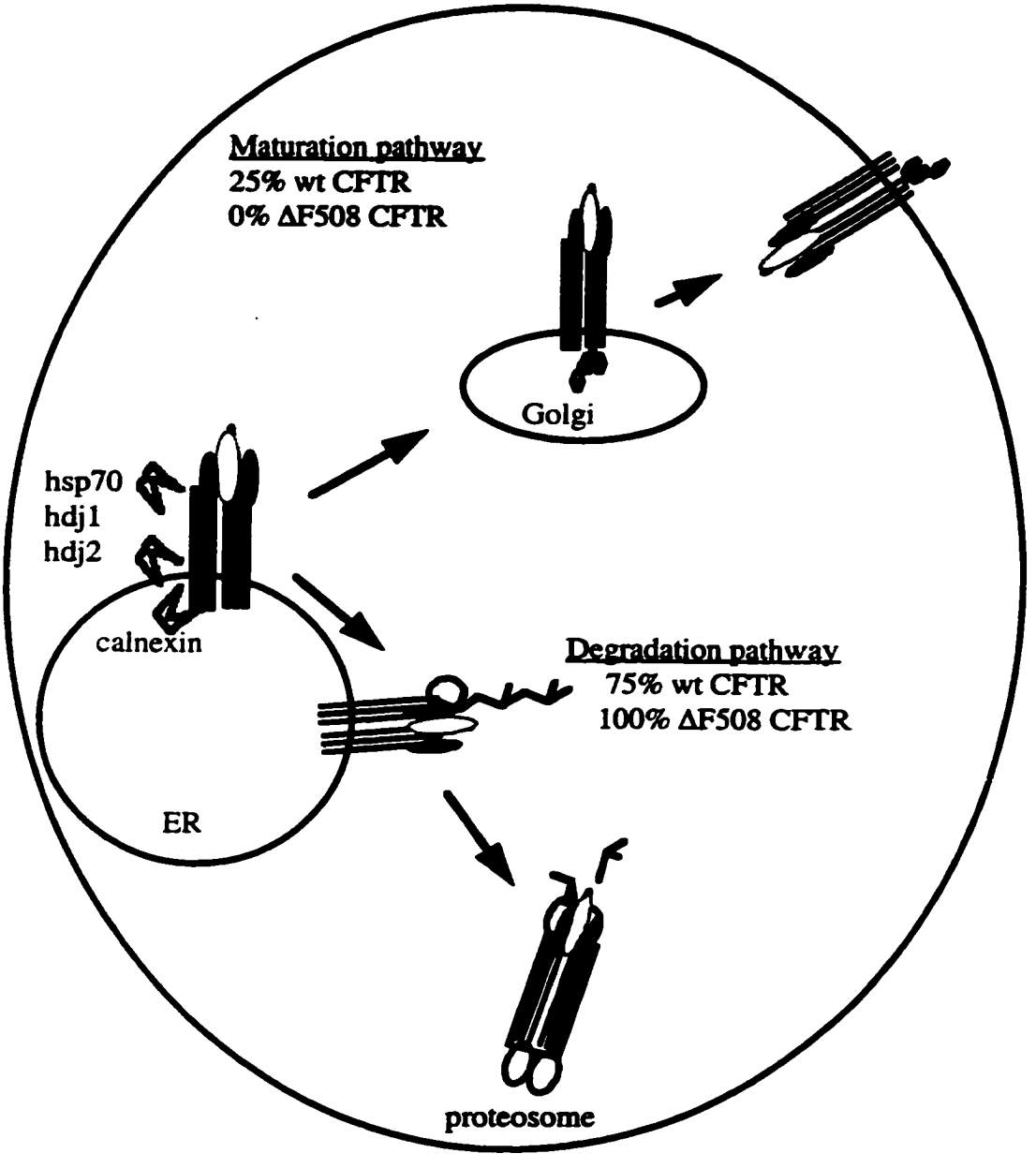


FIG. 2. CFTR maturation and degradation pathways.

protein in the ER but does not redirect the protein toward further maturation or rescue immature CFTR from ultimate degradation (Jensen *et al.*, 1995). Transfection of the dominant negative ubiquitin mutant, K48R, along with CFTR causes early termination of the ubiquitin side chains and results in an ER retained, partially ubiquitinated CFTR that is not a substrate for the proteasome (Ward *et al.*, 1995). However, as with proteasomal blockade, this intervention does not augment CFTR maturation. These studies suggest that an event occurs early in the ER, prior to ubiquitination, that routes CFTR to either the Golgi or the proteasome. Whereas most of the immature, core glycosylated CFTR is unstable in the cell, the portion of CFTR processed beyond the ER to the Golgi reaches a stable state with a half-life of 12-24 hr.

CFTR pore localization. CFTR regulates the conduction of ions across the epithelial membrane. The most direct means by which CFTR accomplishes this function is as an anion channel. CFTR has relatively small chloride conductance of approximately 7-10 pS with a linear I/V curve and an anion permeability sequence of $Br^- > Cl^- > I^-$ (Tabcharani *et al.*, 1990; Berger *et al.*, 1991; Anderson *et al.*, 1992). The two TMDs of CFTR each contain six predicted membrane spanning α-helices, and mutation studies of these helices have suggested that the TMDs comprise the CFTR pore. Mutations in M1, M5, and M6 have been shown to alter anion selection and binding properties (Dean *et al.*, 1990; Anderson *et al.*, 1991b; Sheppard *et al.*, 1993; Mansoura *et al.*, 1998). The CF mutation arginine to histidine at residue 117 (R117H), located near the external region of M2, was found to alter channel kinetics, indicating that this region at least lies close to the

pore. The hypothesis that the pore lies within TMD1 was also supported by the demonstration that TMD1 expressed alone forms a channel in oocytes with conductance properties similar to wild-type CFTR (Schwiebert *et al.*, 1998). Not all of TMD1 appears to be required for channel activity, however, since deletion of M1 through M4 still results in a CFTR protein with near normal conducting properties (Carroll *et al.*, 1995).

Activation. Regulated electrolyte movement is necessary for the proper functions of epithelial organs such as secretion of digestive enzymes by the pancreas. The chloride channel activity of CFTR is regulated by a two tiered mechanism unique within the ABC gene family (Berger *et al.*, 1991). First, phosphorylation of the R-domain primes CFTR, allowing ATP hydrolysis by the NBDs to gate the channel open (Tabcharani *et al.*, 1991; Nagel *et al.*, 1992). This sequence of activation has been well characterized and studies have demonstrated that channels will not open without first being phosphorylated, even in the presence of millimolar concentrations of MgATP (Anderson *et al.*, 1991a). After phosphorylation, channel activity is modulated by ATP until phosphates are removed from CFTR, inactivating the channel.

CFTR mutations leading to CF

Mutations. There are more than 800 known mutations in CF, and numerous other benign variations of CFTR have been detected (Tsui, 1999). CFTR defects resulting in CF can be classified based upon the molecular and physiological consequences of the mutation. Mutations are usually classified as types I through IV (Fig. 3). Class I mutations result in incomplete protein products from premature stop codons, frame shifts, or splice

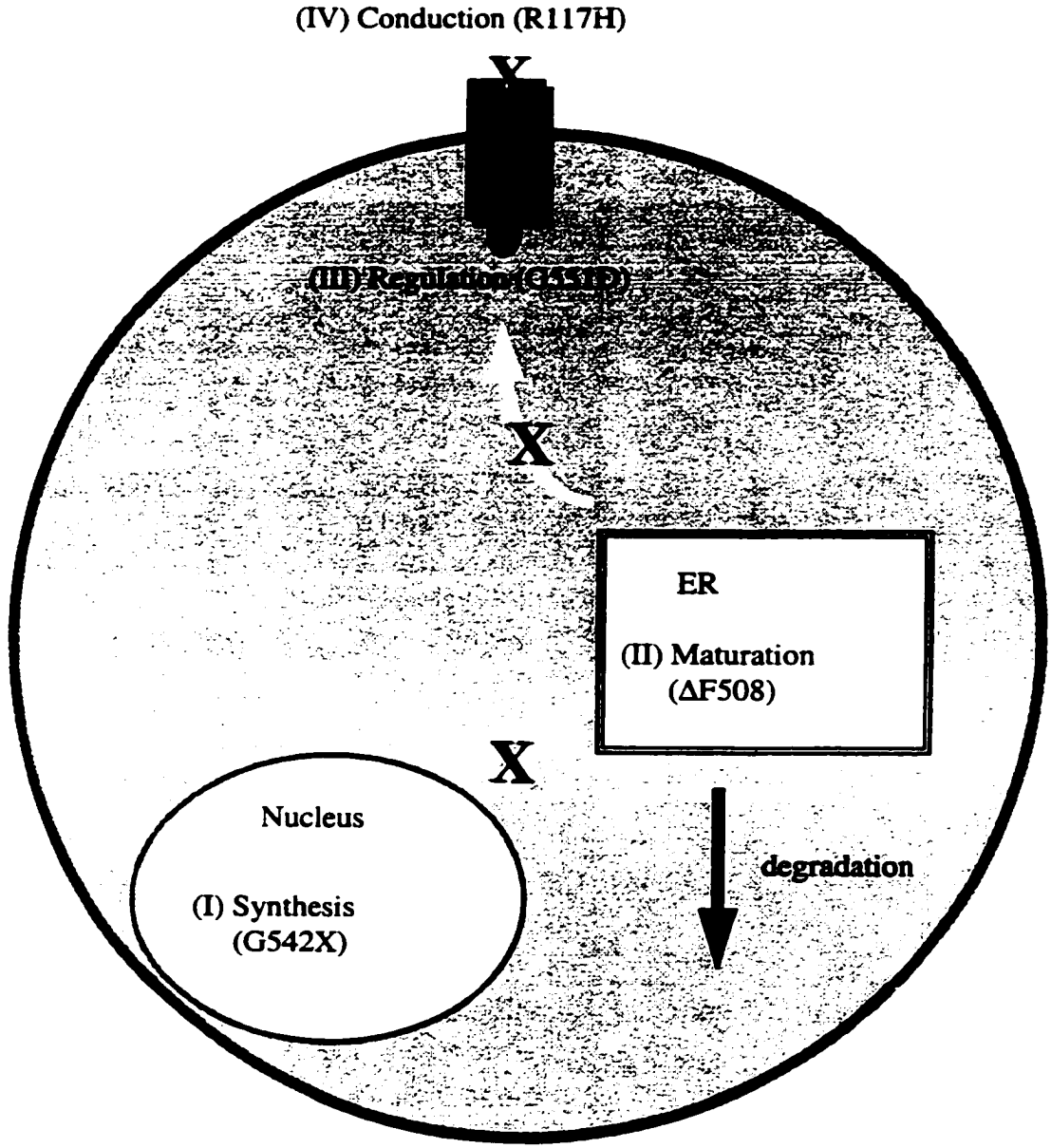


FIG. 3. Classes of CFTR mutations.

variations. The mRNA from some of these mutations (such as R553X) is unstable and minimal protein is expressed (Hammosh *et al.*, 1991). Class II mutations result from defective protein processing which prevents the protein from reaching the plasma membrane. Many mutations in this class occur within NBD1, including the common mutation ΔF508, from which three thymidine nucleotides have been deleted, resulting in the loss of a phenylalanine at position 508 (Riordan *et al.*, 1989). Two other mutations of this class, A455E and P574H, allow the processing of some protein to the membrane, although at levels well below wild-type CFTR (Sheppard *et al.*, 1995). The ability to process even small amounts of CFTR to the membrane results in a less severe phenotype, and most patients with these mutations are pancreatic sufficient (Kristidis *et al.*, 1992; Walker *et al.*, 1997). Class III mutations elicit defects in the regulation of channel function. G551D mutated CFTR reaches the cell membrane but is not easily activated (Li *et al.*, 1996). Class IV mutations such as R117H cause defective ion conduction. These mutations also reach the plasma membrane and appear to have normal regulation. However, once opened, CFTR channels with class IV mutations cannot conduct ions at the level of wild-type CFTR (Sheppard *et al.*, 1993).

ΔF508 mutation. The ΔF508 mutation accounts for approximately 70% of the mutated chromosomes in the CF population (Estivill *et al.*, 1997). ΔF508 is a class II mutation in which all, or nearly all, of the mutated protein targets the proteasome and does not proceed to the Golgi and plasma membrane. The possibility that detectable levels of ΔF508 CFTR may reach the cell surface in some tissues *in vivo* has been suggested recently but not yet confirmed by other laboratories (Kalin *et al.*, 1999). Unlike

wild-type CFTR that is detected as core and mature glycosylated protein bands on Western blots, ΔF508 CFTR is detected only as a single 140-kD (core glycosylated) protein (Cheng *et al.*, 1990). Conditions believed to promote proper protein folding have been demonstrated to increase the amount of ΔF508 CFTR reaching a mature state. For example, studies in which cells expressing the mutated protein were grown at low temperatures, 24-30° C, demonstrated partial correction of the ΔF508 processing defect and increased mutant protein at the plasma membrane (Denning *et al.*, 1992a; Lukacs *et al.*, 1993). These studies support the hypotheses that the ΔF508 protein is defective in folding and that lower temperatures either allow time for the protein to obtain a proper conformation prior to degradation or possibly interfere with chaperone interactions that target the mutant protein to the proteasome. Similar results were obtained when the "chemical chaperone" glycerol was used to stabilize folding intermediates. Glycerol treatment of cells expressing ΔF508 CFTR increased the amount of mutated protein reaching a mature state (Sato *et al.*, 1996). Other drugs such as sodium 4-phenylbutyrate (4PBA) and the organic solvent dimethyl sulfoxide (DMSO) have also been demonstrated to divert small amounts of the mutated CFTR to the plasma membrane and restore CFTR function (Rubenstein *et al.*, 1997; Bebok *et al.*, 1998). The mechanism of action of these compounds, however, is unknown and may not directly involve folding of the ΔF508 protein. Recombinant ΔF508 NBD1 expressed in bacteria had a greater tendency to aggregate than the wild-type NBD1 peptide, suggesting that ΔF508 may also mediate aberrant folding following prokaryotic expression and *in vitro* renaturation protocols (Ko *et al.*, 1993; Qu *et al.*, 1997a; King and Sorscher, 1998).

Molecular chaperones involved in the folding and processing of nascent proteins at the ER have been shown to associate with wild-type and ΔF508 CFTR. Calnexin binds both wild-type and ΔF508 proteins early in processing (Pind *et al.*, 1994). Wild-type CFTR, but not ΔF508, is able to escape the association with calnexin and continue along the maturational pathway. Yang *et al.* (1993) reported similar findings, demonstrating that the cytosolic ER associated chaperone, hsp70, bound immature wild-type and ΔF508 CFTR. The association of hsp70 with the wild-type protein was completed prior to CFTR maturation in the Golgi. Whether the continuous association of hsp70 is required for retention of CFTR in the ER has not been shown. Recently, Meacham *et al.* (1999) established that two members of the hsp40 co-chaperone family, hdj-1 and hdj-2, interact with CFTR. They also showed that hdj-2 binds pre-R-domain truncated CFTR proteins but not truncations immediately after the R-domain. These results suggest that different domains of CFTR fold with variable efficiency and that some may require chaperone involvement to acquire proper conformation. The study also indicates that processing of specific CFTR domains plays an important role in the overall maturation of CFTR and may help explain why certain domains are more susceptible to disease causing mutations.

Mutations that effect regulation. The majority of CF mutations resulting in defective CFTR at the plasma membrane are class III mutations that disrupt regulation. Most of these mutations occur within the NBDs and affect ATP binding or hydrolysis (Welsh *et al.*, 1995). The G551D mutation, detected in approximately 2.5% of CF chromosomes, disrupts ATP hydrolysis required for channel gating (Li *et al.*, 1996).

Since phosphorylation of the R-domain is required for channel opening, mutations within the R-domain may also result in class III defects. Vankeerberghen *et al.* (1998) studied non-PKA site mutations within the R-domain in CFTR expressed in oocytes. Missense mutations within the N-terminal portion of the R-domain (I601F, L610S, A613T, D614G, I618T, L619S, H620P, G628R, and L633P) resulted in processing defects, similar to the ΔF508 mutation (Vankeerberghen *et al.*, 1998). R-domain mutations that did reach the plasma membrane, however, resulted in abnormal channel activity. G622D, R792G, and E822K significantly lowered chloride channel activity, compared with wild-type CFTR. Interestingly, the CF mutation H620Q led to enhanced channel activity compared to wild-type (Wei *et al.*, 1998).

A naturally occurring splice variant of CFTR ending after the R-domain was discovered in normal kidneys and may suggest tissue-specific alternative functions of the protein (Morales *et al.*, 1996; Wilson, 1999). An artificial CFTR construct truncated after the R-domain, D836X, was also examined in HeLa cells (Sheppard *et al.*, 1994). While the resulting channel was observed rarely (and could not be detected by more macroscopic methods (Ostedgaard *et al.*, 1997), patch-clamp analysis suggested that conductive properties of D836X may be similar to those of wild-type CFTR. Regulation of the truncated protein, however, differed from that of full length CFTR. D836X did not require PKA stimulation to activate the channel, although PKA dependent phosphorylation further enhanced activity. The study suggested that D836X may form a dimer in order to achieve an active configuration, although formal evidence that the active molecule required two D836X polypeptides was not demonstrated.

Domain interactions within proteins

ABC proteins. Many prokaryotic members of the ABC gene family are translated as discrete polypeptides that self-assemble through multiple binding interactions to form a functional protein complex. In the prokaryotic histidine permease, two NBDs (HisP) bind to hydrophobic domains HisQ and HisM to form a membrane protein capable of histidine transport (Ames *et al.*, 1992). HisP was crystallized and the resolved structure formed an "L" shape in which the two arms of HisP are predicted to have different functions (Nikaido *et al.*, 1997). In the functional model, arm I comprises the ATP- binding pocket and arm II binds HisM and HisQ and transmits conformational changes to these domains of the protein complex. Mutations within arm II were demonstrated to disrupt the interaction between HisP and the transmembrane subunits (Liu *et al.*, 1999). Function -al consequences of domain binding interactions have also been shown for the prokaryotic maltose permease. MalK must bind the hydrophobic domain of the permease, MalF, in order to hydrolyse ATP (Panagiotidis *et al.*, 1993). The interaction between these two subunits was mapped to a putative helical domain near the N-terminus of MalK (Wilken *et al.*, 1996). Studies of the human P-gly have also identified domain interactions within the protein, including binding between the NBDs and TMDs (Loo and Clarke, 1995).

Ion channels. Domain interactions have been shown to be necessary for the proper function of certain ion channels. For example, functional CLC-0 chloride channels have been reconstituted by co-injection of cRNAs encoding the N-terminal transmembrane region along with the C-terminal portion thought to be cytosolic (Maduke *et al.*, 1998). Moreover, in certain polytopic integral membrane proteins, proper domain interactions within the ER may serve as a requirement for subsequent processing. The Na/K

ATPase has been shown to depend on an α-ß subunit interaction for processing beyond the ER (Geering *et al.*, 1996). Mutations within another ion channel, the Shaker K channel, that cause maturational arrest in the ER can be rescued by additional mutations in a separate transmembrane segment of the protein (Tiwari-Woodruff *et al.*, 1997). These results suggest that interactions between segments of the Shaker K channel occur within the ER and are necessary for normal protein maturation.

Intramolecular interactions involving CFTR domains. Intramolecular interactions within a protein are responsible for the final tertiary structure. Since conformational changes are often necessary for normal protein function, these structural interactions must be dynamic. For example, the Shaker K channel contains an intracellular amino terminal region acting like a ball and chain to block the pore (Hoshi *et al.*, 1990; Heinemann *et al.*, 1994). Voltage changes across the membrane shift the position of the inhibitory domain and allow the channel to conduct potassium (Demo and Yellen, 1991). The R-domain of CFTR may function in a manner similar to the N-terminal region of the Shaker channel and block the CFTR pore. Although CFTR is not voltage dependent, charge changes in the amino acids of the R-domain have prominent effects on channel activity. For example, when serine residues within PKA sites of the R-domain are mutated to aspartic acids, the channel is maintained in an open conformation (Rich *et al.*, 1993a). However, recent studies have indicated that the mechanism of R-domain action is more complex than a simple "ball and chain." The R-domain may also have a positive regulatory role in CFTR Cl^- channel function.

CFTR is expressed as a single polypeptide. However, the protein is composed of domains similar to those of other ABC proteins that contain multiple subunits. Previous studies have investigated interactions between the TMDs of CFTR. Ostedgaard *et al.* (1997) demonstrated a strong association between these regions, leading to functional reconstitution of separately expressed halves of CFTR. While binding between the TMDs was detected, binding interactions among cytosolic domains of the protein (including the R-domain) were not demonstrated.

CFTR activation requires both the phosphorylation of the R-domain and ATP binding by the NBDs. However, the precise relationship between the R-domain and the NBDs in CFTR function is not known. Neville *et al.* (1998) demonstrated that the binding of ATP to a recombinant NBD1-R-domain fusion peptide inhibits R-domain phosphorylation. Interestingly, another study demonstrated that phosphorylation of the R-domain also enhanced NBD1 sensitivity to ATP and increased the rate of channel opening (Winter and Welsh, 1997). Taken together, these reports may suggest a negative feedback mechanism between the R-domain and NBD1, where phosphorylation of the R-domain promotes ATP hydrolysis by NBD1, which, in turn, lowers R-domain susceptibility to further phosphorylation. Recently, the R-domain has been shown to bind to a cytosolic domain not previously thought to be involved with CFTR regulation, the amino terminal tail (Naren *et al.*, 1999). Binding of the R-domain to the N-terminal tail stabilizes CFTR in an open state. Mutations that disrupt the interactions between the R-domain and N-terminal tail also reduce the open time of the channel. These studies signify the emerging importance of domain interactions within CFTR in achieving proper function and regulation.

R-domain function and regulation

R-domain. Although the R-domain has been defined as a single, discrete domain derived from CFTR exon 13, it has been suggested that the domain might be divided into two functionally distinct subregions, RD1 (amino acids 587-672) and RD2 (amino acids 679-798) (Dulhanty and Riordan, 1994b). While relatively little homology exists between distal portions of the R-domains in CFTRs from different species, the amino third of the R-domain is highly conserved (Dulhanty and Riordan, 1994b; Pasyk *et al.*, 1998). Comparisons of CFTRs across species also demonstrate that practically all PKA and PKC sites are conserved, including those in the less conserved carboxy region of the R-domain (Diamond *et al.*, 1991).

R-domain as a channel blocker. The R-domain of CFTR is responsible for basal inhibition of the chloride channel. Studies of exogenous R-domain provided evidence that CFTR channel activity is directly controlled by this portion of CFTR. The addition of recombinant, unphosphorylated R-domain to wild-type CFTR was able to inhibit channel activity in both planar lipid bilayers and inside-out patch-clamp experiments (Ma *et al.*, 1996; Winter and Welsh, 1997). Inhibition from the exogenous R-domain was also shown to be phosphorylation dependent, since phosphorylated R-domain did not block CFTR channel activity.

Lipid bilayer experiments have been performed to examine the ability of regulatory subdomains to block CFTR channel activity (Tasch *et al.*, 1999). While exogenous, full length R-domain peptide completely blocks the channel, smaller subdomain peptides either fail to block or result in a slower, partial block of CFTR. Peptides comprised of

amino acids 588-805 or 672-855 inhibited CFTR channels, although the inhibition took longer to achieve than with full length R-domain, and the channel block was not complete. Smaller R-domain peptides, amino acids 588-746 and 672-805, were unable to confer any block.

PKA phosphorylation and regulation. CFTR is activated by kinases that phosphorylate several sites in CFTR. PKA is the predominant phosphorylating agent of CFTR. CFTR contains at least 10 PKA sites which (with the exception of serine 422) all reside within the R-domain (Fig. 4). Five of the PKA sites have been shown to augment function *in vivo* (serines at 660, 700, 737, 795, and 813, Cheng *et al.*, 1991). The prediction of five active PKA sites correlates with *in vitro* studies demonstrating that approximately 5 moles of phosphate per mole of CFTR are incorporated after PKA stimulation (Picciotto *et al.*, 1992). Removal of individual PKA sites (by replacement of serines with alanines) had little effect on PKA activation of CFTR (Chang *et al.*, 1993). These results have been interpreted to imply functional redundancy in CFTR PKA sites and that phosphorylation of individual sites may not be as important as the cumulative phosphorylation of the R-domain. Recent studies, however, measuring the amount of cAMP dependent stimulation needed to activate CFTR missing individual PKA sites have demonstrated that removal of certain PKA sites renders the protein less active than others. CFTR in which serine 813 was replaced with alanine required higher concentrations of the phosphodiesterase inhibitor 3-isobutyl 1-methyl xanthine (IBMX) to activate than did CFTRs missing serines 670 or 700 (Wilkinson *et al.*, 1997). The study ranked PKA targeted serines in the order of importance to activation as 813 > 660, 795 > 670,

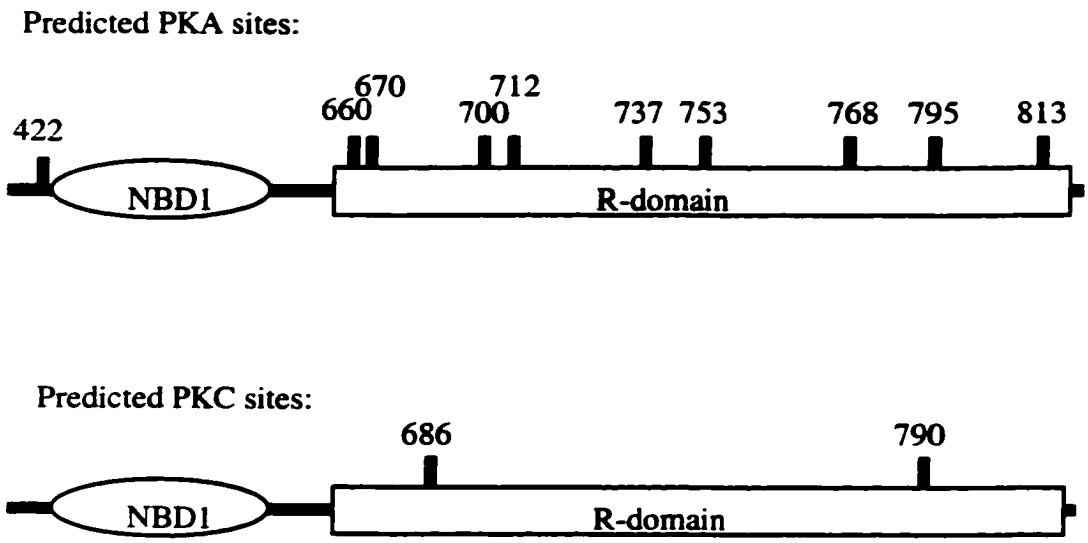


FIG. 4. PKA and PKC phosphorylation sites.

700. Interestingly, two of the serines that were modified, 737 and 768, resulted in a CFTR that was activated more readily than wild-type, suggesting that phosphorylation at these serines may actually inhibit channel activity. The existence of both inhibitory and stimulatory PKA sites indicates that phosphorylation dependent CFTR activation is complex and that other factors such as specific phosphatases might also regulate the overall channel activity of the protein.

Other kinases. While PKA appears to be the prominent kinase activating CFTR, other kinases may also a play role in regulating the channel. For example PKC is able to phosphorylate CFTR at a stoichiometry of approximately 2 moles of phosphate per mole of CFTR (Picciotto *et al.*, 1992). PKC phosphorylates two PKC sites (serine residues 686, 790) and also several PKA sites, including serines 660 and 700. While stimulation of PKC with phorbol esters has been demonstrated to activate CFTR in intact cells (Dechecchi *et al.*, 1993) and in excised membrane patches (Berger *et al.*, 1993; Fischer *et al.*, 1998), it remains unclear whether PKC activates CFTR directly or mediates CFTR stimulation by enhancing the sensitivity of CFTR to PKA. PKC phosphorylation leads to enhanced PKA dependent CFTR activity, indicating that the kinases may act in concert (Chang *et al.*, 1993). The interdependence between PKA and PKC was further demonstrated when Jia *et al.* (1997) showed that inhibition of PKC eliminated PKA activation of CFTR. The authors speculated that constitutive phosphorylation of CFTR by PKC is required for PKA dependent CFTR activation. Another study demonstrated that the isotype of PKC that selectively regulated CFTR, PKC-epsilon, was constitutively active in cells (Liedtke and Cole, 1998). Interestingly, in some systems, PKC-induced

stimulation of CFTR channels can be prevented by inhibition of endogenous basal PKA activity (Yamazaki *et al.*, 1999).

Membrane associated cGMP dependent protein kinase (cGK) type II, found strictly in enterocytes, phosphorylates CFTR as readily as PKA; however, the involvement of cGK in CFTR regulation is uncertain (Picciotto *et al.*, 1992; Berger *et al.*, 1993; Vaandrager *et al.*, 1998).

Phosphatases. When PKA is removed from the bathing solution in patch-clamp studies, CFTR activity decreases (termed "rundown") even in the presence of MgATP (Cliff *et al.*, 1992). Phosphatase inhibitors can slow the rundown of CFTR channel activity and reduce dephosphorylation of the protein, indicating that regulation of CFTR is also under control of phosphatases (Tabcharani *et al.*, 1991; Becq *et al.*, 1993; Berger *et al.*, 1993). The specific phosphatases that influence CFTR are still under investigation, and several recent reports have led to contradictory conclusions. For example, Fisher *et al.* (1998) demonstrated that the addition of exogenous protein phosphatase 2B (PP2B) to patches from 3T3 and Calu-3 cells inactivated CFTR activity. However, other studies have shown that PP2B had little effect, while the addition of PP2A, PP2C, or alkaline phosphatase was able to block channel activity (Luo *et al.*, 1998). Inhibitors of alkaline phosphatase were also shown to slow the rundown of CFTR in excised patches and reduce protein dephosphorylation in isolated membranes (Becq *et al.*, 1994). The phosphatase inhibitors okadic acid, calyculin A, cyclosporin A, and FK506 did not prevent CFTR rundown in airway or intestinal epithelia, providing strong evidence that PP1, PP2A, and PP2B are not the phosphatases regulating CFTR in these tissues (Travis *et al.*,

1997; Fischer *et al.*, 1998). While each of the above phosphatases elicits CFTR rundown, the time course of deactivation differs from that seen with phosphatases endogenous to epithelial cells. PP2C, however, was demonstrated to inactivate CFTR with a time course similar to that observed in epithelial cells (Luo *et al.*, 1998). In addition, PP2Ca was found to be expressed in airway epithelia, and co expression of PP2Ca with CFTR reduced CFTR chloride current and increased the rate of channel inactivation (Travis *et al.*, 1997). These results indicate that several phosphatases, including alkaline phosphatase, can dephosphorylate the CFTR protein in cell free systems. In tissues, PP2C may regulate CFTR; however, the identity of endogenous phosphatases that modulate CFTR activity is still uncertain.

Interactions of CFTR with other regulatory proteins. Cellular proteins involved in trafficking and retention of membrane proteins at the plasma membrane have also been demonstrated to interact with CFTR. The PDZ1 domain of an Na/H exchange regulatory factor binds the C-terminal tail of CFTR and possibly participates in regulation of the channel (Hall *et al.*, 1998; Wang *et al.*, 1998). The last three amino acids of CFTR (T-R-L) comprise a PDZ interacting domain. Removal of these residues disrupts normal targeting of CFTR from the apical membrane and confers CFTR distribution to more lateral membranes (Moyer *et al.*, 1999). Syntaxin, a protein involved in docking and fusion events at the membrane, has also been demonstrated to interact with CFTR and may act as a direct modulator of CFTR function (Naren *et al.*, 1997).

R-domain biochemistry

R-domain biochemistry and conformation. Phosphorylation increases the negative charge of the R-domain and activates the CFTR channel. Phosphorylation of recombinant R-domain peptide (amino acids 645-835) by PKA in cell free systems elicits a shift in the peptide to a higher apparent molecular weight on SDS-PAGE (Picciotto *et al.*, 1992). The change in peptide mobility does not result from the addition of charge, since negative charges would increase, not decrease, mobility on SDS-PAGE. Therefore, these earlier experiments indicate that phosphorylation alters the conformation of the peptide, resulting in slower mobility. A conformational change in recombinant R-domain by phosphorylation was also demonstrated by circular dichroism (CD) spectra (Dulhanty and Riordan, 1994a). PKA dependent phosphorylation changed recombinant R-domain to a less α-helical structure. A similar conformation for recombinant R-domain was observed by CD spectra when the serines within the PKA sites of the R-domain were changed to glutamic acids (to add negative charge directly to the PKA phosphorylation sites) (Dulhanty *et al.*, 1995). PKC was also able to phosphorylate purified recombinant R-domain but had no effect on either the CD spectrum (Dulhanty and Riordan, 1994a) or mobility on SDS-PAGE (Picciotto *et al.*, 1992).

Further evidence that conformational changes in the R-domain effect CFTR function (irrespective of charge alterations) was demonstrated by Cotton and Welsh (1997). Covalent modification of cysteine 832 in the C-terminal portion of the R-domain by the sulfhydryl reagent NEM rapidly and irreversibly augmented CFTR activity following initial PKA activation. These studies demonstrated that conformational changes

(in the absence of charge manipulation) can modify the ability of the R-domain to augment CFTR activity.

Effects of R-domain deletion. The R-domain contains a large number of charged residues and kinase sites that have the potential to incorporate additional negative charge into the domain. Most PKA sites reside in the distal portion of the R-domain in a subdomain defined as RD2 (Dulhanty and Riordan, 1994b). Studies in which all or part of the R-domain was deleted have demonstrated that not all portions of the R-domain are necessary for the channel activity of CFTR. Deletion of amino acids 708-835 (ΔR-CFTR, missing three major PKA sites) led to PKA independent activity, indicating that this region of the R-domain encodes residues necessary for PKA dependent activation (Rich *et al.*, 1991, 1993b). Removal of additional portions of the R-domain (*i.e.*, amino acids 681-835) eliminated all activity, suggesting that proximal portions of the R-domain (amino acids 681-708) are necessary for either proper protein processing or chloride channel activity.

Trans expression of the R-domain. Recent reports have suggested that the mechanism by which the R-domain regulates CFTR is complex and may not simply involve obstructing the pore in the absence of phosphorylation. For example, while unphosphorylated exogenous R-domain was able to inhibit wild-type CFTR, the addition of unphosphorylated R-domain to ΔR-CFTR did not influence channel activity (Ma *et al.*, 1997). Moreover, phosphorylation of the exogenous R-domain increased the activity of ΔR-CFTR, but not that of wild-type CFTR. The addition of phosphorylated R-domain

was also shown to enhance the sensitivity of ΔR-CFTR to ATP and increase the rate of channel opening (Winter and Welsh, 1997). These results indicate that the R-domain may not solely act as an inhibitor of the channel but may also interact with other domains of CFTR to enhance activity.

Goals of this dissertation.

Understanding domain interactions within ABC proteins may help elucidate proper folding pathways, maturational and targeting signals, and functional dynamics of this gene family. Since the discovery of CFTR in 1989, substantial research has led to a detailed understanding of structure and functional correlations between the primary amino acid sequence of CFTR and the chloride channel activity. Comparatively less is known about the tertiary interactions of CFTR and, in particular, the ways in which domain interactions may influence important CFTR properties such as phosphorylation, maturational processing, and the mechanism underlying common mutations such as ΔF508. In analogy to other membrane proteins, including ion channels, disruption of normal CFTR processing and/or function could result if CFTR domains do not properly interact. It is also possible that certain disease associated mutations mediate their effects by disrupting CFTR intramolecular binding. In this dissertation, domain interactions within CFTR that may be involved in processing, function, and regulation of channel activity were investigated. We also studied regulatory subdomains necessary for basal inhibition and PKA dependent activation of CFTR, including the structural and functional consequences of subdomain phosphorylation. Because it is also possible that certain disease associated mutations mediate their effects by disrupting CFTR intramolecular binding, we tested the

ability of a common mutation, ΔF508, to interfere with domain interactions within CFTR. The particular questions addressed in this dissertation include the following: (a) Does CFTR severed at residue 836 reassemble in cells due to the ability of domains to locate and bind one another? (b) Do binding interactions occur early enough in biogenesis (*i.e.*, in the ER membrane) to influence early steps in CFTR synthesis? (c) Do the binding interactions between proximal and distal portions of CFTR influence the regulation and/or phosphorylation of CFTR? (d) What subdomains of the R-domain are necessary for this regulation? (e) Do isolated R-domain and NBD1 interact with distal domains? (f) What is the effect of the ΔF508 mutation on these domain interactions?

METHODS AND MATERIALS

Plasmid construction

All plasmids used in these experiments were based on pTM1, a plasmid in which the T7 RNA polymerase promoter drives expression of inserted genes. The pTM-CFTR has been previously described (Walker *et al.*, 1997). pTM-M837X was produced by amplifying a segment of CFTR with a primer encoding nucleotides 1562 to 1585 (including the unique SphI restriction site) and a reverse primer containing nucleotides 2495 to 2529 (including a stop codon at 2515, followed by a StuI site). The resulting product was inserted between the SphI and StuI sites of pTM-CFTR in order to place a premature stop codon in place of CFTR amino acid 837. pTM-G723X was constructed using a similar strategy but with a stop codon in place of the glycine at amino acid 723. pTM-R-domain was obtained using a PCR product to engineer a start site at methionine 596 and the stop site from pTM-M837X (leading to a vector expressing the R-domain protein, *i.e.*, amino acids 596 to 837).

pTM-Δ1-836 was cloned with PCR amplification by inserting an NcoI site that maintained the methionine at amino acid 836. The reverse primer (from CFTR 2925 to 2955) included the AccI site in CFTR, followed by an engineered SpeI site. The NcoI to SpeI PCR product was then ligated into pTM1. This new plasmid was opened with AccI, and a fragment of CFTR (from nucleotide 2935 to beyond the stop codon) was inserted to produce pTM-Δ1-836, a plasmid encoding all of CFTR after residue 837.

PTM-Δ1 595 was engineered by replacing the KpnI to HpaI fragment from pTM-CFTR with the equivalent fragment from pTM-R-domain and thereby producing a peptide from methionine 596 to the end of CFTR. Δ1-1377 was produced using PCR products that replaced a proline residue at 1377 with a methionine, and the resultant PCR product was ligated into pTM1. pTM-NBD1 and pTM-ΔF508-NBD were engineered using PCR by insertion of an NcoI site encoding a methionine at CFTR position 432 and a stop at 587. The ΔF508-M837X plasmid was produced by inserting the XbaI to SphI fragments from pTM-ΔF508-CFTR into pTM-M837X. All constructs were verified by restriction endonuclease digestion and by nucleotide sequence analysis of key mutations and PCR generated segments used in ligations. pCDNA plamids, also encoding a T7 polymerase promoter to express R-domain fragments 595-813, 595-740, and 708-835, were a generous gift from Drs. Kevin Kirk and A. P. Naren. See Fig. 5 for a pictorial representation of the constructs examined in this dissertation.

Fluorescence measurements of anion permeability

COS7 cells were grown in Dulbecco's modified Eagles medium (DMEM) plus 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were seeded onto glass cover-slips. When the cells reached 50-70% confluence, medium was removed and vaccinia virus encoding the T7 polymerase (vTF7.3) was added at a multiplicity of infection (MOI) of 5-10 in Opti-MEM (GIBCO BRL) (Walker *et al.*, 1997; Clancy *et al.*, 1998). One hour later, 5 ug of pTM1 plasmid expressing all or part of CFTR was mixed with 20 ug 1:1 DOTAP/DOPE (Avanti Polar Lipids, Birmingham, AL) in Opti-MEM and added to the cells. After 4 hr, the medium was replaced with fresh DMEM/10% FBS. Twelve to

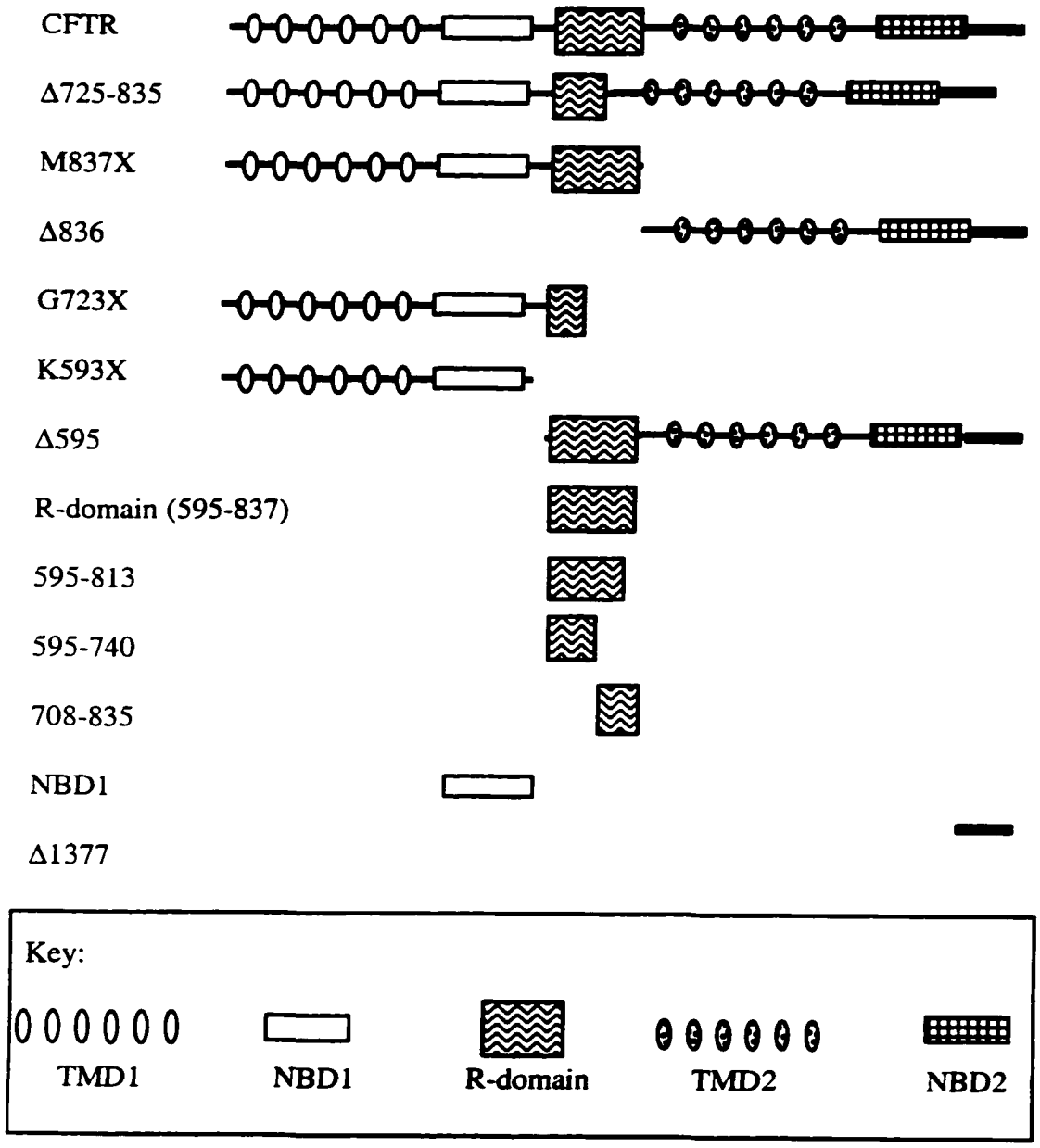


FIG. 5. CFTR truncations and domains used in these experiments.

16 hr posttransfection, the coverslips were washed with phosphate-buffer saline (PBS) and loaded with 10 mM 6-methoxy-N-(3-sulfopropyl)quinolonium (SPQ), a halide sensitive, nonpermeable fluorescent dye, by hypotonic shock (50% Opti-MEM, 50% water for 10 min as previously described) (Clancy *et al.*, 1998. Fig. 6). The cells were then incubated for 5 min in an iodide solution (buffer A: 130 mM NaI, 4 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $Mg(NO_3)_2$, 10 mM glucose, 20 mM HEPES) that quenches SPQ fluorescence. Coverslips were mounted on a fluorescence microscope and individual cellular fluorescence was monitored every 20 sec. After 3 min, the iodide quenching buffer was replaced by a nitrate dequenching buffer (buffer B: 130 mM $NaNO_3$ replacing the NaI in buffer A). At 8 min, buffer B plus 20 μM forskolin/100 μM IBMX was added to activate PKA in the cells. After 13 min, buffer A was returned. To test the effect of kinase inhibition on constitutive activity, cells on coverslips were pretreated with 1 μM staurosporine (Calbiochem) in Opti-Mem for 30 min and then loaded with SPQ in hypotonic Opti-Mem plus 1 μM staurosporine. Cellular fluorescence was measured as above, except that no PKA agonists were added in the experiment.

To test the effect of PKA inhibition, the above protocol was modified so that measurements of fluorescence were limited to the last quenching step. Shortening the protocol was performed since (a) the final quenching step was found to be the most sensitive indicator of halide permeability and (b) limiting the duration of the experiment minimized washout of the inhibitor. Cells on coverslips were loaded with SPQ (10 mM) in non hypotonic Opti-Mem for 4 hr. In some experiments, 100 μM Rp-8-CPT-cAMPS (Gjertsen *et al.*, 1995, Biolog, La Jolla, CA), a type I specific kinase inhibitor, was added

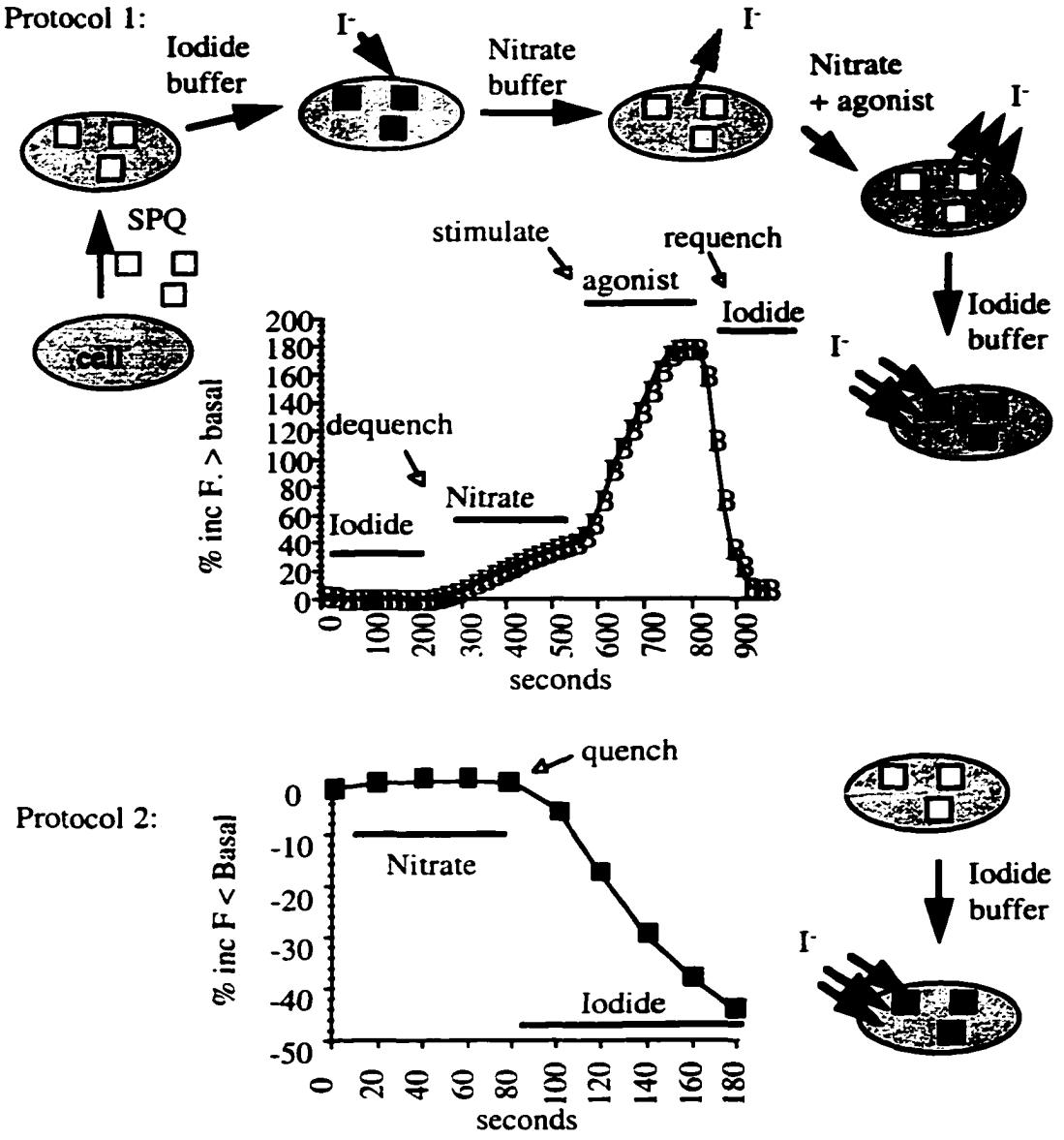


FIG. 6. Halide permeability assay. Protocol 1: Cells with SPQ are bathed in iodide to quench SPQ fluorescence, switched to a nitrate buffer to elicit iodide efflux (dequench), and then switched to nitrate + agonist to open CFTR (stimulate). In the final step, iodide is restored to allow cellular uptake to quench SPQ fluorescence (requench). Protocol 2: Cells with SPQ bathed in nitrate are switched to iodide to monitor basal halide influx (quench).

to the Opti-Mem 45 min prior to fluorescence measurements. Cells were placed in buffer B (see above) and the coverslips mounted on a fluorescence microscope. Measurements of cellular fluorescence were taken every 20 sec. After approximately 1 min, buffer B was replaced with buffer A (see above).

Immunoprecipitation

COS7 cells were seeded into 6-well plates. When 50-70% confluent, the cells were infected and then transfected as described above. After 24 hr, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 1% NP-40, 0.5% deoxycholic acid [DOC], 0.1% SDS) or PBS + 0.5% triton X-100 for at least 20 min. Both buffers included the protease inhibitor cocktail, Complete (Boehringer Mannheim). The lysates were then spun 5 min at 14,000 rpm to remove debris, and the soluble fraction was incubated with Protein G or A agarose for 1 hr to remove nonspecific proteins binding to the beads. The precleared lysates were added to protein A beads plus a polyclonal (rabbit) antibody specific to CFTR NBD1 (Walker *et al.*, 1997) or to protein G beads plus a monoclonal antibody specific to either the C-terminal tail or the regulatory domain of CFTR (Genzyme Corporation, Cambridge, MA). Three hours later, beads were pelleted and washed three times in RIPA buffer (5 min per wash). Protein sample buffer containing SDS was added; the beads were incubated at 50° C for 15 min; and the eluted proteins were studied by 8%, 10%, or 16% SDS-PAGE. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and the membrane was blocked with 3% dry milk in PBS for 20 min, stained with primary antibody for 2-4 hr, and then washed three times with PBS containing 0.1% Tween-20. An alkaline phosphatase conjugated secondary antibody

(Southern Research Inc., Birmingham, AL) was administered for 1 hr. The blot was then washed three times with PBS + 0.1% Tween-20 and developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) in carbonate buffer (pH 8.8) according to manufacturer's protocol (Boehringer Mannheim). In some experiments, cells were treated with 20 μM forskolin (Calbiochem) 20 min prior to lysis to stimulate PKA activity in the cells. Removal of phosphates from immunoprecipitated proteins was performed by treatment with alkaline phosphatase (Sigma) for 1 hr at 37° C prior to SDS-PAGE. In other studies, 100 U of endoglycosidase H (Endo H, New England Biolabs) was administered to immobilized proteins for 1 hr at 50° C to remove specific glycan chains from the proteins.

To test the effect of kinase inhibition, cells were pretreated with inhibitors prior to immunoprecipitation (as described above). Cells were treated with 1 μM staurosporine (kinase inhibitor) for 30 min, 100 μM Rp-8-CPT-cAMPS (PKA specific inhibitor), 5 μM chelerythrine (PKC specific inhibitor), or 1 μM bisindolylmaleimide I (Bin-1, PKC specific inhibitor, Calbiochem) for 45 min. In some experiments, 20 μM forskolin was administered 10 min prior to lysing for immunoprecipitation.

In vitro phosphorylation

COS7 cells were seeded into 6-well plates. When 50-70% confluent, the cells were infected/transfected as described above. After the final wash step in the immunoprecipitation procedure described above, the purified proteins bound to beads were washed once in PKA buffer (50 mM Tris, 1 mM, 0.1 mg/ml BSA, pH 7.5). The proteins were then

incubated with 0.5 µl PKA (1-3 mg/ml, Promega) and 10 µCi gamma ^{32}P-ATP in 100 µl of PKA buffer at 30° C. After 45 min, the bead/protein complexes were washed twice in PBS and heated in 20 µl sample buffer, and the proteins were separated by 8% SDS-PAGE. The gel was dried and the radioactive protein bands visualized using phosphoimagery. The radioactivity of the proteins was quantified using IP Lab software.

RESULTS

CFTR expressed as two separate halves maintains activity

When CFTR truncated immediately after the regulatory domain (M837X, Fig. 5) was coexpressed with the remaining portion of the protein (Δ1-836, Fig. 5), enhanced halide permeability was observed as assayed using the halide sensitive dye SPQ (Fig. 7). This increased permeability was noted only when both M837X and Δ1-836 were coexpressed and not when either fragment was expressed alone. The halide efflux recorded from cells coexpressing M837X and Δ1-836 differed from that of cells expressing full length CFTR. While wild-type CFTR responded to PKA stimulation (e.g., by adenylate cyclase activation through forskolin) with an increase in anion transport, cells coexpressing M837X and Δ1-836 had high basal halide permeability that did not require forskolin. The activity recorded from cells expressing M837X plus Δ1-836 was similar to that reported for an R-domain deleted CFTR (ΔR-CFTR). To establish that in our assay a ΔR-CFTR construct also produced highly constitutive activity, we expressed CFTR missing amino acids 725-835 (Δ725-835 CFTR, Fig. 5) in COS7 cells and assayed halide movement by SPQ fluorescence. High constitutive activity was observed in cells expressing Δ725-835 CFTR similar to that seen with coexpression of M837X and Δ1-836 (Fig. 8).

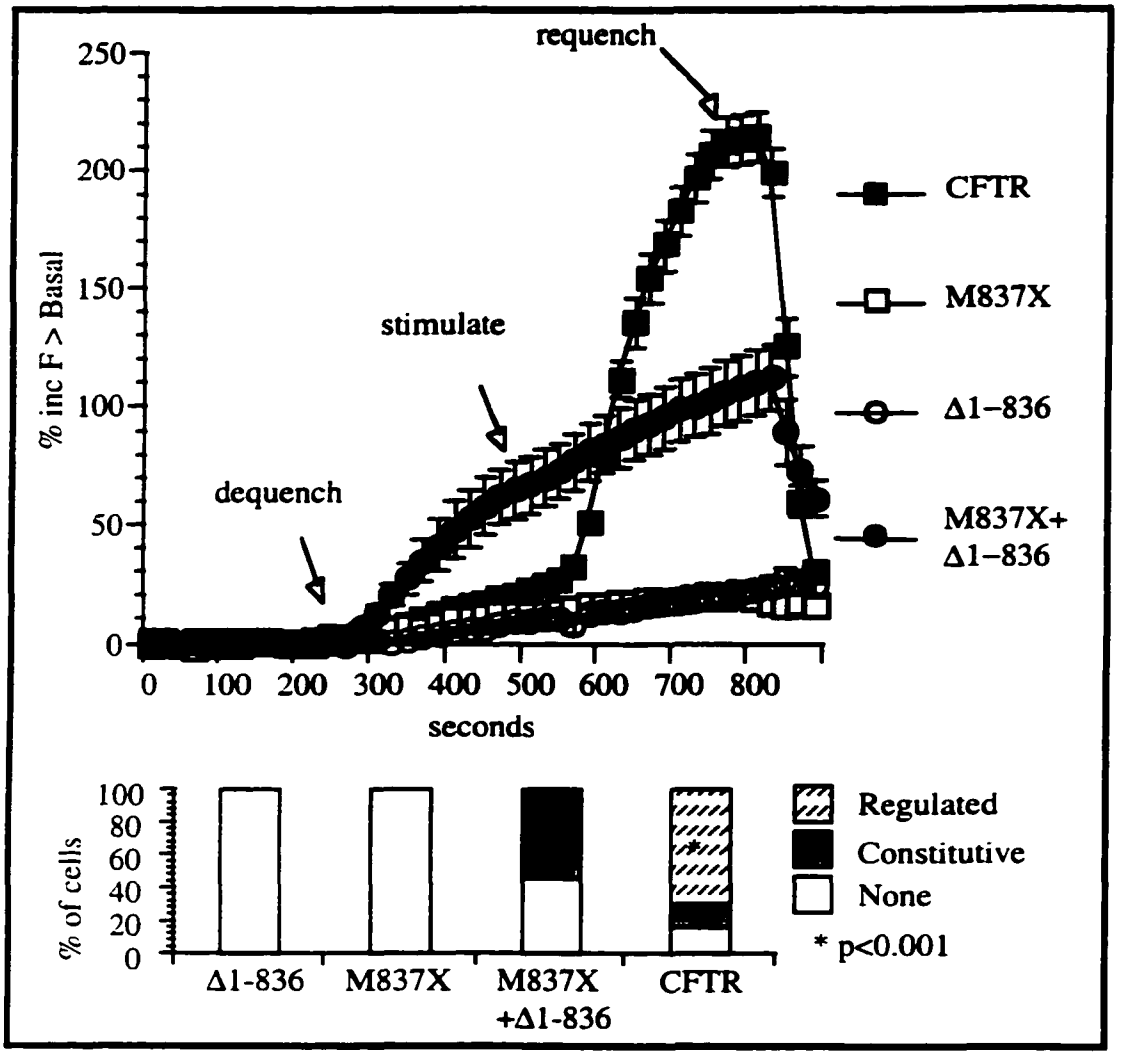


FIG. 7. M837X plus Δ1-836 produces basal halide efflux. CFTR , M837X, Δ1-836, or M837X+Δ1-836 was expressed and halide efflux assayed using the halide sensitive fluorescent dye, SPQ. The cells began in an iodide buffer. Then, at 200 sec, the buffer was replaced with nitrate buffer (dequench); at 500 sec, forskolin and IBMX were added (stimulate); and at 800 sec, iodide buffer was returned (requench). Enhanced halide efflux was detected in cells coexpressing M837X+Δ1-836 (upon dequench, solid circles, $p < 0.001$) and expressing CFTR (upon stimulation, solid squares, $p < 0.001$). No enhanced halide movement was detected when either M837X (open squares) or Δ1-836 (open circles) was expressed alone. %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 100 and 200 sec). Error bars = SEM. N >250 cells per condition. The bar graph shows the percentage of cells producing regulated, constitutive, or no activity. Positive activity = 100% increase in the rate of fluorescence from baseline to 300-400 sec (constitutive) or to 600-700 sec (regulated).

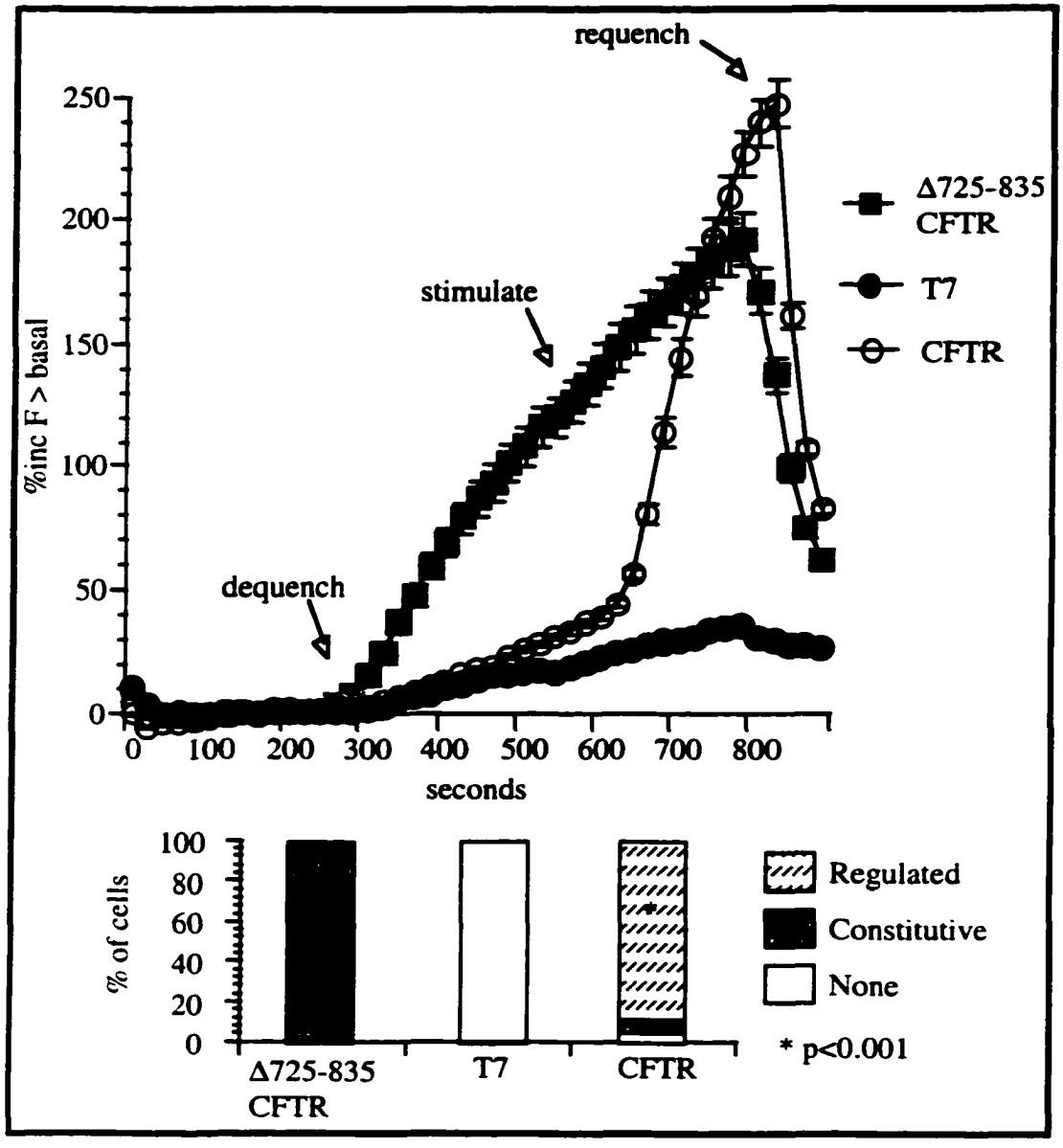


FIG. 8. CFTR missing the R-domain also produces constitutive activity. CFTR, Δ725-835 CFTR, or T7 alone was expressed in cells and halide efflux assayed as in Fig. 7. Enhanced halide efflux was detected in cells expressing Δ725-835 CFTR (upon dequench, solid squares, $p < 0.001$) and in cells expressing of CFTR (upon stimulation, open circles, $p < 0.001$). %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 100 and 200 sec). Error bars = SEM. N >100 cells studied per condition. The bar graph shows the percentage of cells producing regulated activity (responding to forskolin), constitutive activity, or no activity. Positive activity = 100% increase in the rate of fluorescence from baseline to 300-400 sec (constitutive) or to 600-700 sec (regulated).

CFTR halves bind one another early in processing.

The functional complementation of the two halves of CFTR suggested a physical interaction between the fragments. To investigate the association of M837X and Δ1-836, coimmunoprecipitation studies were performed using antibodies specific to either NBD1 (which recognizes M837X) or the C-terminal tail (which recognizes Δ1-836). When Δ1-836 was expressed alone in cells and immunoprecipitated from cellular lysates with the C-terminal antibody, Δ1-836 could be detected on Western blots (Fig. 9A, lane 2). However, when the antibody specific to NBD1 was used for immunoprecipitation, Δ1-836 could not be detected (Fig. 9A, lane 1). When Δ1-836 was coexpressed with M837X, Δ1-836 could be immunoprecipitated with the antibody specific to NBD1, even under stringent conditions (RIPA buffer with 0.5% SDS, Fig. 9A, lane 3). A control protein, ß-galactosidase (ß-gal) did not coimmunoprecipitate with Δ1-836 (Fig. 9A, lane 6). These results demonstrate that M837X physically binds Δ1-836. Immunoprecipitated Δ1-836 was observed as two bands (Fig. 9B, lane 1). Since the glycosylation sites for CFTR (amino acids 894 and 900) are contained within Δ1-836, we verified that glycosylation was the cause of the higher molecular weight form of Δ1-836. Treatment of purified Δ1-836 with Endo H eliminated the higher molecular weight band but did not affect the lower form, demonstrating that the higher molecular weight form was glycosylated (Fig. 9B, lane 2). Interestingly, both the unglycosylated and glycosylated forms of Δ1-836 coimmunoprecipitated with M837X, indicating that the interaction between the two proteins occurred early in processing (*i.e.*, within the ER).

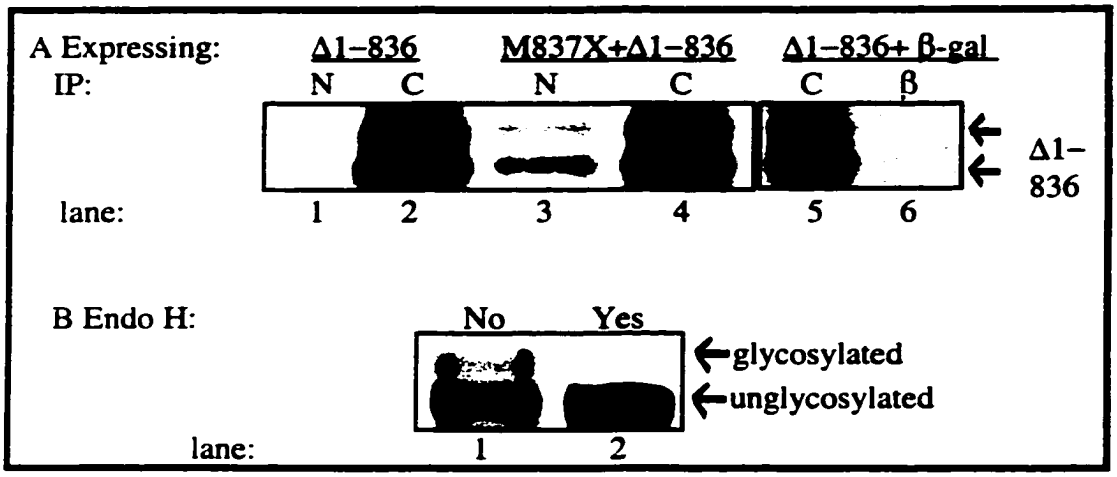


FIG. 9. M837X and Δ1-836 interact early in processing. Panel A. Δ1-836, M837X+Δ1-836, or Δ1-836+β-gal was expressed in COS7 cells and immunoprecipitated from lysates using antibodies specific to M837X (N), Δ1-836 (C), or β-gal (β). The Western blot was stained to detect Δ!-836. Δ1-836 coimmunoprecipitated with M837X (lane 3) but did not bind β-gal (lane 6). Panel B. Δ1-836 was expressed in COS7 cells and immunoprecipitated from lysates using an antibody to Δ1-836 (C). Half the immunoprecipitated protein was treated with Endo H prior to PAGE. The Western was stained to detect Δ1-836. The higher MW Δ1-836 band was sensitive to Endo H (lane 2). IP = immunoprecipitating antibody.

Interactions between M837X and Δ1-836 have biochemical consequences

To demonstrate that M837X could also be detected after binding to Δ1-836, the two fragments were coexpressed and physical interactions assayed as above. M837X coimmunoprecipitated with Δ1-836 (Fig. 10A, lane 4) but not with the control ß-gal protein (Fig. 10A, lane 6). Interestingly, the M837X protein that coimmunoprecipitated with Δ1-836 was greatly enriched for an apparent higher molecular weight conformation (resulting in an M837X doublet on Western blots, Fig. 10A,* lane 4). Direct immunoprecipitation of M837X indicated that the total level of shifted protein was negligible when M837X was expressed alone (Fig. 10A, lane 1), and more evident when M837X was coexpressed with Δ1-836 (Fig. 10A, lane 3).

The specificity of the association of the two halves was further supported by the following observations. First, the binding interaction led to the reconstitution of function (Fig. 7). Second, no binding to either Δ1-836 or M837X was observed by a control protein, ß-gal, expressed under the same conditions (Fig. 9A, lane 6; Fig. 10A, lane 6; Fig. 10B, lanes 1 and 3). Third, binding was observed only when the two portions of CFTR were expressed in the same cells but not when lysates from cells expressing one portion were mixed with lysates from cells expressing the other portion (data not shown). These results, together with the data presented in the following sections, indicate the specificity of domain binding interactions between M837X and Δ1-836 during CFTR processing.

The M837X mobility shift is phosphorylation dependent

As shown in Fig. 10, a slightly larger M837X protein band coimmunoprecipitated with Δ1-836. To determine whether the basis of this higher molecular weight band was

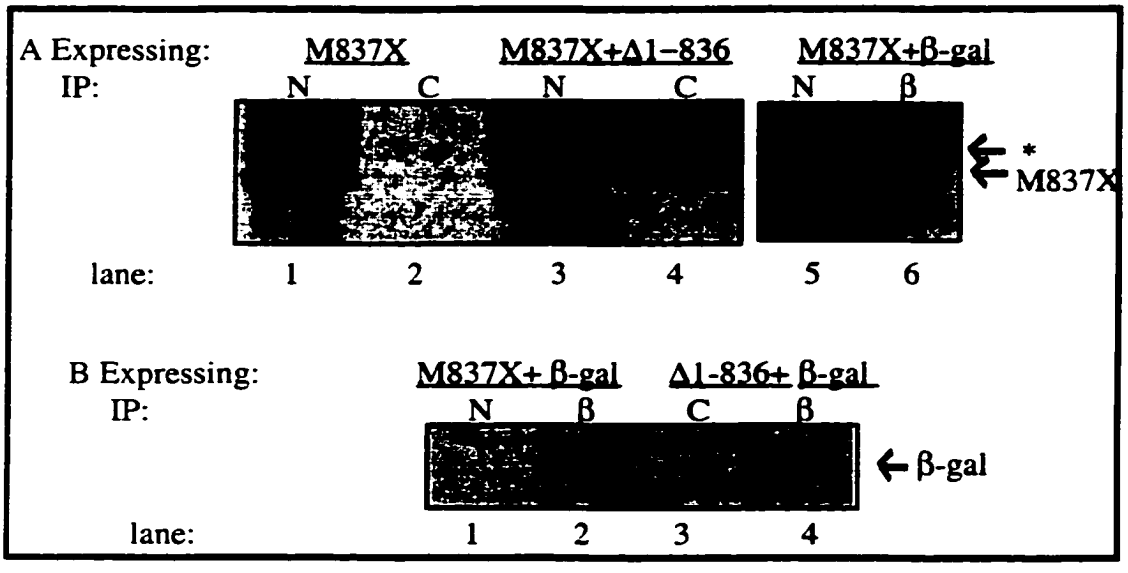


FIG. 10. M837X binding to Δ1-836 results in a reduced mobility form of M837X. Panel A. M837X, M837X+Δ1-836, or M837X+ β-gal) was expressed in COS7 cells and immunoprecipitated from lysates using antibodies specific to M837X (N), Δ1-836 (C), or β-gal (β). The Western was stained to detect M837X. M837X coimmunoprecipitating with Δ1-836 is detected as a doublet (* = reduced mobility form of M837X, lane 4). Panel B. M837X+ β-gal or Δ1-836+ β-gal was expressed in COS7 cells and immunoprecipitated as described as above. Neither portion of CFTR bound the control protein, β-gal. IP = immunoprecipitating antibody.

due to protein phosphorylation, we investigated the effect of PKA stimulation on the M837X protein. Forskolin treatment of cells expressing M837X alone produced an M837X protein band of reduced mobility (*, Fig. 11, lane 1) similar to that observed bound to Δ1-836 (*, Fig. 11, lane 5). Treatment of purified protein with alkaline phosphatase eliminated the higher molecular weight band detected either after PKA stimulation or in complex with Δ1-836 (Fig. 11, lanes 2 and 6). Alkaline phosphatase treatment did not, however, alter the lower molecular weight M837X protein. These results indicate that the interaction of Δ1-836 with M837X confers a phosphorylation dependent reduction in the mobility of M837X. *In vitro* phosphorylation of immunoprecipitated M837X also confirmed a mobility shift in the protein after coexpression with Δ1-836 or treatment with forskolin (Fig. 12, lanes 2 and 3). Since both PKA stimulation and interactions with distal domains resulted in the reduced mobility of the M837X protein, we assayed both of these interventions in concert. Forskolin treatment of the M837X bound to Δ1-836 resulted in nearly a total shift of M837X to the reduced mobility form, indicating that these events are additive (Fig. 12, lane 4).

We next examined whether kinase activity results in reduced mobility of the M837X protein. We tested the potent, broad spectrum kinase inhibitor staurosporine for its ability to block the forskolin-induced mobility shift of M837X. Staurosporine pretreatment was able to block completely the forskolin effect on the mobility of M837X (Fig. 13, lane 3).

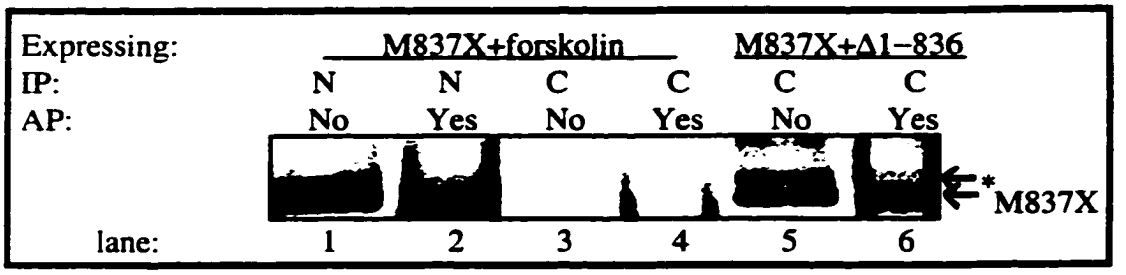


FIG. 11. Forskolin treatment also produces a reduced mobility form of M837X. Lysates from cells expressing M837X pretreated with forskolin (prior to lysis) or cells coexpressing M837X+Δ1-836 (without forskolin treatment) were immunoprecipitated as in Fig. 10, except that half of the immunoprecipitated proteins were incubated with alkaline phosphatase (AP) prior to gel electrophoresis. M837X from cells treated with forskolin and M837X coexpressed with Δ1-836 resulted in a reduced mobility M837X protein band (*, lanes 1 and 5). The shifted M837X protein band was eliminated by treatment with alkaline phosphatase (lanes 2 and 6). IP = immunoprecipitating antibody.

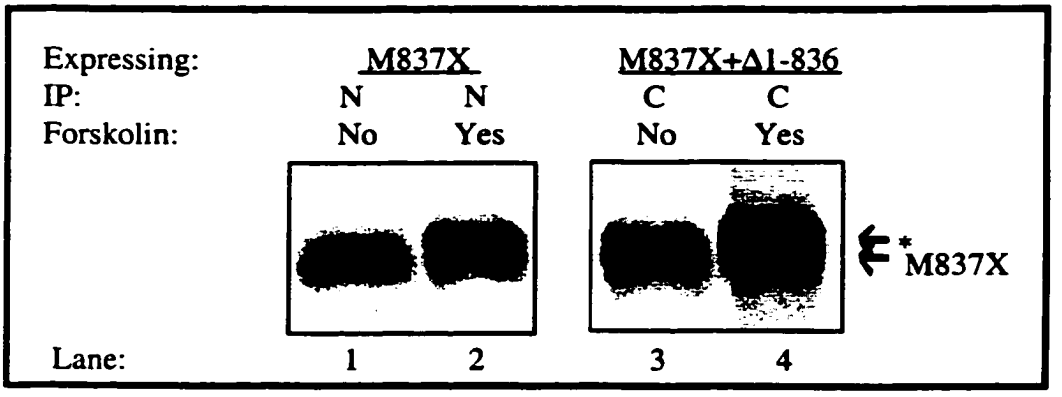


FIG. 12. The reduced mobility of M837X was verified by *in vitro* phosphorylation. M837X or M837X+Δ1–836 was expressed in COS7 cells pretreated with forskolin and immunoprecipitated from lysates as described in Fig. 10. The immunoprecipitated protein was incubated with PKA catalytic subunit and ^{32}P-ATP. Forskolin treatment (lane 2) or binding to Δ1–836 (lane 3) increased the amount of M837X with reduced mobility (*) in an additive manner (lane 4). IP = immunoprecipitating antibody.

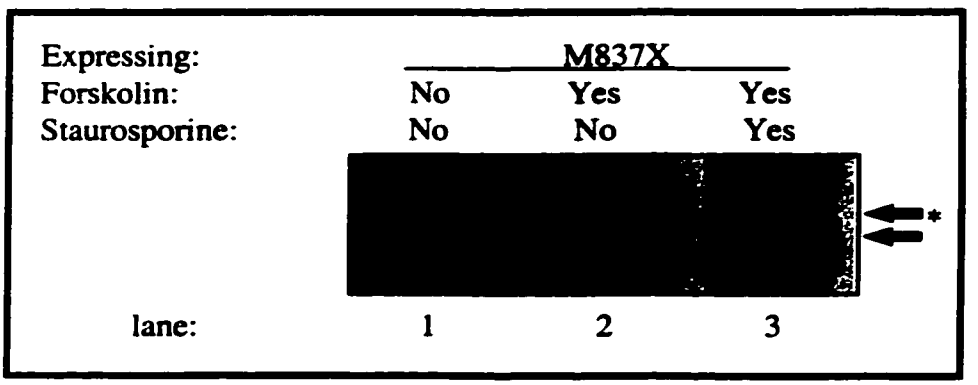


FIG. 13. Staurosporine inhibits the forskolin-induced mobility shift of M837X. COS7 cells expressing M837X were pretreated with staurosporine and then stimulated with forskolin. M837X was immunoprecipitated from lysates and visualized by Western blotting. Staurosporine inhibited the forskolin-induced mobility shift of M837X (*, compare lane 2 and lane 3).

Kinase inhibitors block high basal activity and the mobility shift of M837X

The increased halide efflux conferred by M837X and Δ1-836 might be due to disabled R-domain function and absent PKA dependent regulation of CFTR (as demonstrated for ΔR-CFTR, Rich *et al.*, 1991), or to a novel mechanism involving augmented susceptibility of the R-domain to phosphorylation by kinases (resulting in enhanced R-domain basal phosphorylation and CFTR activation). Since interactions between M837X and Δ1-836 led to a phosphorylation dependent mobility shift and since forskolin induced a similar shift of M837X that could be blocked by kinase inhibitors, we tested the effect of kinase inhibition on the high basal activity of M837X and Δ1-836. When cells expressing M837X plus Δ1-836 were treated with the potent but nonselective kinase inhibitor staurosporine for 30 min, halide permeability was dramatically reduced (Fig. 14). Removal of the kinase inhibitor followed by a 2-hr incubation in normal media allowed partial restoration of activity. Since PKA is a predominant activator of CFTR, we also tested the effects of a PKA specific inhibitor, Rp-8-CPT-cAMPS, on the constitutive activity of M837X and Δ1-836. Rp-8-CPT-cAMPS is a reversible cAMP analog; therefore, we modified our previous halide permeability assay to focus on the last quenching buffer substitution, because (a) this step appears to be very sensitive to CFTR function (Figs. 7 and 8), (b) limiting the duration of the protocol minimized washout of the reversible PKA antagonist, and (c) this modified protocol conserved very costly kinase inhibitory reagents. When cells coexpressing M837X and Δ1-836 were treated with Rp-8-CPT-cAMPS, a significant decrease in halide permeability was detected compared to untreated cells (Fig. 15). The inhibition by Rp-8-CPT-cAMPS was completely reversible, and removal of the inhibitor followed by incubation in normal media for 2 hr com-

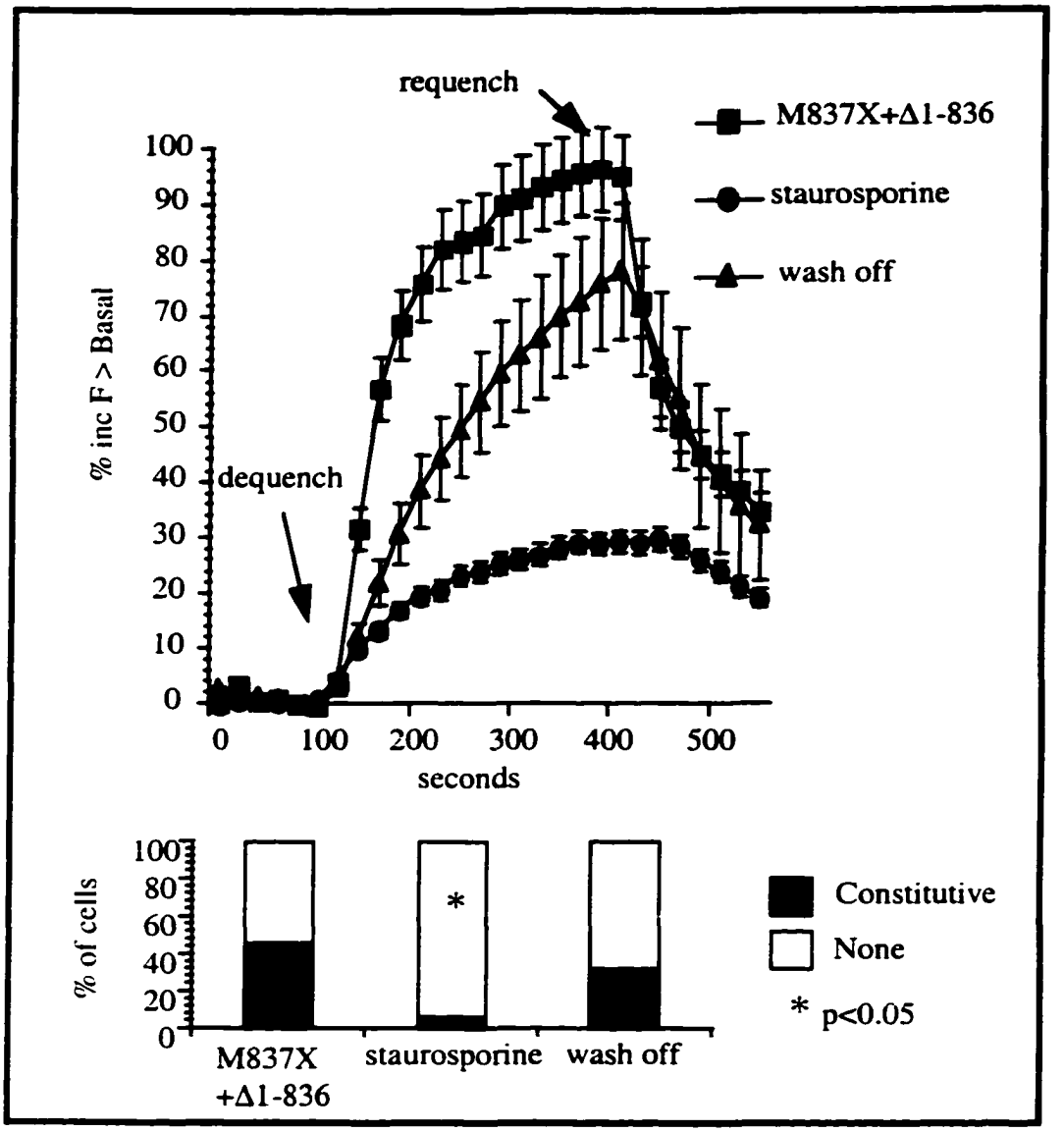


FIG. 14. Staurosporine reduces the halide permeability of M837X+Δ1–836. M837X+Δ1–836 was expressed in cells and halide efflux assayed as in Fig. 7. The enhanced basal halide efflux detected in cells expressing M837X+Δ1–836 (squares) was reduced after staurosporine treatment (circles, $p < 0.05$). The inhibition was partially reversed after removal of the staurosporine and 2-hr incubation in inhibitor free media (triangles). %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 0 and 100 sec). Error bars = SEM. N >100 cells studied per condition. The bar graph shows the percentage of cells assayed producing constitutive activity or no activity.

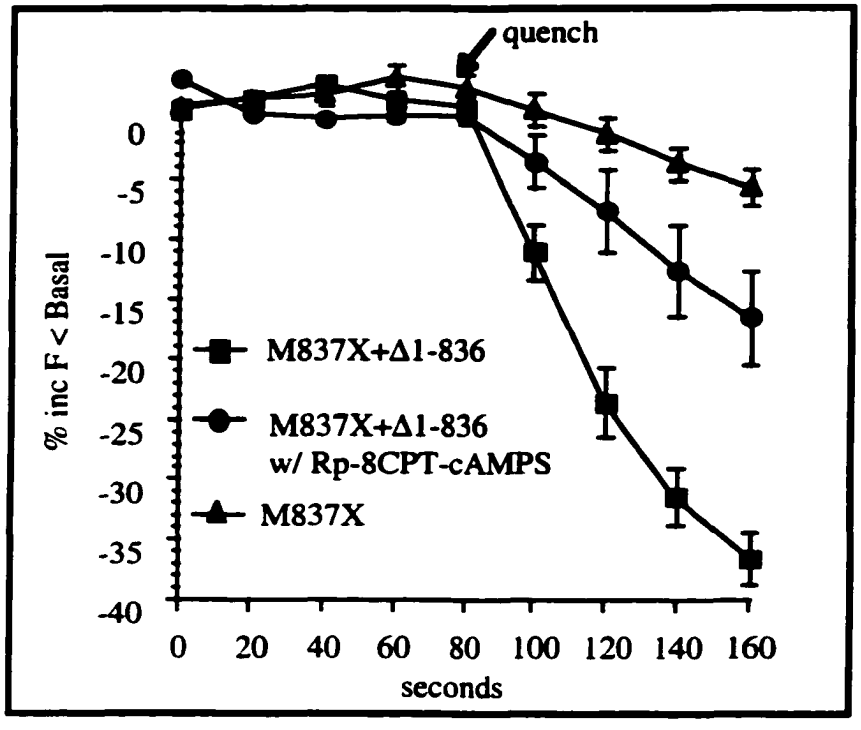


FIG. 15. The high basal halide permeability is PKA dependent. M837X (triangles) or M837X+Δ1–836 (squares) was expressed in COS7 cells, and half of the cells expressing M837X+Δ1–836 were treated with Rp-8-CPT-cAMPS, a PKA antagonist (circles). Halide influx was assayed by monitoring SPQ fluorescence. After 60 sec, the nitrate buffer was replaced with iodide buffer, and the rate of fluorescence quenching was monitored. Rp-8-CPT-cAMPS inhibited the halide permeability of M837X+Δ1–836 ($p < 0.05$). % inc F < Basal = the percentage decrease in fluorescence below baseline (average between 20 and 60 sec). Error bars = SEM. N > 150 cells studied per condition.

pletely restored the activity of M837X and Δ1-836 (Fig. 16). These results indicate that enhanced PKA dependent phosphorylation of M837X is responsible for the high basal halide efflux produced following coexpression of M837X plus Δ1-836.

Since kinase inhibitors were able to reduce the high basal activity of M837X plus Δ1-836, we tested the ability of kinase blockers, including the PKC specific inhibitors, Bin-1 and chelerythrine, for their effects upon the mobility shift of M837X bound to Δ1-836. Western blots of M837X bound to Δ1-836 purified from cells pretreated with the kinase inhibitors demonstrated that different kinase inhibitors had variable effects on the shifted M837X protein band (Fig. 17). Staurosporine dramatically inhibited the mobility shift of the M837X protein bound to Δ1-836 (Fig. 17, lane 4). Rp-8-CPT-cAMPS also reduced the amount of protein shifted to the higher apparent molecular weight but to a lesser degree than staurosporine (Fig. 17, lane 2). The PKC inhibitors had no effect (Fig. 17, lanes 1 and 3). To verify that PKC phosphorylation was not involved with the molecular weight shift of M837X, we treated cells expressing M837X with PMA (phorbol 12-myristate 13-acetate) to stimulate PKC activity. PMA treatment did not alter the mobility of M837X (Fig. 18).

Amino acids 723-837 are required for PKA dependent activity

Since the activity of M837X and Δ1-836 was shown to be PKA dependent and the R-domain contains most of the PKA consensus sites, we tested the effect of removal of all or part of the R-domain on the enhanced halide permeability of the two halves. When a CFTR truncated immediately before the R-domain (K593X, Fig. 5) was coexpressed with Δ1-836, no enhanced halide permeability was detected by SPQ fluorescence

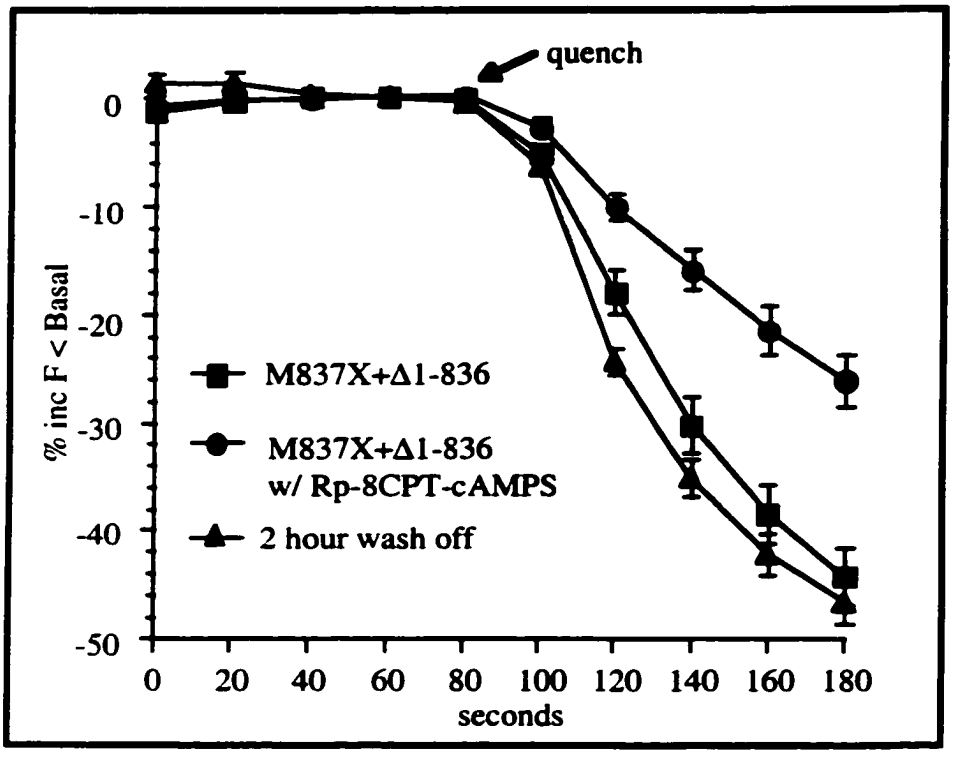


FIG. 16. Rp-8-CPT-cAMPS inhibition is reversible. M837X+Δ1–836 was expressed in COS7 cells and halide influx measured as in Fig. 16. Following treatment with Rp-8-CPT-cAMPS, the cells were washed and incubated in inhibitor free media for 2 hr. Removal of the Rp-8-CPT-cAMPS restored normal halide permeability to the cells expressing M837X and Δ1-836. % inc F < Basal = the percentage decrease in fluorescence below baseline (average between 20 and 60 sec). Error bars = SEM. N > 150 cells studied per condition.

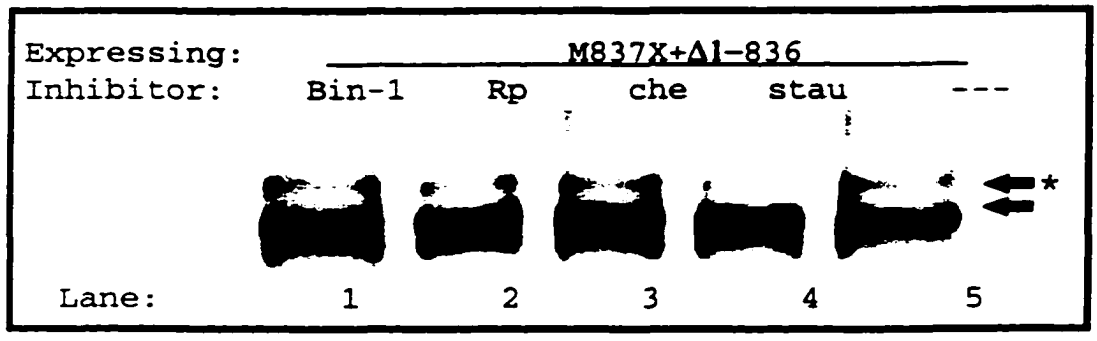


FIG. 17. PKA inhibitors reduce the amount of shifted M837X bound to Δ1–836. COS7 cells expressing M837X+Δ1–836 were treated with staurosporine (a kinase inhibitor), Rp-8-CPT-cAMPS (a PKA inhibitor), Bin-1, or Che (PKC inhibitors) and the proteins immunoprecipitated with an antibody specific to Δ1–836. The PKC inhibitors had no effect on the shifted mobility of M837X bound to Δ1–836 (*, lanes 1 and 3). Rp-8-CPT-cAMPS caused a minor reduction in the upper band (lane 2, by phosphoimagery, the shifted band is appx. 33% reduced compared to lane 5), and staurosporine produced a considerable reduction in the mobility shift of M837X bound to Δ1–836 (lane 4).

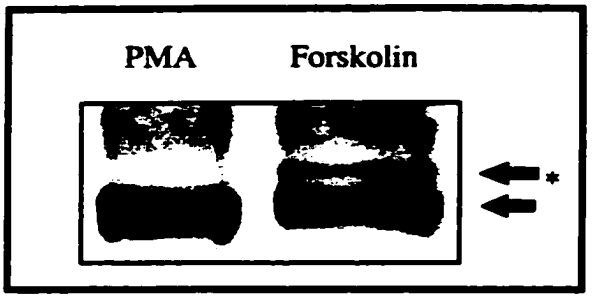


FIG. 18. A PKC agonist does not induce a mobility shift of the M837X protein. COS7 cells expressing M837X were treated with PMA to stimulate PKC activity or with forskolin to stimulate PKA activity. Forskolin induced a mobility shift in M837X; however, PMA did not affect the protein.

(Fig. 19). These results imply that the R-domain or a portion of the R-domain is required for the activity of the two halves. Studies from other laboratories in which portions of the R-domain were removed from contiguous CFTR constructs suggested that the carboxyl region of the R-domain may not be required for CFTR function; therefore, we tested a CFTR truncated midway in the R-domain for the ability to complement Δ1-836 functionally. G723X (missing the last 114 amino acids of the R-domain, Fig. 5) coexpressed with Δ1-836 led to enhanced halide permeability similar to M837X plus Δ1-836 (*i.e.*, no PKA stimulation was required to induce activity, Fig. 19; compare to Fig. 7).

Functional complementation of Δ1-836 by G723X suggested a physical interaction between these two constructs; therefore, we tested G723X and the nonfunctional K593X for their ability to coimmunoprecipitate with Δ1-836. Both G723X and K593X coimmunoprecipitated with Δ1-836 (Fig. 20A, lane 4, and Fig. 20B, lane 2). However, in contrast to M837X, neither protein was detected as an apparent higher molecular weight form when bound to Δ1-836. Since G723X did not exhibit a mobility shift when bound to Δ1-836, we speculated that G723X might lack the necessary regions of the R-domain to alter conformation in response to phosphorylation. While PKA activation changed the mobility of M837X (Fig. 11), forskolin treatment of cells expressing G723X did not alter the mobility of this protein, indicating that the C-terminal tail of the R-domain is necessary for the conformational changes required to curb mobility (Fig. 20C, compare lanes 2 and 4).

G723X is missing two PKA phosphorylation sites, serines at positions 795 and 813, that greatly influence CFTR activation (Wilkinson *et al.*, 1997). To verify that G723X was capable of being phosphorylated, we performed *in vitro* phosphorylation

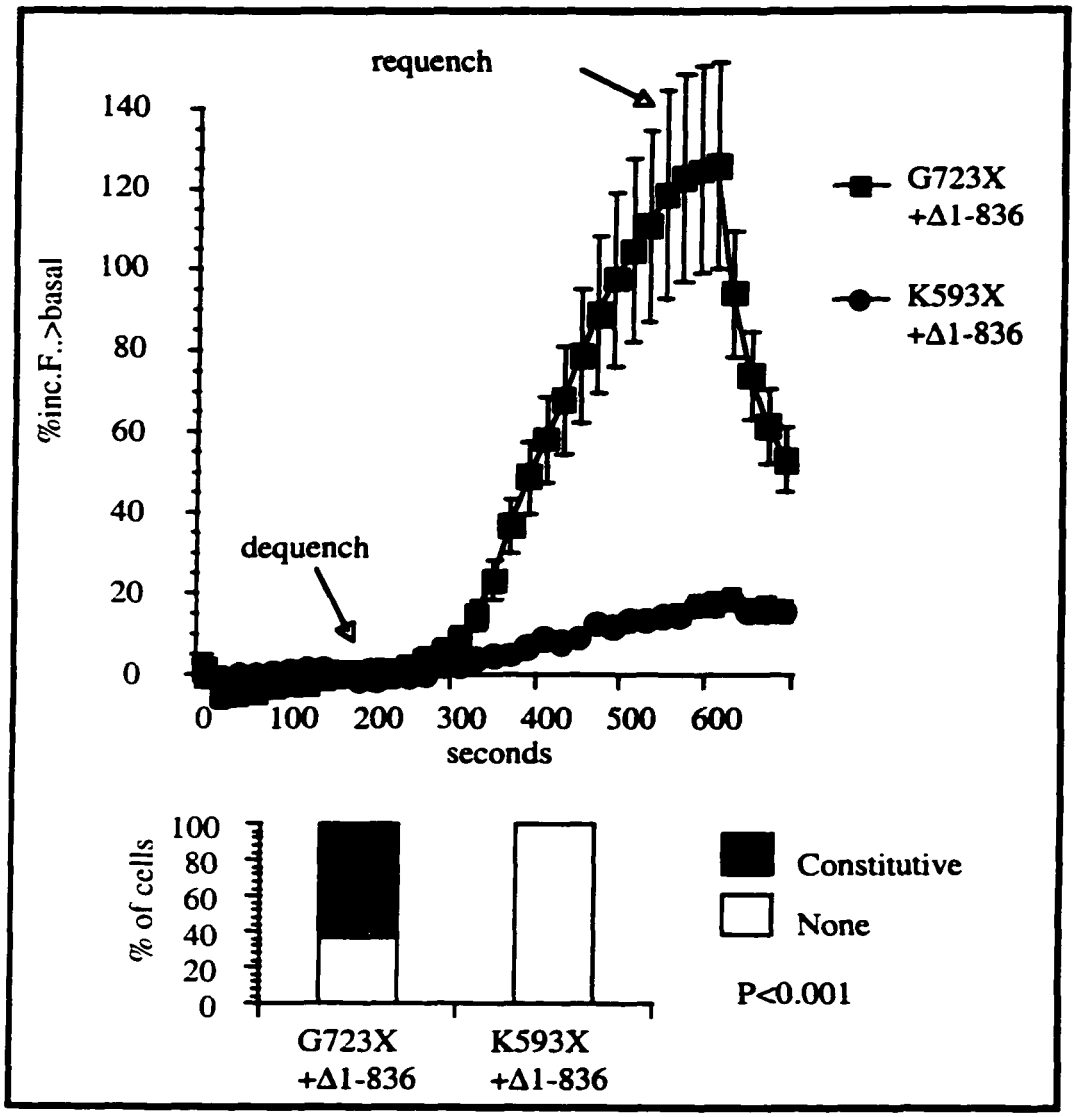


FIG. 19. G723X plus Δ1-836 produces halide efflux, but K593X plus Δ1-836 does not. G723X+Δ1-836 or K593X+Δ1-836 was expressed in cells and halide efflux assayed as described in Fig. 7. G723X produced high basal halide efflux when coexpressed with Δ1-836 (squares, $p < 0.01$) similar to M837X+Δ1-836 (Fig. 7). K593X, however, failed to increase halide permeability when coexpressed with Δ1-836 (circles). Stimulation with forskolin had no effect on halide permeability (data not shown). %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 100 and 200 sec). Error bars = SEM. N > 150 cells studied per condition. The bar graph shows the percentage of cells producing constitutive activity or no activity.

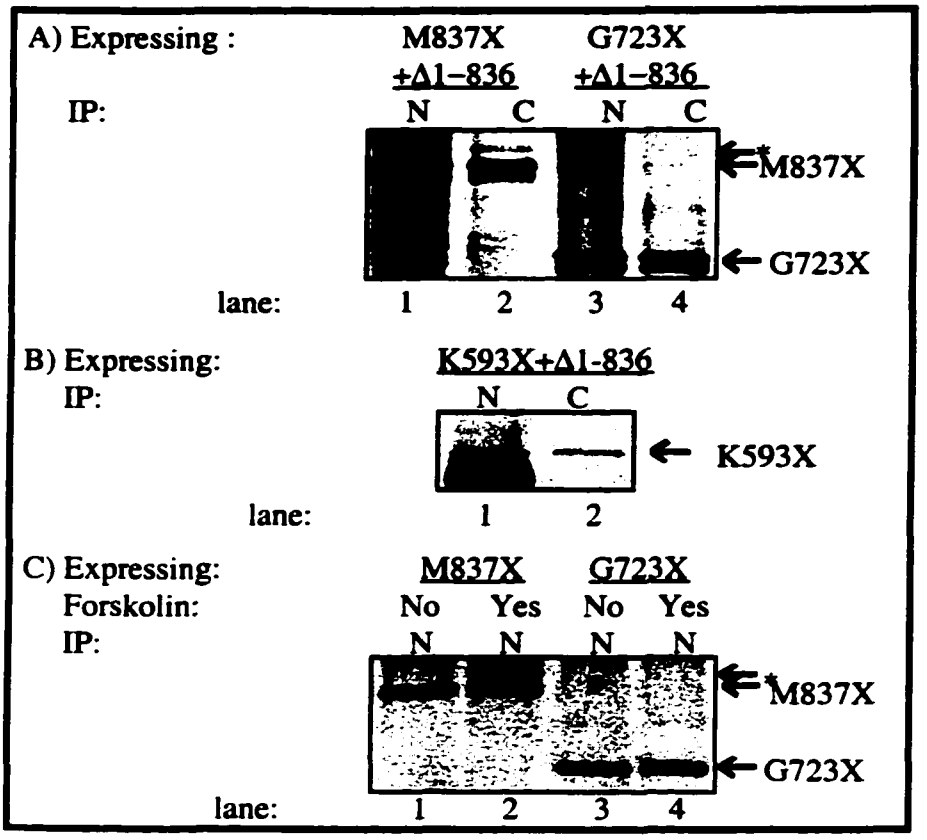


FIG. 20. G723X binds Δ1-836, but this interaction does not result in a mobility shift. Panel A. M837X+Δ1-836 or G723X+Δ1-836 was expressed in cells and the proteins immunoprecipitated as described in Fig. 10. While both G723X and M837X bound Δ1-836 (lanes 2 and 4), only the interaction of M837X with Δ1-836 produced a shift in the mobility of the protein (*, lane 2). Panel B. K593X+Δ1-836 was expressed in cells and the proteins immunoprecipitated as described above. K593X bound Δ1-836 (lane 2), but the interaction did not alter the mobility of K593X. Panel C. Cells expressing M837X or G723X were treated with forskolin prior to lysing and immunoprecipitated. M837X, but not G723X, was detected with reduced mobility in response to forskolin (*, compare lane 2 and lane 4). IP = immunoprecipitating antibody.

studies. *In vitro* phosphorylation of G723X resulted in a single phosphorylated protein band under all three of the conditions tested: basal, following treatment with forskolin, and after coimmunoprecipitation with Δ1-836 (Fig. 21, lanes 1, 4, and 5).

Although coexpression of G723X plus Δ1-836 results in a functional CFTR protein that resembles Δ725-835 CFTR, we have shown that M837X plus Δ1-836 is not functionally identical to a contiguous, wild-type CFTR molecule (Fig. 7). Therefore, to compare the PKA independent function of Δ725-835 CFTR with G723X and Δ1-836, we tested the effect of Rp-8-CPT-cAMPS on the constitutive activity of both, as in Fig. 15. Both the enhanced halide permeability of Δ725-835 CFTR (Fig. 22) and that of G723X plus Δ1-836 (Fig. 23) were found to be insensitive to PKA inhibition. These results confirm that amino acids 723-837 are necessary for both the conformational change that occurs in the R-domain in response to phosphorylation and the PKA dependent functional activation of M837X plus Δ1-836.

PKA regulation is restored if the R-domain is attached to Δ1-836

In Fig. 19, the enhanced halide permeability of M837X plus Δ1-836 was abolished by removal of the R-domain from M837X (*i.e.*, no function could be detected in cells expressing K593X and Δ1-836). To determine if this function could be restored by expressing the R-domain on the distal half of CFTR, we expressed K593X with Δ1-595, a CFTR beginning immediately before the R-domain (Fig. 5). Coexpression of Δ1-595 with K593X did not result in enhanced halide permeability (Fig. 24). These results and those from Fig. 19 suggest that attachment of the R-domain to the proximal regions of CFTR is required for activity. To investigate the effect of the R-domain attached to the distal half

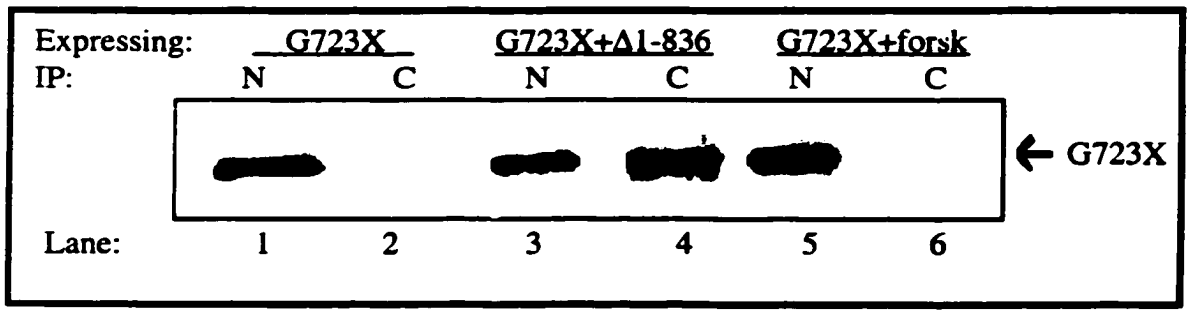


FIG. 21. G723X can be phosphorylated by PKA. G723X or G723X+Δ1–836 was expressed in COS7 cells, and half the cells expressing G723X alone were treated with forskolin. Proteins were immunoprecipitated from lysates as described in Fig. 10. The immunoprecipitated proteins were incubated with PKA catalytic subunit and ^{32}P-ATP. G723X could be phosphorylated by PKA (lanes 1, 3, 4, and 5). However, no shift in mobility was detected in G723X bound to Δ1–836 (lane 4) or in response to forskolin treatment (lane 5). IP = immunoprecipitating antibody.

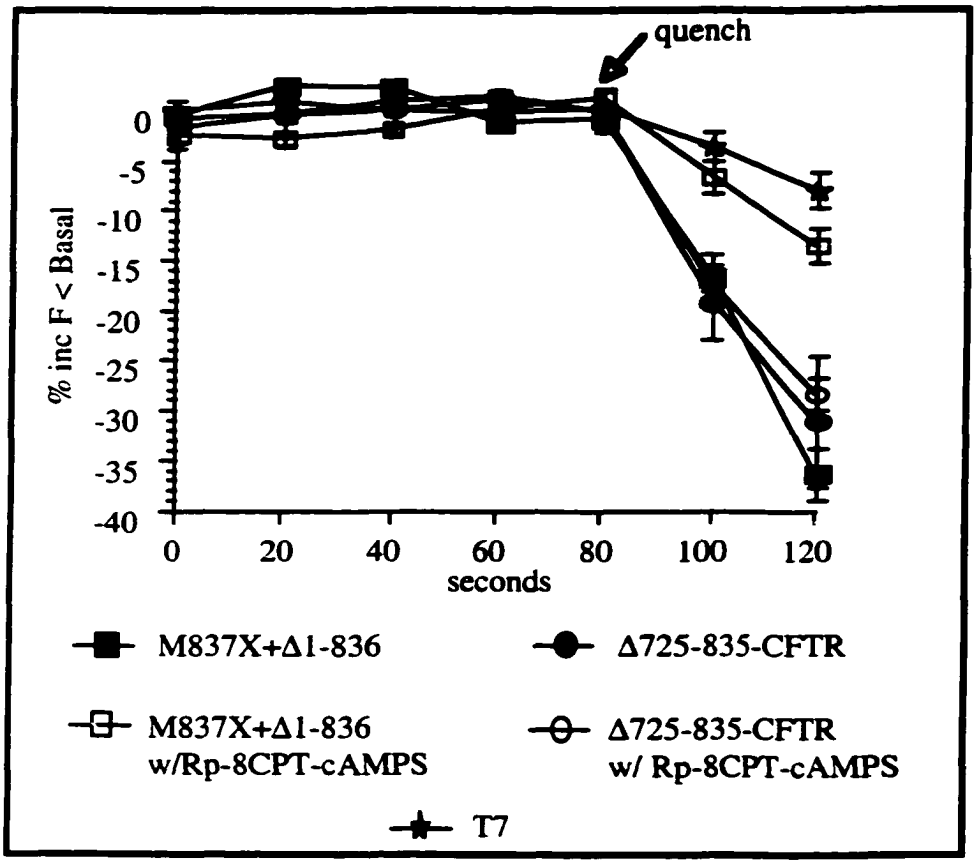


FIG. 22. The halide permeability produced by ΔR-CFTR is not PKA dependent. M837X+Δ1-836 or Δ725-835-CFTR was expressed in COS7 cells and half the cells treated with Rp-8-CPT-cAMPS. Halide influx was assayed as in FIG. 16. Rp-8-CPT-cAMPS inhibited the enhanced halide permeability of M837X+Δ1-836 ($p < 0.05$) but not that of Δ725-835-CFTR. T7 = control cells expressing T7 polymerase alone and no CFTR domains. %inc F < Basal = the percentage decrease in fluorescence below baseline (average between 20 and 60 sec.) Error bars = SEM. N > 150 cells studied per condition.

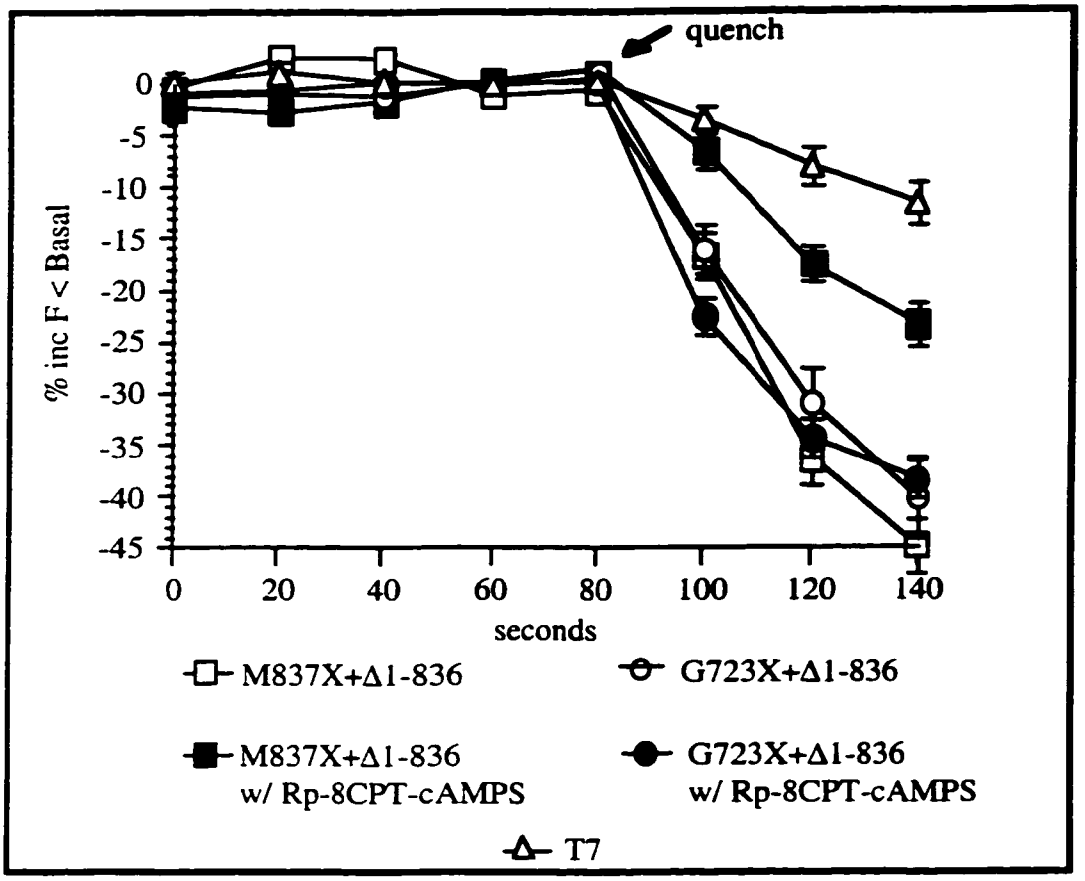


FIG. 23. The halide permeability produced by G723X+Δ1-836 is not PKA dependent. M837X+Δ1-836 or G723X+Δ1-836 was expressed in cells and half the cells treated with Rp-8-CPT-cAMPS. Halide influx was assayed as described in Fig. 16. Rp-8-CPT-cAMPS inhibited the enhanced halide permeability of M837X+Δ1-836 ($p < 0.05$) but not that of G723X+Δ1-836. T7 = control cells expressing T7 polymerase alone and no CFTR domains. % inc F < Basal = the percentage decrease in fluorescence below baseline (average between 20 and 60 sec). Error bars = SEM. N > 100 cells studied per condition.

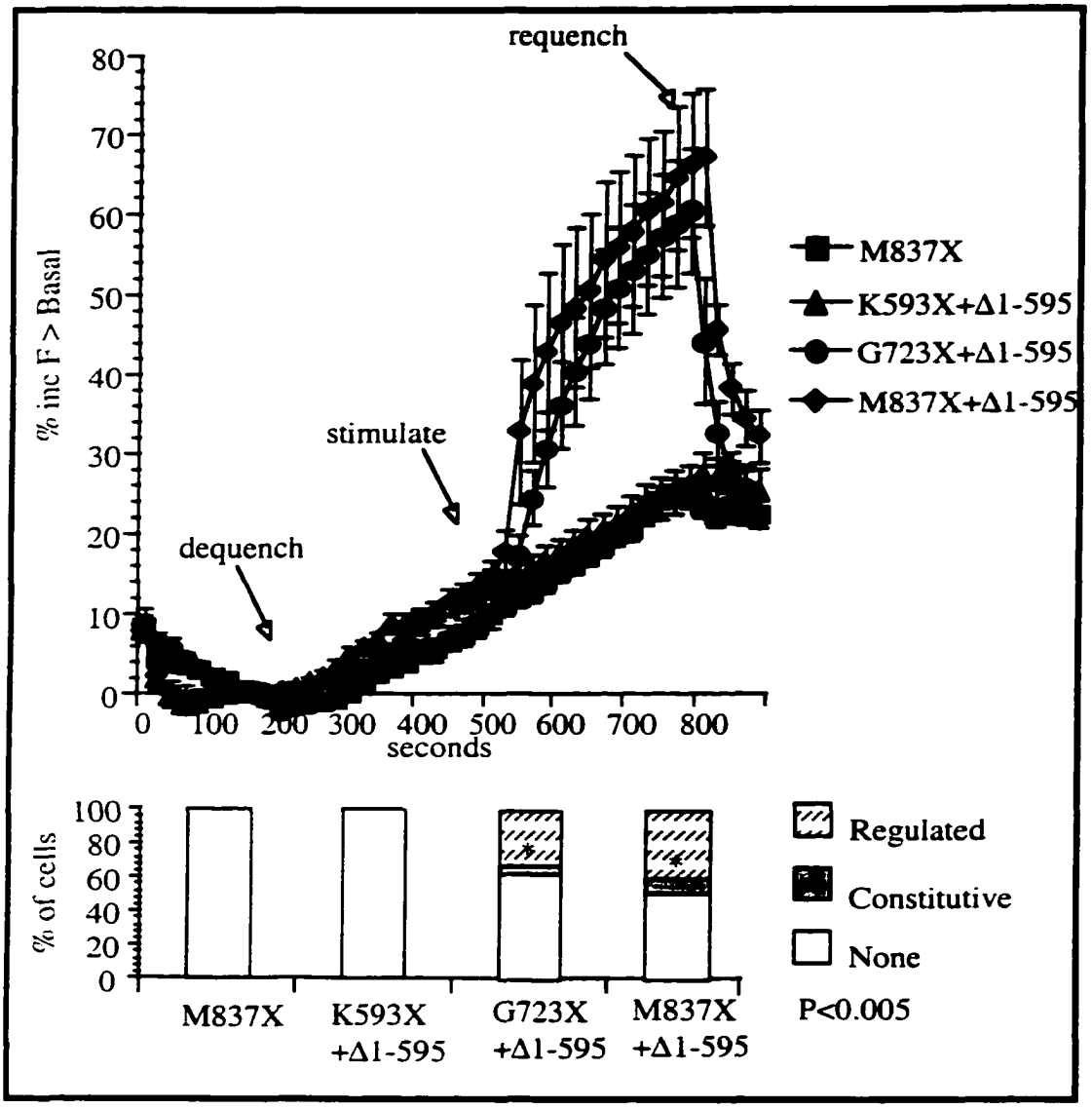


FIG. 24. G723X+Δ1-595 reconstitutes regulated function. M837X, K593X+ Δ1-595, G723X+Δ1-595, or M837X+Δ1-595 was expressed and halide permeability assayed as in Fig. 7. Enhanced halide efflux following the addition of forskolin was detected in G723X+Δ1-595 (circles) and M837X+Δ1-595 (diamonds, $p < 0.01$). No enhanced halide permeability was detected in K593X+Δ1-595 (triangles) %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 100 and 200 sec). Error bars = SEM. N >100 cells per condition. The bar graph shows the percentage of cells assayed producing regulated, constitutive, or no activity.

of CFTR, we coexpressed G723X (containing a portion of the R-domain) or M837X (containing the complete R-domain) with Δ1-595. Enhanced halide permeability was detected with G723X plus Δ1-595 and with M837X plus Δ1-595 (Fig. 24). Interestingly, in contrast to the coexpression of G723X or M837X with Δ1-836 (see Figs. 7 and 19), the activity detected from either of these constructs with Δ1-595 was not predominantly constitutive but required forskolin in a manner similar to wild-type CFTR. These results suggest that R-domain configuration strongly influences constitutive phosphorylation in the half-molecule model. The results also show that certain CFTR constructs can locate and bind one another, and can confer a cAMP dependent halide permeability that resembles the wild-type CFTR molecule.

Isolated R-domain binds distal domains

The phosphorylation dependent mobility shift of M837X depends on expression of subdomains within the R-domain and is enhanced by the interaction of M837X with Δ1-836. One possible explanation for these results could be a direct association of the R-domain with regions in Δ1-836. To examine interactions between the R-domain and Δ1-836, COS7 cells expressing isolated R-domain alone (amino acids 595-836, R-domain, Fig. 5), Δ1-836 alone, or the R-domain plus Δ1-836 were lysed and the proteins coimmunoprecipitated using antibodies specific to either the R-domain or the C-terminal tail (which recognizes Δ1-836). When the R-domain was coexpressed with Δ1-836 but not when expressed alone, the R-domain could be immunoprecipitated using the C-terminal specific antibody (Fig. 25A, lanes 1, 2, and 3). The complementary experiment demonstrated that Δ1-836 could also be coimmunoprecipitated with an antibody to the

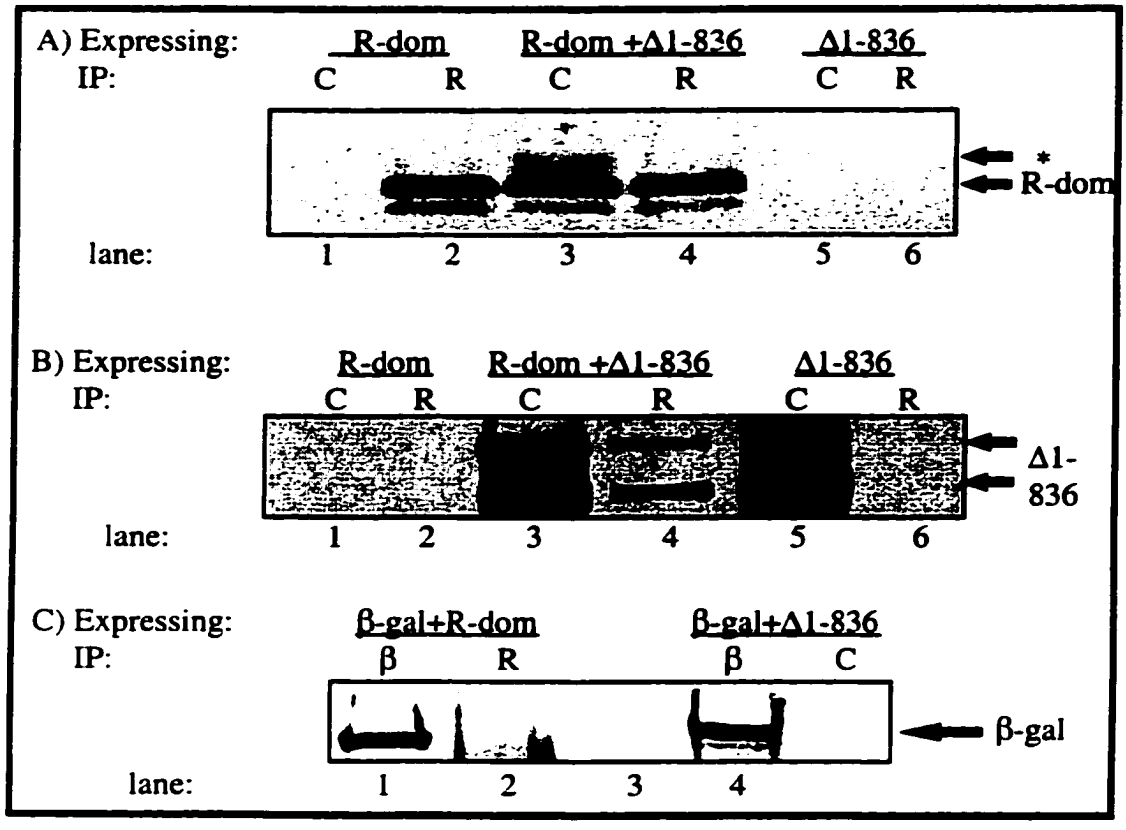


FIG. 25. The R-domain binds to Δ1-836. Panel A. R-domain (R-dom), R-dom+ Δ1-836, or Δ1-836 was expressed in cells and immunoprecipitated using antibodies specific to Δ1-836 (C) or the R-domain (R). The Western blot was stained to detect the R-domain. The R-domain was detected in association with Δ1-836 as a doublet (* = reduced mobility form, lane 3). Panel B. R-dom, R-dom+Δ1-836, or Δ1-836 was expressed and immunoprecipitated as in panel A. The Western was stained to detect Δ1-836. Δ1-836 coimmunoprecipitated with the R-domain (lane 4). Panel C. β-gal+R-dom, or β-gal+Δ1-836 was expressed and immunoprecipitated using antibodies specific to β-gal (β), R-domain (R), or Δ1-836 (C). Neither the R-domain nor Δ1-836 was detected in association with β-gal (lanes 2 and 4). IP = immunoprecipitating antibody.

R-domain (Fig. 25B, lane 4). Neither the R-domain nor the Δ1-836 peptides were detected coimmunoprecipitating with a control ß-gal protein (Fig. 25C). Interestingly, some of the R-domain that associates with Δ1-836 was detected as an apparent higher molecular weight protein (Fig. 25A,* lane 3). To examine whether phosphorylation was the cause of this shift in protein mobility, cells expressing the R-domain alone were treated with forskolin. Forskolin treatment produced a reduction in the mobility of the R-domain similar to that detected in the R-domain coimmunoprecipitating with Δ1-836 (Fig. 26, * lanes 1 and 4). Phosphorylation was verified as the cause of the altered protein mobility. Alkaline phosphatase treatment of the immunoprecipitated protein resulted in the collapse of the apparent higher molecular weight R-domain into the lower form (Fig. 26, lanes 2 and 6).

To characterize further the region within the R-domain necessary for phosphorylation-induced changes in protein mobility, we expressed smaller R-domain peptides, amino acids 595-813, 595-740, or 708-835 (Fig. 5), in COS7 cells and treated the cells with forskolin to stimulate PKA activity. Interestingly, all of the peptides that included the carboxy portion of the R-domain resulted in a mobility shift upon forskolin treatment (Fig. 27). The R-domain peptide 595-740 (missing the last half of RD2, (Dulhanty and Riordan, 1994b)) did not, however, produce a shift in mobility upon forskolin treatment.

The ΔF508 mutation eliminates the constitutive activity of M837X and Δ1-836

Since the mechanism blocking transport of ΔF508 CFTR to the plasma membrane is still not completely understood, we tested the effect of this mutation on the enhanced halide efflux produced by M837X and Δ1-836. Inclusion of the ΔF508 mutation in

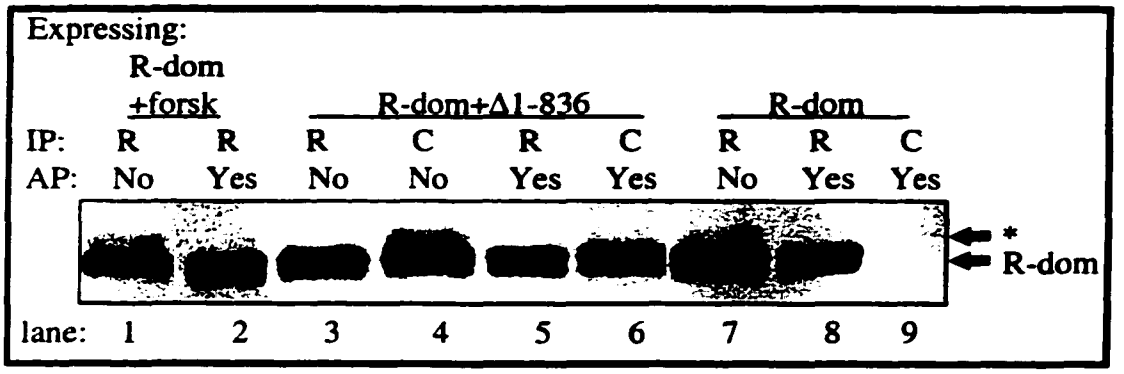


FIG. 26. Forskolin treatment also produces a reduced mobility form of the R-domain. Lysates from COS7 cells expressing either the R-domain alone, pretreated with forskolin (R-dom+forsk), or R-dom+Δ1-836 (without forskolin treatment) were immunoprecipitated as in Fig. 26. Half of the immunoprecipitated samples were incubated with alkaline phosphatase (AP) prior to PAGE. The R-domain from cells treated with forskolin and the R-domain coexpressed with Δ1-836 resulted in a R-domain protein with reduced mobility (*, lanes 1 and 4). The shifted R-domain protein was eliminated by treatment with alkaline phosphatase (lanes 2 and 6). IP = immunoprecipitating antibody.

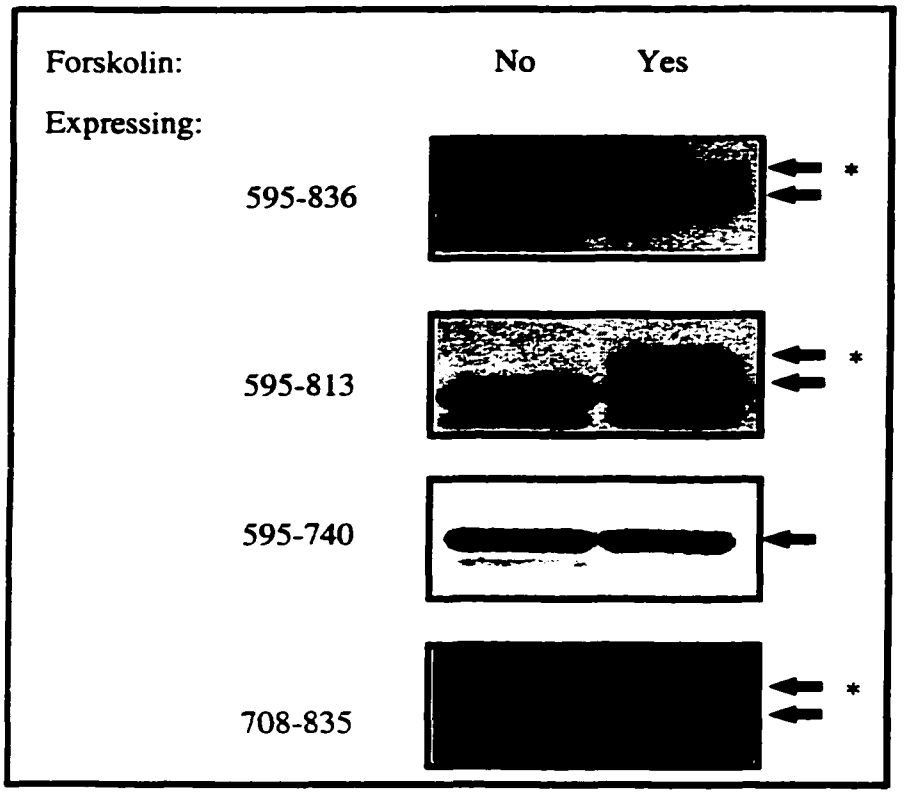


FIG. 27. The mobility shift of the R-domain is dependent on amino acids 741-813. CFTR 595-836, 595-813, 595-740, or 708-835 was expressed in COS7 cells and half the cells treated with forskolin. The proteins were immunoprecipitated with anti-R-domain antibodies and detected by Western blotting. Forskolin treatment resulted in a mobility shift of 708-835 but not 595-740, indicating that the C-terminal portion of the R-domain is necessary for the PKA-induced mobility shift of the R-domain.

M837X completely abrogated the increased halide movement observed after coexpression of wild-type M837X with Δ1-836 (Fig. 28). The possibility that the ΔF508 mutation disrupted the function of M837X and Δ1-836 by abolishing the binding between the fragments was also tested. The ΔF508 mutation did not block the physical binding of M837X and Δ1-836 (Fig. 29). The ΔF508-M837X protein that was detected coimmunoprecipitating with Δ1-836 appeared as two bands, indicating that the phosphorylation dependent mobility shift of M837X was maintained in the ΔF508 mutated protein.

NBD1 binds distal domains.

Although the ΔF508 mutation did not disrupt interactions between two large segments of CFTR (Fig. 29), we also considered the possibility that an interaction between NBD1 and distal domains might be hindered by the mutation. Strong interactions between the TMD1 and TMD2 of CFTR have been demonstrated previously and could mask a defect in NBD1 binding to other CFTR domains by our immunoprecipitation protocol. To verify that NBD1 binds more distal CFTR domains, NBD1 (Fig. 5) was coexpressed with Δ1-836 and binding interactions were assayed by coimmunoprecipitation using antibodies specific to either NBD1 or the C-terminal tail (which recognizes Δ1-836). NBD1 was detected coimmunoprecipitating with Δ1-836 (Fig. 30A, lane 4). The complementary experiment demonstrated that Δ1-836 could also be found in association with NBD1 (Fig. 30B, lane 3). Since both unglycosylated and glycosylated forms of Δ1-836 were detected binding NBD1, the interaction occurred early in processing.

To identify specific regions within Δ1-836 that bind NBD1, NBD1 was coexpressed with the C-terminal tail of CFTR, Δ1-1377 (amino acids 1378-1480, begin

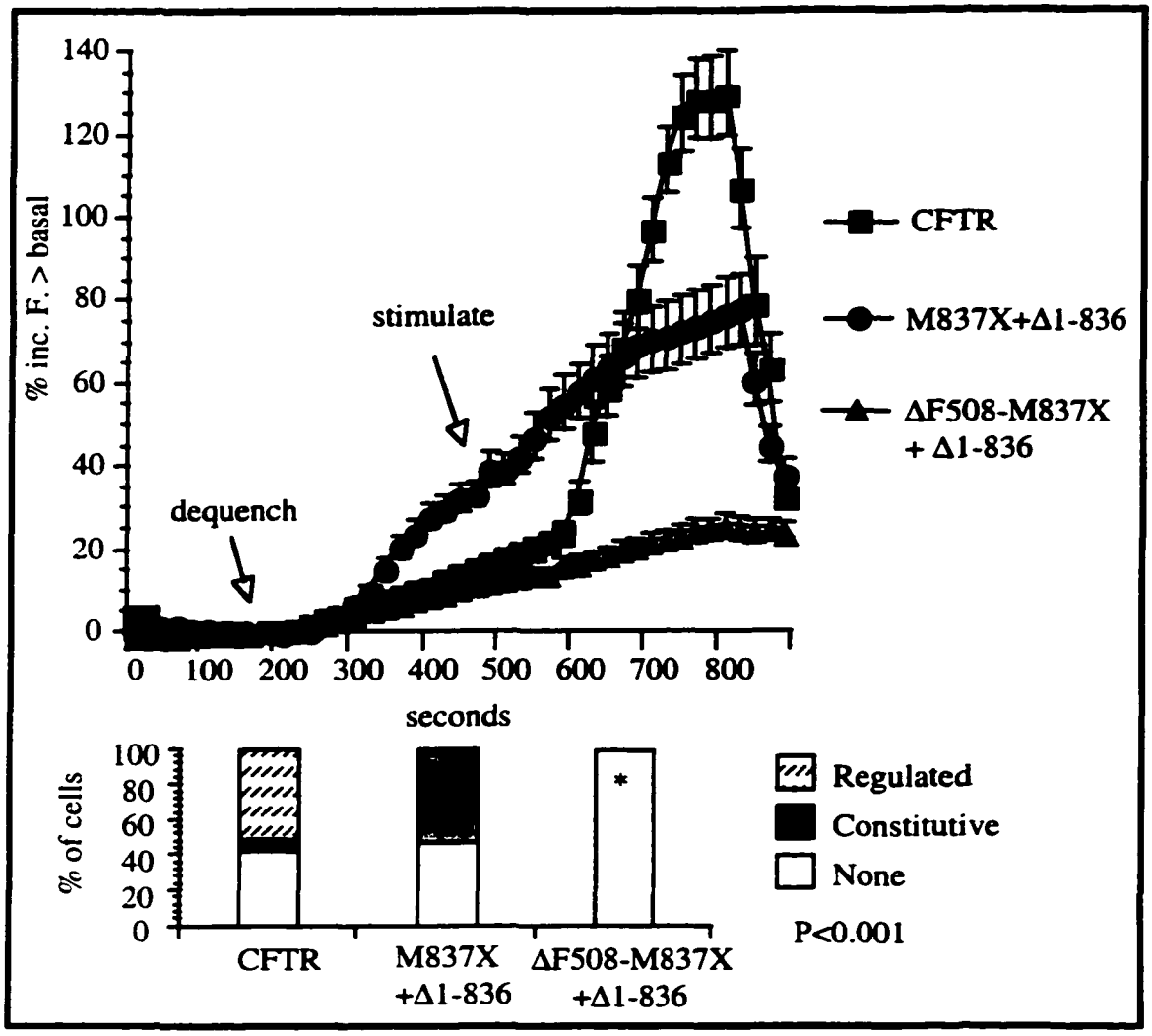


FIG. 28. The ΔF508 mutation abrogates the halide permeability of M837X+Δ1-836. CFTR , M837X+Δ1-836, or ΔF508-M837X+Δ1-836 was expressed in COS7 cells and halide movement assayed as described in Fig. 7. Enhanced halide permeability was not observed after coexpression of ΔF508-M837X and Δ1-836. %inc. F. > basal = the percentage increase in fluorescence above baseline (average between 100 and 200 sec). Error bars = SEM. N >200 cells studied per condition. The bar graph shows the percentage of cells assayed producing regulated activity (responding to forskolin), constitutive activity, or no activity.

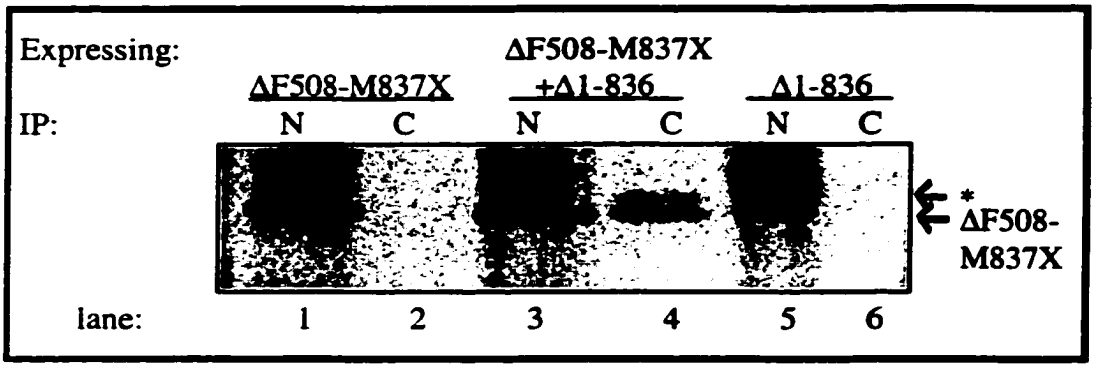


FIG. 29. The ΔF508 mutation does not abolish binding between M837X and Δ1-836. ΔF508-M837X, ΔF508-M837X+Δ1-836, or Δ1-836 was expressed in COS7 cells and immunoprecipitated as in Fig. 10. ΔF508-M837X was detected bound to Δ1-836 in a reduced mobility form similar to wild-type M837X (*, lane 4). IP = immunoprecipitating antibody.

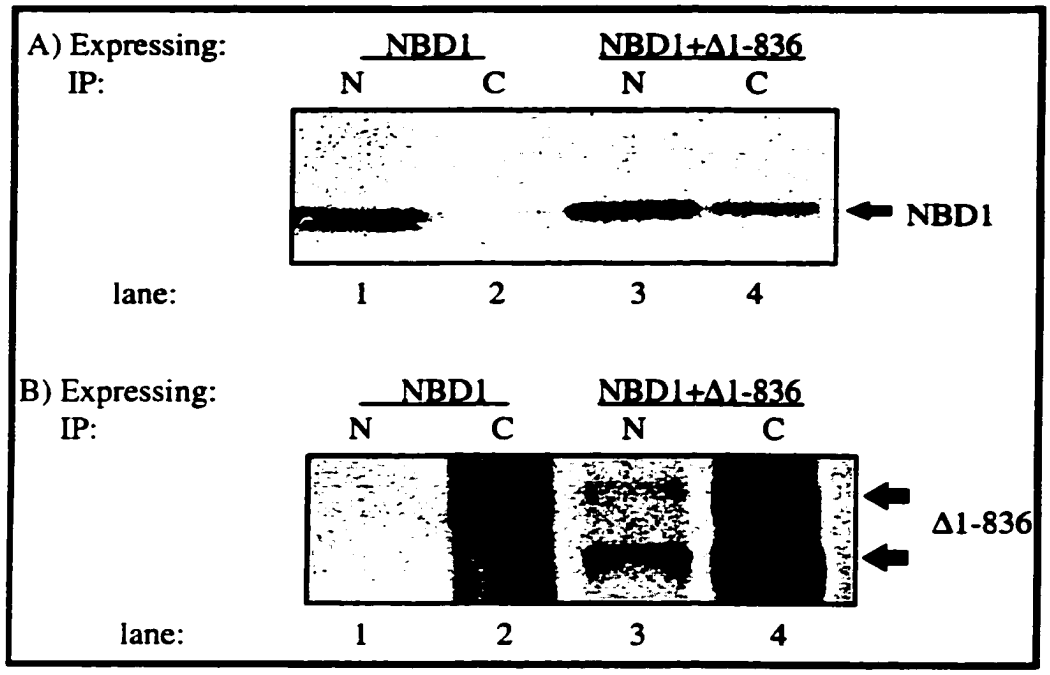


FIG. 30. NBD1 binds Δ1-836. Panel A. NBD1 or NBD1+Δ1-836 was expressed in COS7 cells and immunoprecipitated from lysates using an antibody specific to NBD1 (N) or Δ1-836 (C). The Western blot was stained to detect NBD1. NBD1 coimmunoprecipitated with Δ1-836 (lane 4). Panel B. NBD1 or NBD1+Δ1-836 was expressed in COS7 cells and immunoprecipitated as in panel A. The Western blot was stained to detect Δ1-836. Δ1-836 could be detected in association with NBD1 (lane 3). IP = immunoprecipitating antibody.

ning after NBD2, Fig. 5). When NBD1 was coexpressed with Δ1-1377, coimmunoprecipitation of NBD1 in association with Δ1-1377 indicated a strong interaction between the two peptides (Fig. 31A, lane 3, Fig. 31B, lane 6). Another domain of CFTR, the R-domain, was also tested for interactions with Δ1-1377. While Δ1-1377 could be immunoprecipitated with NBD1, no interactions between the R-domain and Δ1-1377 were observed (Fig. 31B, lane 4).

The ΔF508 mutation does not affect NBD1 binding to distal domains

Fig. 31 demonstrates that NBD1 binds distal domains, including the C-terminal tail of CFTR. We tested the influence of the ΔF508 mutation on these interactions. ΔF508-mutated NBD1 was coexpressed with Δ1-836, and binding between the peptides was assayed by coimmunoprecipitation. The ΔF508 mutation did not inhibit the binding interaction between NBD1 and Δ1-836 (Fig. 32A, lane 4). We also tested the effect of the ΔF508 mutation on the interaction between NBD1 and Δ1-1377. ΔF508-mutated NBD1 bound Δ1-1377 in a manner indistinguishable from wild-type NBD1 (Fig. 32B, lane 6, and Fig. 32C, lane 5). These results suggest that cellular recognition of the ΔF508 mutation does not result from the loss of binding interactions between NBD1 and the C-terminal tail of CFTR.

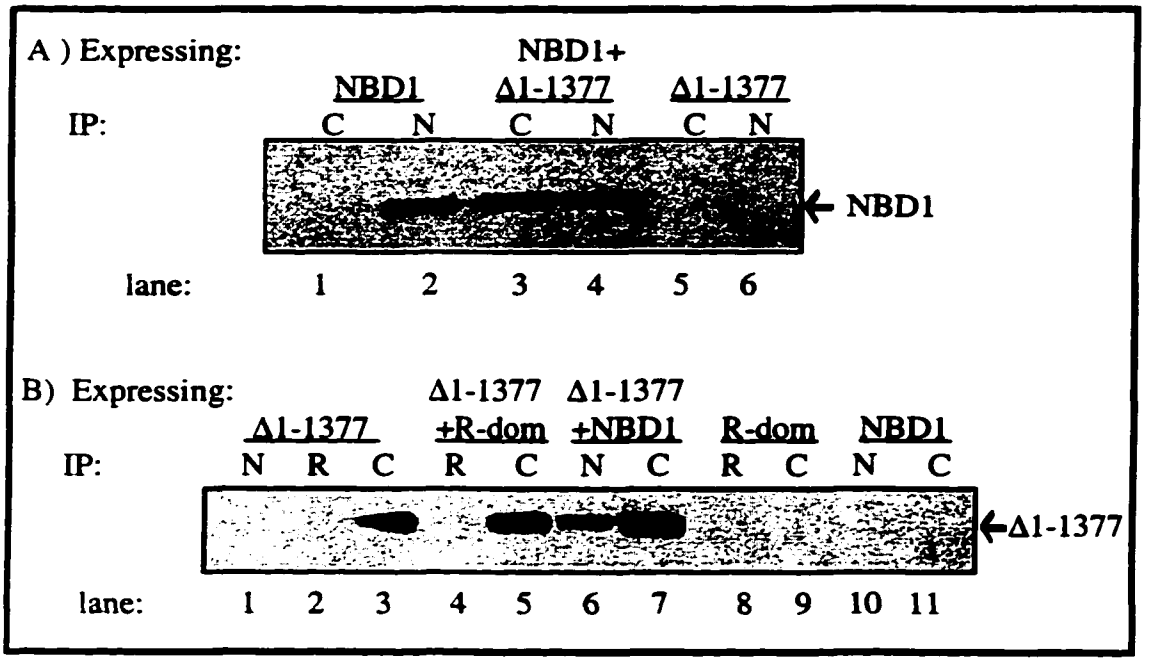


FIG. 31. NBD1, but not the R-domain, binds the C-terminal tail of CFTR. Panel A. NBD1, NBD1+Δ1-1377, or Δ1-1377 was expressed in COS7 cells and immunoprecipitated from lysates using antibodies specific to Δ1-1377 (C) or NBD1 (N). The Western blot was stained to detect NBD1. NBD1 was detected coimmunoprecipitating with Δ1-1377 (lane 3). Panel B. Δ1-1377, Δ1-1377+R-domain, Δ1-1377+NBD1, R-domain, or NBD1 was expressed in COS7 cells and immunoprecipitated from lysates using antibodies specific to NBD1 (N), Δ1-1377 (C), or the R-domain (R). The Western blot was stained to detect Δ1-1377. Δ1-1377 was detected coimmunoprecipitating with NBD1 (lane 6), but not with the R-domain (lane 4). IP = immunoprecipitating antibody.

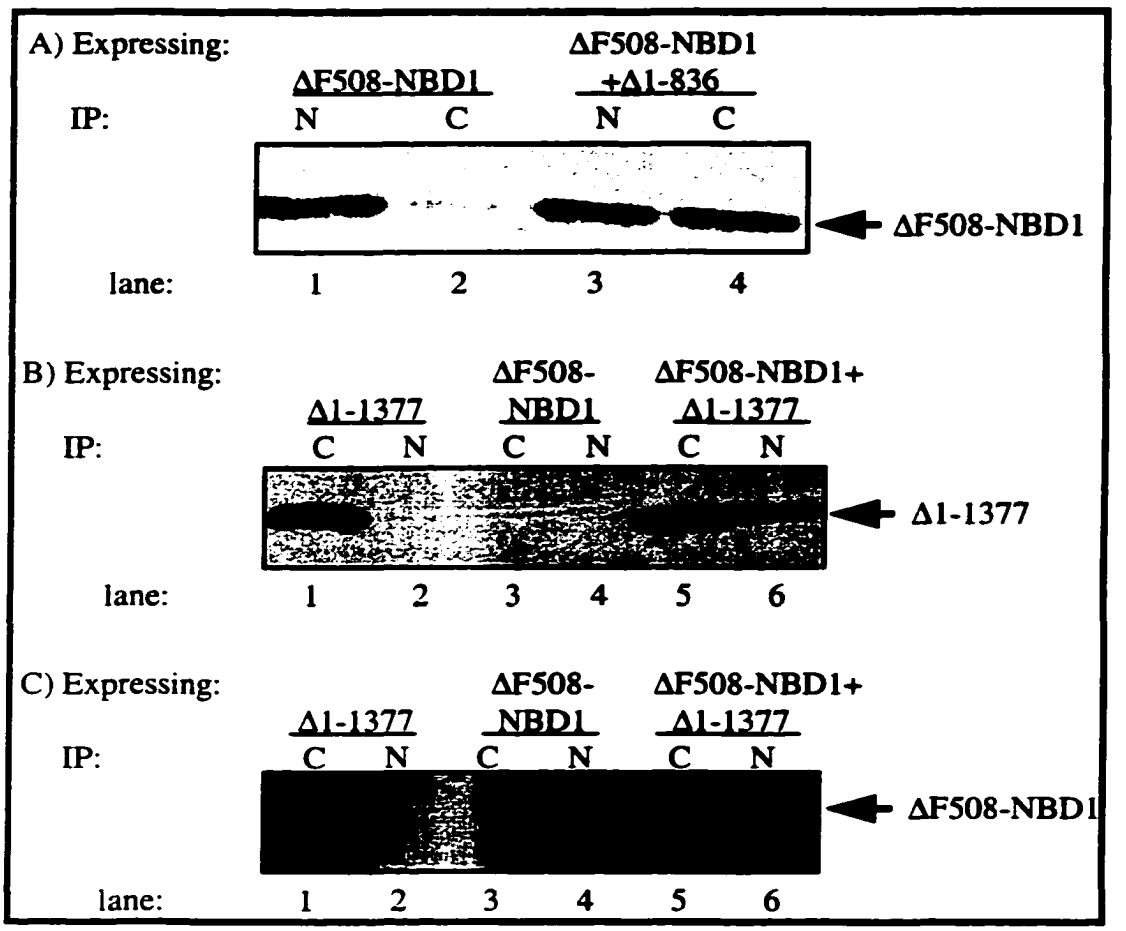


FIG. 32. The ΔF508 mutation does not alter NBD1 binding to Δ1-836 or Δ1-1377. Panel A. ΔF508-NBD1 or ΔF508-NBD1+Δ1-836 was expressed in COS7 cells and immunoprecipitated as in FIG. 31. ΔF508-NBD1 coimmunoprecipitated with Δ1-836 (lane 4). Panel B. Δ1-1377, ΔF508-NBD, or ΔF508-NBD1+Δ1-1377 was expressed in COS7 cells and immunoprecipitated as in Fig. 31. Δ1-1377 coimmunoprecipitated with ΔF508-NBD1(lane 6).
Panel C. Δ1-1377, ΔF508-NBD1, or ΔF508-NBD1+Δ1-1377 was expressed in COS7 cells and immunoprecipitated as in panel B. Δ1-1377 coimmunoprecipitated with ΔF508-NBD1 (lane 5). IP = immunoprecipitating antibody.

DISCUSSION

Domain interactions within CFTR

Many prokaryotic members of the ATP-binding cassette gene family are transcribed on separate genes that bind post translationally through multiple domain interactions to form the functional protein. For example, the histidine permease is composed of two hydrophobic subunits, HisM and HisQ, and two copies of the NBD, HisP. Interactions between these domains are necessary for protein assembly and function. Mutations disrupting normal binding between HisP and the TMDs lead to functional consequences, including constitutive ATP hydrolysis (Liu *et al.*, 1999). Because of the homology between the domains of CFTR and some prokaryotic members of this gene family, we tested the hypothesis that intramolecular domain binding interactions within CFTR were an important aspect in the assembly and function of CFTR. The experiments shown in Figs. 7, 9, and 10 demonstrate that CFTR domains synthesized on different ribosomes integrate into the ER membrane, locate each other prior to entering the Golgi, and bind in a fashion that leads to high level function at the plasma membrane. Intramolecular binding within CFTR has been described previously between the two membrane spanning domains of CFTR (Ostedgaard *et al.*, 1997), and between the cytosolic R-domain and N-terminal tail (Naren *et al.*, 1999). The experiments presented here establish that intramolecular binding also occurs between the R-domain and downstream regions of CFTR and that this association has functional consequences.

Consequences of intramolecular binding by the CFTR R-domain

The experiments presented here were designed to test the interaction of the R-domain of CFTR with distal regions of the protein and to determine whether these interactions play a role in R-domain phosphorylation and CFTR activation. When the R-domain (amino acids 596-837) was coexpressed with downstream elements of CFTR (residues 836 to 1480), physical binding between these two portions of CFTR was established by coimmunoprecipitation (Fig. 25). The interaction of Δ1-836 with the R-domain also conferred a mobility shift of the R-domain protein on SDS-PAGE. Dulhanty and Riordan (1994a) demonstrated that a reduction in mobility on SDS-PAGE of recombinant R-domain was associated with specific conformational changes. In our studies in living cells, a similar reduced mobility of the R-domain bound to Δ1-836 was dependent on phosphorylation and could be eliminated by alkaline phosphatase treatment of the immunoprecipitated protein (Fig. 26). These biochemical consequences of R-domain interactions were also observed with M837X, an amino terminal CFTR peptide that includes the R-domain. M837X coexpressed with Δ1-836 or after cellular pretreatment with forskolin resulted in phosphorylation dependent, reduced mobility on SDS-PAGE (Figs. 7, 10, 11, 12, and 13). These studies indicate, therefore, that the R-domain binds to distal regions of CFTR and that this interaction results in a phosphorylation dependent change in the R-domain mobility.

Phosphorylation dependent activation of CFTR

CFTR is activated upon phosphorylation of the R-domain by PKA. Cleaving the CFTR molecule at residue 836 markedly alters the protein so as to release CFTR from

tonic R-domain inhibition (Fig. 7). The cellular phenotype under these conditions resembles that of the Δ725-835 CFTR (Fig. 8). However, the mechanism underlying the activity of M837X and Δ1-836 differs fundamentally from Δ725-835 CFTR, since only in the former situation was constitutive function due to phosphorylation events that could be inhibited by blocking PKA (Figs. 15 and 22). Kinase activity was also required for the phosphorylation dependent reduction in mobility of M837X. Cellular pretreatment with the potent kinase inhibitor staurosporine blocked the forskolin-induced mobility shift of the M837X protein (Fig. 13), in addition to the constitutive activity of M837X and Δ1-836 (Fig. 14). The PKA specific inhibitor Rp-8-CPT-cAMPS was also able to diminish reversibly the halide permeability of M837X and Δ1-836 (Figs. 15 and 16), indicating that this constitutive function requires PKA activity. Since basal PKC phosphorylation of CFTR has also been suggested to be necessary for CFTR activity, we tested the effects of PKC inhibitors on the mobility shift of M837X coimmunoprecipitated with Δ1-836. Fig. 17 demonstrates that, while the nonspecific kinase inhibitor staurosporine blocked the molecular weight shift of the M837X bound to Δ1-836 and the PKA inhibitor Rp-8-CPT-cAMPS diminished the amount of shifted band, the PKC inhibitors chelethérine and Bin-1 did not alter the amount of the shifted protein band at all. While PKA inhibitors diminish both the activity of the two halves and the mobility shift of the first half, treatment with Rp-8-CPT-cAMPS did not completely eliminate either of these events. While we have demonstrated that functional PKA is important for the mobility shift and the high basal activity of the two halves, we cannot rule out the possibility that other kinases are involved. PKC phosphorylation may, for example, enhance the sensitivity of M837X to PKA phosphorylation.

The mechanism leading to the mobility shift of the R-domain and the high activity of the two halves under basal conditions relies on interactions between the R-domain and distal domains. Since the R-domain is believed to have not only an inhibitory effect on CFTR channel activity but a stimulatory role, as well, it is possible that binding of the R-domain to downstream domains stimulates halide channel activity instead of simply alleviating a block in the pore of the protein. A stimulatory role could result from interacting with the NBDs and enhancing the binding to or hydrolysis of ATP. Interactions between M837X and Δ1-836 resulted in phosphorylation events leading to the loss of R-domain participation in CFTR blockade (Fig. 7). Interestingly, when the R-domain was reattached to the second half of CFTR (Δ1-595) and coexpressed with M837X, forskolin activation of the complex was restored (Fig. 24). The R-domain, therefore, appears to require proper anchoring to distal domains in order to regulate the channel competently. This result may also suggest that an improperly anchored R-domain is more readily phosphorylated or, alternatively, is less accessible to phosphatases that normally inhibit CFTR activity.

In summary, based on the observations that (a) the phosphorylation dependent higher molecular weight M837X was dramatically increased when bound to Δ1-836, (b) a high basal halide permeability was produced by coexpression of M837X with Δ1-836, and (c) this activity was suppressed by PKA inhibitors, we conclude that PKA dependent R-domain phosphorylation and CFTR activation were promoted by R-domain interactions with downstream elements of CFTR. The increased susceptibility of the R-domain to PKA phosphorylation in the two-subunit model of CFTR (M837X and Δ1-836) may reflect greater accessibility of the R-domain to endogenous PKA or blockade of phosphatase action. In either case, the results presented here offer a means by which

R-domain function in living cells can be monitored biochemically and mutations that disrupt PKA regulation of CFTR can be better characterized.

Functional subdomain within the R-domain of CFTR

Although the R-domain has been defined as a single discrete domain derived from CFTR exon 13, Dulhanty and Riordan (1994b) suggested that the R-domain might be divided into two functionally distinct subregions, RD1 (amino acids 587-672) and RD2 (amino acids 679-798). The amino terminal third of the R-domain, RD1, appears necessary for chloride channel function (Rich *et al.*, 1993b). This region exhibits strong homology between CFTRs from several different species and also contains sequence homology to the linker region of another ABC polypeptide, the multi-drug-resistant (MDR) protein. In the present study, we show that the C-terminal portion of the R-domain (RD2), while not necessary for channel activity, plays a critical role in CFTR inhibition under basal conditions. If the carboxy terminus of the R-domain (the last 114 amino acids) is omitted from M837X, the PKA dependent activity when coexpressed with Δ1-836 is lost. G723X plus Δ1-836 still results in constitutive halide permeability (Fig. 19); however, the basal activity resembles the PKA independent activity of Δ725-835-CFTR (Fig. 23). Furthermore, in contrast to M837X, G723X failed to exhibit a reduced mobility on SDS-PAGE after either coexpression with Δ1-836 or cellular forskolin treatment (Fig. 20). The portion of the R-domain from amino acids 723 to 837 is therefore required for both PKA dependent activity and biochemically altering the mobility of the R-domain upon phosphorylation in cells. Screening different portions of R-domain for the phosphorylation dependent mobility shift isolated a smaller region necessary for this change. While

PKA treatment of a peptide of amino acids 595-813 resulted in a reduced mobility of the peptide, the same treatment of a peptide of amino acids 595-740 did not (Fig. 27). The region from 740 to 813 is therefore necessary for the conformational change in response to PKA and is likely to contribute to the "switch" regulating initial PKA dependent CFTR activation. There are two dibasic (R-R/K-X-S) PKA sites, serines 795 and 813, within the region from 740 to 813 that have been implicated in the regulation of CFTR. A peptide of 708-835 also exhibited a shift in mobility upon PKA phosphorylation, verifying that the C-terminal region is sufficient for the mobility shift and does not require upstream regions of the R-domain for this effect.

Identification of domain binding sites within CFTR

A goal of this dissertation was to observe binding interactions involving cytosolic domains that may influence the proper maturation of the protein. Both the R-domain and NBD1 were found to bind regions of CFTR downstream of residue 836 (Figs. 25 and 31). NBD1, but not the R-domain, binds strongly to Δ1-1377, the C-terminal tail of CFTR beginning after NBD2 and including the C-terminal PDZ binding domain (Fig. 32). While NBD1 binds to Δ1-1377, the R-domain strongly interacts with residues between 836 and 1377, possibly at NBD2 (Rich *et al.*, 1991). The binding of these domains to Δ1-836 occurs at a time when folding and processing determine the fate of nascent CFTR molecules (*i.e.*, in the ER, Figs. 25 and 30). Δ1-1377 could not be coimmunoprecipitated with soluble R-domain and NBD1 did not bind a control protein (ß-gal), supporting the specificity of the NBD1/Δ1-1377 interaction. ER localized binding between NBD1 or the R-domain and other portions of CFTR has not been described previously.

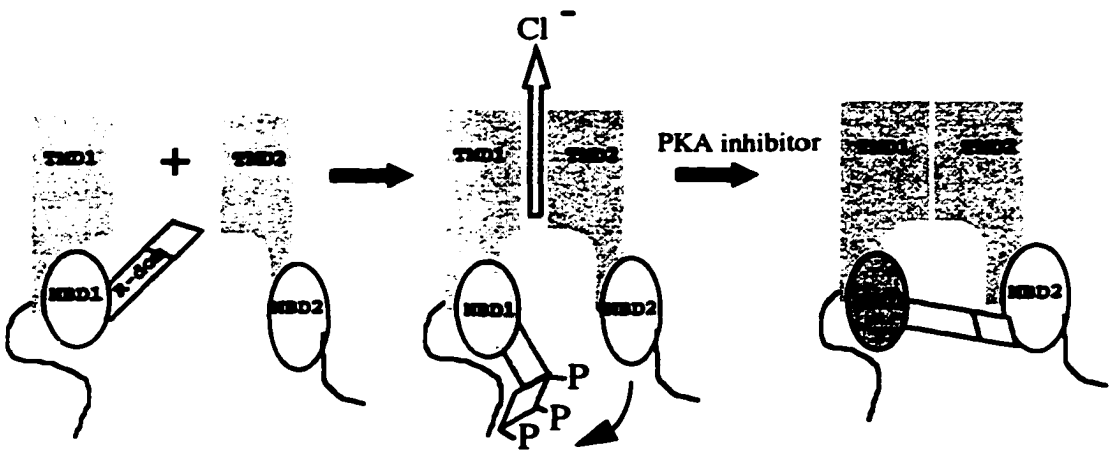
The effect of the ΔF508 processing mutant on CFTR domain interactions

Domain:domain recognition is believed to be an important aspect of protein folding, as well as a criterion for entry into the Golgi and subsequent protein maturation. Because ΔF508 within NBD1 disrupts protein folding and maturation (Gregory *et al.*, 1991; Lukacs *et al.*, 1994; Qu *et al.*, 1997b; Zhang *et al.*, 1998), we tested whether steady state binding of NBD1 to more downstream domains was influenced by this mutation. The omission of F508 faithfully reproduced the loss of activity in a CFTR molecule severed at residue 836, suggesting that cellular mechanisms recognize the mutation in this CFTR model (Fig. 28). While disrupted processing of the ΔF508-mutated two halves would be expected to be responsible for the loss of activity, we cannot rule out that the mutated protein traffics to the membrane but is functionally defective. Domain binding between the large half fragments of CFTR, M837X, and Δ1-836 was not detectably disrupted by the mutation. However, the existence of other binding interactions between the halves (e.g., involving the R-domain or TMDs) would be expected to mask the influence of the ΔF508 mutation in this respect. We therefore tested the ΔF508 mutation on the binding of isolated NBD1 to distal regions of CFTR. The binding of isolated NBD1 to Δ1-836 or to Δ1-1377 was not abrogated by ΔF508 (Fig. 32). These results indicate that the ΔF508 mutation can still be recognized in cells when CFTR domains self-assemble; however, a defect in the domain interactions investigated here cannot be implicated as underlying the ΔF508 processing abnormality. The results do not exclude disruption of other domain binding interactions by ΔF508 (e.g., between NBD1 and NBD2) or the possibility that more sensitive detection methods might indicate novel binding defects.

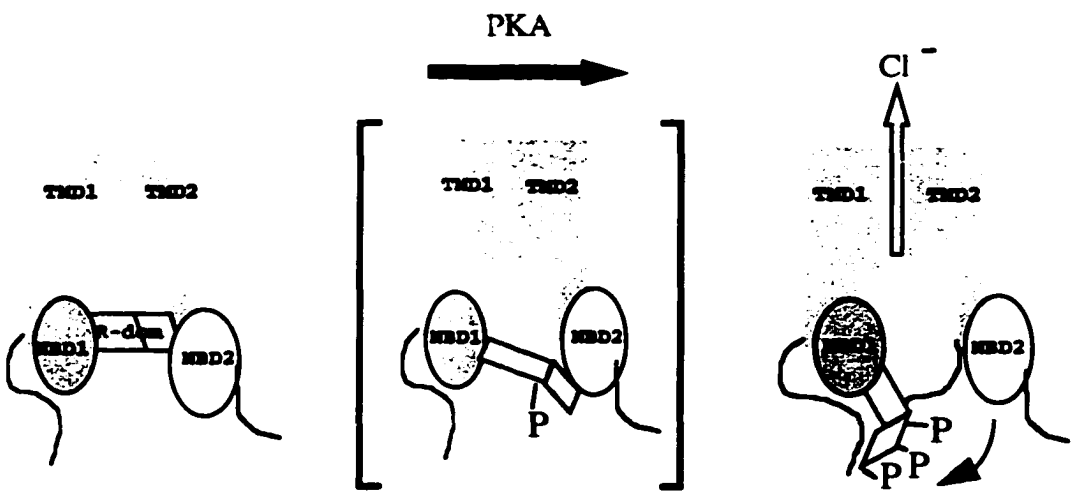
Summary

Coexpression of amino (M837X) and carboxy (Δ1-836) portions of CFTR produces predominantly unregulated halide efflux. The addition of kinase inhibitors renders the cleaved protein inactive and indicates specificity of the phosphorylation dependent activating mechanism. Therefore, we suggest a model in which the anchoring of the R-domain to downstream regions (beginning at TMD2) maintains CFTR in an inactive basal state. Cutting the R-domain anchor (expressing CFTR as M837X and Δ1-836) or activation by PKA confers enhanced R-domain phosphorylation and results in a conformational change in the M837X protein that leads to activation. This model suggests that in full length (wild-type) CFTR, initial activation could also be expected to require domain interactions and R-domain conformational changes (Fig. 33).

Intramolecular binding within CFTR is complex. The second half of CFTR (after residue 836) has affinity for both the R-domain and isolated NBD1. The binding interactions involving the R-domain alter the conformation of the protein, indicating that the binding has functional consequences. Therefore, while CFTR is synthesized as a single polypeptide chain, our results suggest that CFTR domains are highly interactive. This process resembles the self-assembly and function of several prokaryotic members of the ATP binding cassette gene family and establishes the importance of domain binding in the structure and activity of eukaryotic members of the ABC gene family, as well. Because domain interactions are required for proper maturation of other membrane proteins, we examined whether a CFTR processing mutation disrupted the interactions of NBD1 with other portions of CFTR. The ΔF508 mutation did not disrupt physical binding of



M837X + Δ1–836
PKA dependent constitutive activity



Wild-type CFTR
PKA regulated activity

FIG. 33. Model of M837X and Δ1–836 constitutive activity.

M837X or NBD1 to Δ1-836 but did abolish the functional interactions so that halide permeability at the cell surface is no longer observed.

In conclusion, this dissertation provides new biochemical evidence for a PKA-dependent structural change in the R-domain as a direct result of phosphorylation in living cells. This change correlates with CFTR activation and is strongly facilitated by binding between the R-domain and CFTR residues after amino acid 836. Moreover, we have characterized a subdomain within the regulatory domain of CFTR (residues 723-837) that is necessary for PKA dependent activation. Finally, these experiments demonstrate that constitutive CFTR activity can be accomplished by at least two mechanisms: (a) direct modulation of the R-domain (by serine mutations or partial R-domain deletions) or (b) enhanced susceptibility of CFTR to steady state PKA phosphorylation.

FUTURE STUDIES

The experiments described in this dissertation provide a means by which intramolecular binding interactions within CFTR can be more fully explored. We have established binding between two large regions of CFTR (M837X and Δ1-836) in order to examine the complex mechanism by which the CFTR polypeptide fits together in the ER and plasma membrane. In the future, it will be important to localize regions of the second half of CFTR that bind the R-domain and determine the portion of Δ1-836 (minimal amino acid sequences) necessary to facilitate phosphorylation of the R-domain. An improved understanding of R-domain activity would result from such studies, and polypeptide activators of CFTR might also be developed in this way.

Studies of other ion channels such as the Shaker K^+ channel and the Na/K ATPase have shown that domain binding is an important prerequisite for proper maturation. Class II CFTR mutations, including ΔF508 CFTR, are not properly trafficked. The functional defect appears to be faithfully reproduced in the CFTR half-molecule model. In our studies, we have begun to lay a foundation for understanding the ways in which NBD1 and Δ1-1377 may form binary complexes during proper folding. Although our initial experiments have not identified a gross defect in domain binding caused by the ΔF508 mutation, detailed measurements of binding, stoichiometry, and affinity have not yet been performed and should be done in order to evaluate the possible role of ΔF508 in domain

binding and maturational processing. Other class II CFTR mutations could also be studied by this approach.

Finally, our experiments suggest the importance of PKA sites in the interval between amino acids 740 and 837 in phosphorylation and activation. The half-molecule model presented here offers a direct means for evaluating consensus PKA sites in this region (serines at 795 and 813) for a role in mediating an R-domain conformational change. Site directed mutagenesis could be used to determine which serine(s) play a role in R-domain conformational changes. Clinically important mutations in the R-domain that result in plasma membrane targeted CFTR with diminished channel activity (e.g., D614G, I618T, G622D, R792G, and E822K) can also be examined by these methods. Similarly, R-domain point mutations that augment CFTR function (e.g., H620Q and A800G) could be tested within M837X for their ability to elicit a mobility shift of the protein.

In summary, the experiments described here set the stage for future studies designed to improve our understanding of CFTR phosphorylation and activation and possibly of the mechanisms by which mutations that disrupt processing (class II) or regulation (class III) cause disease.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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