

The regulation of CFTR by protein-protein interactions

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ABSTRACT

William R. Thelin: The regulation of CFTR by protein-protein interactions

(Under the direction of Dr. Sharon L. Milgram)

Cystic fibrosis (CF) is an autosomal recessive disease resulting from the misregulation of epithelial ion transport. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane chloride channel expressed in polarized epithelial cells. To identify factors that regulate CFTR activity, we utilized biochemical and proteomics approaches to identify novel CFTR binding proteins.

We find that the C-terminus of CFTR directly interacts with the serine/threonine phosphatase PP2A. PP2A is a heterotrimeric phosphatase composed of a catalytic subunit and two divergent regulatory subunits (A and B), which mediate the cellular localization and substrate specificity of the enzyme. By mass spectrometry, we identified the exact PP2A regulatory subunits associated with CFTR as A α and B' ϵ , and find that the B' ϵ subunit binds CFTR directly. PP2A subunits localize to the apical surface of airway epithelia and PP2A phosphatase activity co-purifies with CFTR in Calu-3 cells. In functional assays, PP2A inhibition blocks the rundown of basal CFTR currents and increases channel activity in excised patches of airway epithelia and in intact mouse jejunum. Moreover, PP2A inhibition increases the pericilliary liquid in cultures of well differentiated human bronchial epithelial

cells by a CFTR-dependent mechanism. Thus, PP2A is a relevant CFTR phosphatase in epithelial tissues and may be a clinically relevant drug target for CF.

Additionally, the N-terminus of CFTR directly interacts with two actin binding proteins, filamin A and filamin B. In polarized epithelial cells, filamins are highly localized to the sub-apical compartment where they likely interact with CFTR at or near the plasma membrane. We find that CFTR and filamins specifically interact by co-immunoprecipitation and that a disease-causing mutation in CFTR, serine 13 to phenylalanine (S13F), disrupts this interaction. Consistent with the loss of cytoskeletal anchorage, S13F CFTR displays decreased cell surface levels and less confinement at the plasma membrane relative to wild-type CFTR. Furthermore, S13F CFTR is more rapidly degraded compared to wild-type CFTR which correlates with the accumulation of S13F CFTR in the lysosomes. Taken together, these data suggest the filamins regulate the cell surface stability and endocytic trafficking of CFTR.

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LIST OF ABBREVIATIONS

A2BR	Adenosine Receptor
ABP	Actin binding protein
AMPK	Adenosine monophosphate kinase
AP2	Adaptor protein 2
ASL	Airway surface liquid
ATP	Adenosine 5'-triphosphate
β 2-AR	Beta 2-aderenergic receptor
BHK	Baby hamster kidney
CAL	CFTR-associated ligand
cAMP	3'-5'-cyclic adenosine monophosphate
cGK-II	cGMP dependent protein kinase II
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
ER	Endoplasmic reticulum
ERAD	ER associated degradation
FLN	Filamin
GCC	Guanylyl cyclase C
GFP	Green fluorescent protein
HSP	Heat shock protein
HEK	Human embryonic kidney
Isc	Short circuit current
LC	Liquid chromatography
MS	Mass spectrometry
NBD	Nucleotide binding domain
NHERF	Sodium hydrogen exchange regulatory factor
R Domain	Regulatory domain
PCL	Pericilliary liquid
PDE	Phosphodiesterase
PDZ	Post-synaptic density 95, Discs large, ZO-1
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PP1	Protein phosphatase type 1
PP2	Protein phosphatase type 2
SPT	Single particle tracking
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SNX	Sorting nexin
TCZ	Transient confinement zone
TOF	Time-of-flight

Chapter 1: The physiology of CFTR

Epithelial cells provide a physical barrier which regulates the flow of substances between an organism and its environment. Epithelia function to prevent bacteria and other harmful substances from accessing the blood stream. Furthermore, epithelial cells must balance their protective function with the selective uptake of nutrients and electrolytes. The ability of epithelial cells to function as a barrier and transport tissue is derived from their polarized nature. The apical (luminal) plasma membrane of epithelial cells is physically separated from the basolateral (serosal) membranes by tight junctions (Nelson et al., 1992; Wollner and Nelson, 1992). Receptors, ion channels, and lipids are transported to selective membrane domains, giving rise to the polarized distribution of signaling and transport molecules. This segregation of cell surface proteins allows epithelia to achieve the vectorial transport of water, ions, and other solutes.

The lungs are chronically exposed to pathogens and other noxious particles through normal tidal breathing. Airway epithelial cells provide a first line of innate host defense by regulating an elaborate clearance mechanism. In the lungs, the luminal surface is covered by a thin film known as the airway surface liquid (ASL) composed of a mix of gel and sol (Widdicombe, 1989). The mucus gel is primarily composed of mucins, which are high molecular weight glycoproteins secreted by goblet cells. The mucus layer traps bacteria and inhaled particles preventing them from accessing the airway cells. The high carbohydrate content and oligosaccharide diversity of mucins is thought to facilitate bacterial adhesion

(Thornton et al., 1991). In addition to a purely passive role, antimicrobial peptides known as defensins are secreted into the mucus where they are proposed to disrupt bacterial cell walls by forming membrane pores (Chilvers and O'Callaghan, 2000). Mucus clearance is regulated by ciliated epithelial cells which propel the mucus through the upper airways where it is swallowed or expectorated. The normal function of airway cilia is dependent upon the composition of periciliary fluid (PCL) underlying the mucus layer. The PCL provides a low viscosity fluid which facilitates ciliary movement and is critical for mucociliary clearance (Tarran et al., 2001).

Diseases which disrupt mucociliary clearance, such as cystic fibrosis (CF) or primary ciliary dyskinesia, significantly increase susceptibility to life-threatening infections. CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator, an apical membrane chloride channel (Riordan et al., 1989). The loss of CFTR function results in an imbalance of salt and water transport in airway epithelial cells. This imbalance prevents the efficient clearance of mucus in the lungs ultimately leading to the formation of thick mucus plugs in the lower airways (Quinton, 1999). Opportunistic pathogens, such as *Pseudomonas aeruginosa*, colonize the mucus causing lung infections (Kubesch et al., 1993). In CF patients, chronic lung infection is the primary cause of morbidity.

Prior to the cloning of the gene encoding CFTR, many pioneering studies had identified that CF was caused by the loss of a cAMP-dependent chloride conductance which likewise altered the transport of other ions such as sodium and bicarbonate (Poulsen et al., 1994; Smith et al., 1996; Stutts et al., 1997). In addition to a loss of apical chloride efflux, CF epithelia exhibit significantly increased sodium absorption. Currently, there are two

prominent hypotheses which attempt to link the defect in ion transport to chronic lung infections. The “compositional” hypothesis suggests that, in the absence of CFTR, epithelial cell absorption of NaCl is decreased, thereby resulting in an increased NaCl concentration in the ASL. At NaCl concentrations exceeding 100 mM, the function of the antimicrobial defensins is impaired. Thus, the “compositional” hypothesis predicts that in the absence of functional defensins, bacteria are able to colonize the mucus layer leading to infection. Smith et al. found that bacterial did not efficiently grow when placed on the apical surface of well-differentiated primary airway epithelial (WD-PAE) cells (Smith et al., 1996). However, the mucus from CF cultures did not inhibit bacterial growth suggesting that the antimicrobial properties were impaired. Consistent with the “compositional” hypothesis, adding water to the apical surface of the CF cultures to decrease the salt concentration restored the ability of the mucus to inhibit bacterial growth (Smith et al., 1996). A second hypothesis, known as the “volume” hypothesis suggests that the ASL height, not ASL composition, is impaired in CF airways. The “volume” hypothesis proposes that a dominant role of CFTR is to negatively regulate the activity of the epithelial sodium channel (ENaC). Based on observations that the activity of the epithelial sodium channel is significantly increased in CF airways, this model predicts that isotonic salt transport results in a volume-depleted ALS (Stutts et al., 1997). Furthermore, decreases in ASL volume affect mucociliary clearance two-fold. First, the PCL height is reduced which impairs ciliary movements. Secondly, the mucus layer becomes more viscous and is therefore more difficult to clear. Thus, mucus accumulates in the lower airway which supports bacterial colonization. Recently, Mall et al. provided strong support for the “volume” hypothesis by producing a CF-like phenotype in transgenic mice over-expressing ENaC subunits (Mall et al., 2004). One could imagine that

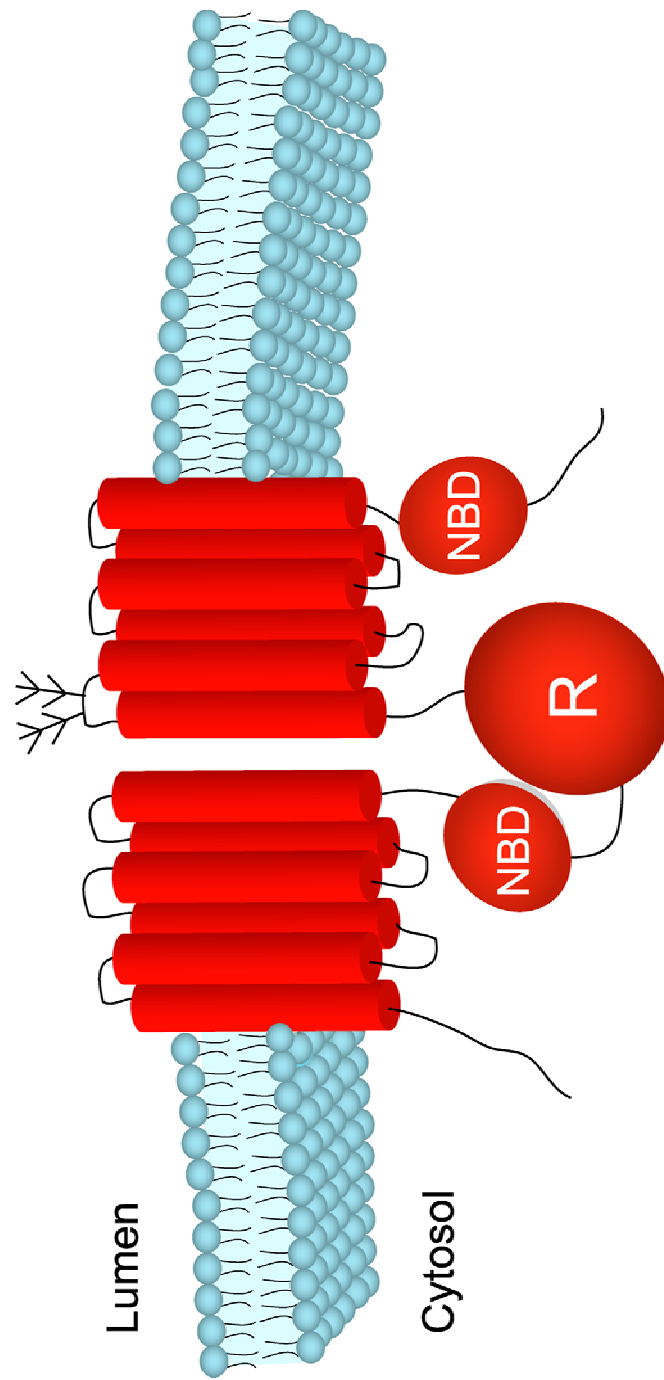
measuring the salt concentrations of ASL derived from normal or CF airways would distinguish between the “compositional” and “volume” models. However, the thin layer of ASL has proven difficult to collect and analyze (Tarran et al., 2001). Alternatively, newer approaches in which the ASL of WD-PAE cultures is labeled with fluorescent dextrans and measured by confocal microscopy has provided a powerful means to study ASL volume. Using this approach Tarran et al. found that the inhibition of CFTR results in a dramatic decrease in ASL height (from 13 μM to 7 μM), while CFTR activation increases ASL height (Tarran et al., 2005). These data provide strong evidence that CFTR activity is directly coupled to the regulation of ASL volume.

In 1989, Riordan et al. reported the cloning of the gene encoding CFTR which led to a rapid increase in our understanding of this disease (Riordan et al., 1989). Based on sequence similarity, it was evident that CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily. ABC transporters include a large number of proteins (48 ABC transporter genes in humans alone) found in both prokaryotic and eukaryotic organisms which transport a diversity of substrates including ions, peptides, lipids, and other solutes. CFTR, like other ABC transporters, is topologically composed of two membrane-spanning domains (MSD), which form the membrane pore, and two nucleotide binding domains (NBDs) (Riordan et al., 1989). The NBDs contain sequences predicted to bind and hydrolyze MgATP, such as the Walker A, Walker B, and LSGGQ motifs. In addition, CFTR is dually glycosylated on asparagine residues 894 and 900 in the 4th extracellular loop, which may have a role in CFTR folding and degradation (Cheng et al., 1990) (Figure 1.1).

Since the identification of the gene encoding CFTR, more than 1,000 different disease-causing mutations have been identified in CF patients. The CFTR mutations are

Figure 1.1. CFTR is an ABC transport protein

Similar to other ABC transporters, CFTR contains two membrane spanning domains composed of six transmembrane domains and two nucleotide binding domains which hydrolyze ATP to ADP. Unlike other ABC transporters, CFTR has a unique cytosolic regulatory domain which regulates channel gating.



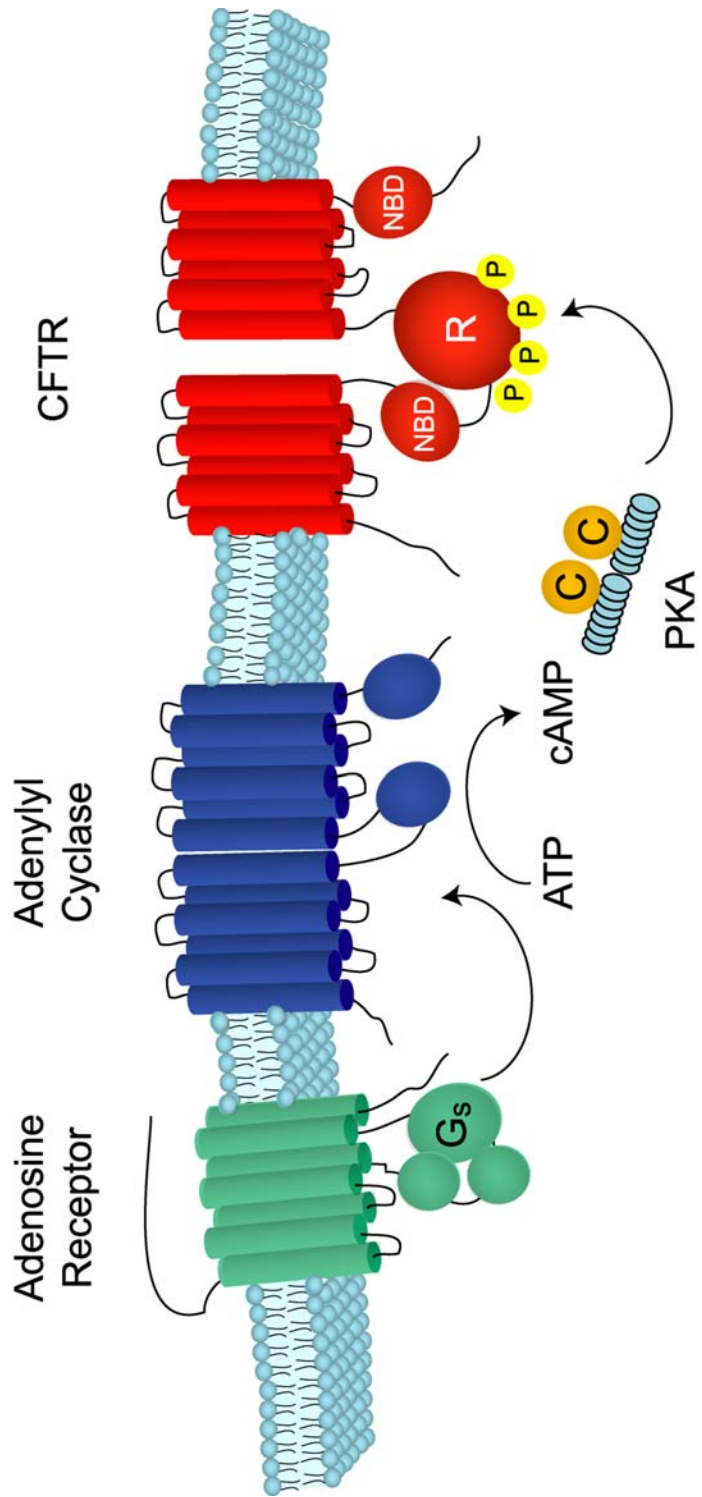
functionally categorized by defects in early biosynthesis and folding (Class I, II, and V) or chloride channel activity (Class III and IV) (Welsh and Smith, 1993; Zielenski and Tsui, 1995). Class I mutations are caused by nonsense mutations which prematurely truncate the protein. Class II mutations interfere with the folding of CFTR in the endoplasmic reticulum (ER). As a result, CFTR is retained in the ER where it is eventually targeted for degradation by the ER-associated degradation pathway (ERAD). Class II mutations are the most common and include the $\Delta F508$ (deletion of phenylalanine 508) mutation found in ~70% of all CF patients. Class III and IV mutations affect channel regulation at the cell surface. For example, the G551D, a class III mutation, occurs in the LSGGQ motif in NBD1 and severely impairs channel gating (Delaney et al., 1996; Oceandy et al., 2003; Oceandy et al., 2002). Class IV mutations, such as R117H, result in altered channel conductance. Finally, class V mutations result in reduced CFTR mRNA and, therefore, reduced protein (Zielenski and Tsui, 1995). More recently, Haardt et al. proposed a new class of CFTR mutations (Class VI) which cause the premature degradation of CFTR in a post-ER compartment (Haardt et al., 1999). The class VI mutations are based on the observation that CFTR proteins lacking at least the last 80 C-terminal amino acids are rapidly retrieved from the cell surface and degraded through a ubiquitin-dependent pathway.

Initial studies in cells which lacked a CFTR-like activity including Chinese Hamster ovary (CHO) (Anderson et al., 1991b), HeLa (Gregory et al., 1990), NIH-3T3 (Anderson et al., 1991b), and *Xenopus* oocytes (Bear et al., 1991) demonstrated that the expression of CFTR produced a unique cAMP-dependent chloride conductance. Furthermore, in excised membrane patches, the chloride channel activity of CFTR was shown to require the activity of the cAMP-dependent protein kinase (PKA) and MgATP (Anderson et al., 1991a). For

other ABC transporters, ATP hydrolysis at the NBDs provides the energy to actively transport substances across the plasma membrane. Initially, it was not clear why CFTR required the NBDs as chloride is passively transported through CFTR down a favorable concentration gradient. However, it is now clear that the hydrolysis of ATP by the NBDs provide the driving force for channel opening (Baukrowitz et al., 1994). The requirement for PKA activity comes from the unique CFTR regulatory domain (R domain). The CFTR R domain is a 272 amino acid stretch which separates NBD1 from MSD2. The R domain contains multiple di-basic PKA consensus phosphorylation motifs. As such, PKA can phosphorylate the CFTR R domain at least 10 times (Seibert et al., 1995). The phosphorylation of the R domain increases the rate of ATP hydrolysis by the NBDs and thereby increases channel opening (Howell et al., 2004) (Figure 1.2). The phosphorylation of the R domain is thought to induce a conformational change in CFTR which may promote interactions between NBD1 and NBD2 (Howell et al., 2004). In studies designed to examine the phosphorylation-dependent structural changes in R domain structure, Ostedgaard et al. found that the R domain is relatively unstructured in solution (Ostedgaard et al., 2000). Presently, it is not clear whether the R domain functions as an activation domain or inhibitory domain. The addition of exogenous R domain proteins blocked CFTR channel activity in patches, suggesting an inhibitory function (Ma et al., 1996). However, the R domain likely functions to stimulate channel activity as its phosphorylation potentiates CFTR activity to higher levels than CFTR mutants which do not require phosphorylation for gating (Baldursson et al., 2001). While the importance of the R domain with respect to CFTR channel activity is well appreciated, the mechanism by which the R domain functions is not understood. Current studies using structural, biochemical, and electrophysiological

Figure 1.2 CFTR is activated downstream of adenosine receptor activation

In airway epithelia, the activation of CFTR is thought to be predominately regulated by extracellular adenosine. Activation of the adenosine receptor leads to the generation of cAMP by membrane bound adenylyl cyclases. The catalytic subunit of PKA binds to cAMP and is released from the regulatory subunits where it can phosphorylate the CFTR R domain. The CFTR R domain can be phosphorylated at least 10 times by PKA which potently stimulates channel opening.



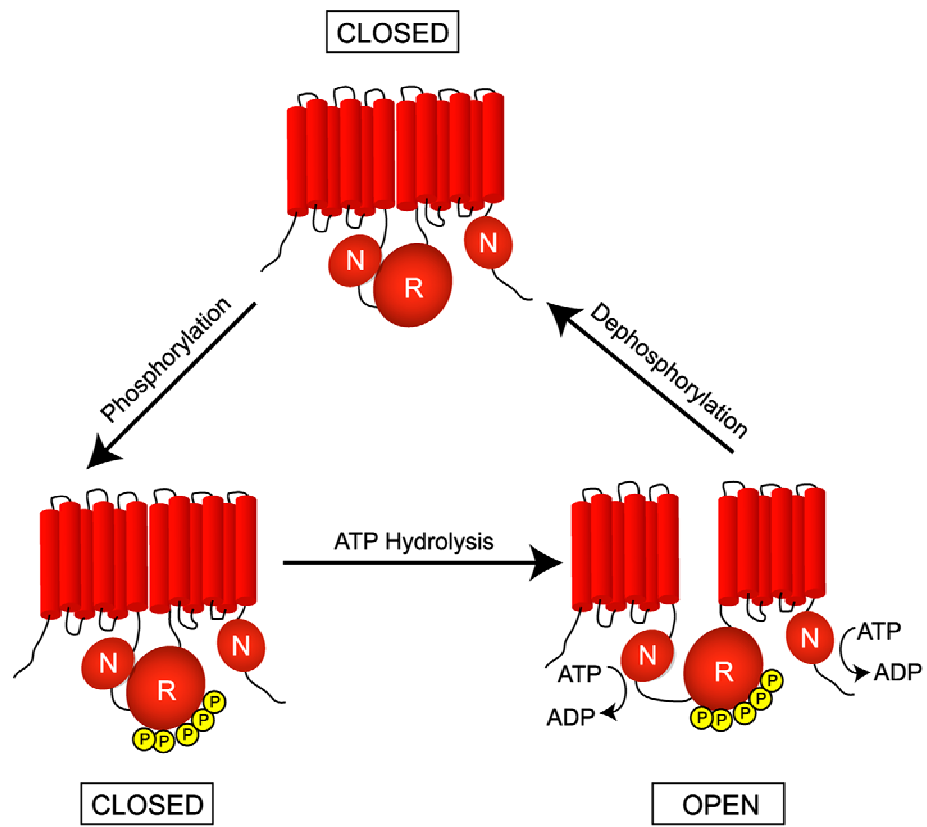
approaches are addressing how the R domain physically associates with the NBDs and how R domain phosphorylation influences these interactions.

The channel activity of CFTR is tightly controlled by a balance of kinase and phosphatase activity. CFTR is predominantly activated by PKA phosphorylation. However, other kinases have also been shown to regulate CFTR including Ca^{2+} -dependent and independent PKC isoforms (Chappe et al., 2003; Dahan et al., 2001; Liedtke et al., 2002), the cGMP dependent protein kinase II (cGKII) (Dahan et al., 2001), and the tyrosine kinase c-Src (Fischer and Machen, 1996). When added exogenously to patch preparations, PKC stimulates CFTR activity. However, the magnitude of CFTR activation by PKC alone is only ~15% of that by PKA (Chang et al., 1993). More importantly, Jia et al. showed that PKC phosphorylation was critical for the potentiation of CFTR channel activity by PKA (Jia et al., 1997). Based on these studies, they proposed a model whereby the constitutive phosphorylation of CFTR by PKC is required for PKA dependent activation of the channel. In addition, cGKII phosphorylation of CFTR activates the channel comparably to PKA activation (Dahan et al., 2001). However, the kinetics of cGKII phosphorylation is slower than PKA which has been predicted to reflect the initial requirement of membrane association for activated cGKII. The addition of the tyrosine kinase c-Src in excised patches increases CFTR gating; however, the physiological significance of Src-mediated CFTR regulation is not clear (Fischer and Machen, 1996).

The signaling pathways which activate CFTR *in vivo* are tissue dependent. In the airways, CFTR is predominantly activated by adenosine receptor (A2B) signaling, a Gs coupled G-protein coupled receptor (Eidelman et al., 1992). Activation of the A2B receptor by leads to the generation of cAMP by membrane bound adenylate cyclases. PKA is

Figure 1.3 CFTR channel gating reflects the balance of endogenous kinase and phosphatase activity

The phosphorylation of the CFTR R domain by kinases such as PKA, PKC, and cGK-II cause a conformational change in the channel increasing the efficiency of ATP hydrolysis by the NBDs. ATP hydrolysis provides the driving force for channel opening. The dephosphorylation of CFTR by serine-threonine phosphatases such as PP2A, PP2B, and PP2C deactivate the channel.



activated in response to the elevated cAMP levels, leading to CFTR phosphorylation and increased channel gating (Figure 1.3). CFTR can also be potently activated by the B₂-adrenergic receptor (β_2 AR) similarly to the A₂B receptor. The β_2 AR can activate CFTR in tissues such as sweat ducts. However, it is not clear whether the β_2 AR is a physiologically relevant activator of CFTR in the airways. In the intestines, CFTR is alternatively activated through cyclic GMP signaling pathways. Guanylate cyclase C (GCC) is an integral membrane guanylate cyclase expressed in the gut. GCC converts GTP into cGMP, in response to ligands such as guanylin or uroguanylin (Li et al., 1995b). CFTR is activated downstream of GCC activation by the cooperative actions of cGKII and PKA (Tien et al., 1994). In addition to its endogenous ligands, GCC is activated by heat stable enterotoxins (STa) elaborated by strains of pathogenic E. coli (Schultz et al., 1990). STa activation of GCC results in apical chloride efflux through CFTR, as well as, the inhibition of sodium transport through the sodium hydrogen exchange regulatory factor 3 (NHE-3). The net result of this signaling pathway is increased salt in the intestinal lumen. This likewise provides a driving force for water movement into the lumen resulting in secretory diarrhea (Zhang et al., 1999). In less developed countries, secretory diarrhea is a life-threatening health concern.

While the activation of CFTR has been extensively studied, much less is known about channel deactivation. Once it was clear that CFTR activity required kinase activity, phosphatases were obvious candidates for negative channel regulators. Work from many labs suggests that multiple phosphatases including PP2A, PP2B, PP2C, and alkaline phosphatase are involved in the deactivation of CFTR (Luo et al., 1998; Reddy and Quinton, 1996). Based on studies using exogenous phosphatases, PP2A and PP2C are the most effective at dephosphorylating CFTR *in vitro* (Berger et al., 1993). Furthermore, when added

to patch preparations, purified PP2A and PP2C both inactivate maximally phosphorylated CFTR (Luo et al., 1998). However, PP2C altered channel openings, while PP2A shortened CFTR burst duration. These data suggest that PP2A and PP2C distinctly regulate CFTR channel activity. Cell permeable phosphatase inhibitors have been used to study CFTR deactivation in intact cells. In human sweat ducts, cardiac myocytes, 3T3 fibroblasts, and Hi-5 insect cells, inhibitors of PP2A, such as okadaic acid and calyculin A, increase CFTR channel activity (Becq et al., 1994; Luo et al., 1998; Yang et al., 1997). In contrast, PP2A inhibitors were observed to have little effect on the rapid deactivation of maximally phosphorylated CFTR in T84 intestinal epithelial cells and human airway epithelial cells (Travis et al., 1997; Zhu et al., 1999). However, the role of PP2A in the regulation of CFTR in polarized epithelia has not been well characterized. In baby hamster kidney cells (BHK), PP2C co-immunoprecipitates with exogenous CFTR (Zhu et al., 1999). Additionally, PP2C overexpression in Fischer rat thyroid cells (FRT) decreases CFTR chloride conductance (Travis et al., 1997). However, the ability of endogenous PP2C to regulate CFTR in native epithelial tissues is unclear as no PP2C inhibitors have been identified. Furthermore, studies in NIH-3T3 fibroblasts suggest that the inhibition of PP2B also stimulates CFTR channel activity (Fischer et al., 1998). However, it is not clear if PP2B is a relevant CFTR phosphatase *in vivo*.

To date, many signaling molecules able to regulate CFTR have been identified. However, the ability of many of these protein kinases and phosphatases to modulate CFTR activity has been studied in heterologous expression systems, which may not accurately recapitulate the epithelial-specific regulation of CFTR. In polarized epithelia, CFTR is tightly regulated in a defined apical compartment which reflects the organization of CFTR

into protein complexes with regulatory factors. Furthermore, it is now appreciated that all aspects of CFTR biology are regulated by direct and indirect protein interactions. The study of CFTR protein interactions has provided a great deal of insight into the processes which regulate CFTR biogenesis, trafficking, and channel gating. The remainder of this chapter recounts much of what is known regarding the proteins that directly interact with CFTR.

Multiple protein interactions regulate the CFTR folding and stability

Genetic and biochemical screens for CFTR-interacting proteins have provided significant insights into the chaperone proteins which associate with CFTR including heat shock protein 40 (Hsp40), Hsp70, Hsp90, cysteine string protein (CSP), and calnexin (Farinha et al., 2002; Loo et al., 1998b; Meacham et al., 1999; Pind et al., 1994; Yang et al., 1993; Zhang et al., 2002). The majority of CFTR mutations, including the $\Delta F508$ mutation, result in channel misfolding, highlighting the importance of chaperones in CFTR biogenesis. Chaperones dually function to promote the folding of nascent protein chains and, conversely, the degradation of mis-folded proteins. During protein folding, chaperones such as Hsp70 interact with hydrophobic regions which are generally buried within the native protein. By masking exposed hydrophobic amino acid stretches, chaperone proteins prevent protein aggregation and coordinately facilitate proper folding. In Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, the overexpression of the human Hsp40 protein, Hdj-1, and Hsp70 stabilizes the ER-form of wild-type CFTR, but not $\Delta F508$ CFTR (Farinha et al., 2002; Meacham et al., 1999; Yang et al., 1993). Loo et al. demonstrated that, in CHO and BHK cells stably expressing wild-type CFTR, treatment with geldanamycin or herbimycin A caused Hsp90 to dissociate from nascent CFTR proteins (Loo et al., 1998b). Furthermore,

the loss of Hsp90 binding accelerated the degradation of the ER-form of CFTR via the proteasome. Alternatively, overexpression of CSP with wild-type CFTR in HEK-293 cells stabilized and retained CFTR in the ER (Zhang et al., 2002). Thus, the modulation of chaperone levels impacts the folding and ER-exit of CFTR.

CFTR mutants that fail to achieve a stable conformation are retained in the ER and ultimately degraded via the ER-associated degradation pathway (ERAD). CFTR degradation by ERAD depends upon the retention of non-native CFTR in the ER followed by proteosomal targeting. Several mechanisms have been proposed to regulate the ER retention of CFTR which include sorting motifs and the glycosylation state of CFTR. CFTR contains four arginine-based RXR motifs which have been shown to function as ER retention signals (Chang et al., 1999). The introduction of arginine to lysine mutations in all four motifs (RXR to RXK) in the context of $\Delta F508$ CFTR allowed ~90% of the mutant protein to escape the ER. However, the importance of these motifs for normal CFTR biogenesis is not understood. In addition, the ER resident chaperone calnexin is implicated in the ER-retention of CFTR (Pind et al., 1994). Calnexins interact with the N-linked oligosaccharide chains of CFTR in a calcium-dependent manner. The processing of the glycosylation sites of native CFTR by glucosidases results in the disassociation of CFTR and calnexin. However, for non-native CFTR, the glycosylation sites are not fully processed, leading to a prolonged interaction with calnexin and ER-retention. Once retained in the ER, chaperones such as Hsp70 can recruit ubiquitination machinery including the E2 ubiquitin ligase UbcH5a (Younger et al., 2004) and the E3 ubiquitin ligase CHIP (Meacham et al., 2001). The addition of a poly-ubiquitin chain targets CFTR for proteosomal degradation (Ward et al., 1995).

One caveat to the majority of CFTR-chaperone studies is that these interactions have predominantly been studied using heterologous expression systems where CFTR matures inefficiently (Ward and Kopito, 1994). In most heterologous systems, only ~30% of wild-type CFTR exits the ER. However, in epithelial cell lines where CFTR is endogenously expressed, CFTR is efficiently processed with nearly 100% of newly synthesized protein reaching a post-ER compartment (Varga et al., 2004). The efficient processing of CFTR in epithelia, but not other cell types, may reflect lower expression levels in epithelia and/or the appropriate complement of chaperones and other regulatory proteins necessary for efficient biogenesis. It will be important in future studies to characterize the interactions between CFTR and chaperones in relevant epithelial cells.

The observation that most disease-causing mutations in CFTR, including the $\Delta F508$ mutation found in ~70% of CF patients, are retained and degraded in the ER has significantly increased the interest in understanding the processes which regulate CFTR biosynthesis. Maneuvers such as using chemical chaperones (such as glycerol) or reduced temperatures (<30°C) can facilitate the ER-exit of non-native CFTR where to allow its function at the plasma membrane, albeit at a lower capacity (Brown et al., 1996; Denning et al., 1992). Thus, rescuing non-native CFTR is currently being investigated as an avenue for CF therapeutics. Recently, inhibitors of the calcium-adenosine triphosphate (SERCA) pump, such as curcumin and thapsigargin, have received a great deal of attention as they have been proposed to increase the export of $\Delta F508$ CFTR from the ER (Egan et al., 2002; Egan et al., 2004). Prolonged treatment with SERCA pump inhibitors depletes luminal calcium from the ER and presumably causes the disassociation of calnexin from non-native CFTR. While Egan et al. show that curcumin functionally rescues $\Delta F508$ CFTR in cell culture and the

Δ F508 mouse model; other groups have been unable to validate these findings (Grubb et al., 2005; Song et al., 2004).

Despite the controversies over SERCA pump inhibitors, the rescue of mutant CFTR proteins such as Δ F508 is still under investigation as a potential method to treat CF. However, recent studies have suggested that this approach is complicated by the fact that temperature-rescued Δ F508 CFTR exhibits altered channel gating and decreased stability at the cell surface (Clarke et al., 2004; Egan et al., 2002; Egan et al., 2004; Gentzsch et al., 2004; Swiatecka-Urban et al., 2005a). Thus, the identification of factors which regulate the membrane trafficking and channel activity of CFTR will not only further our understanding of normal CFTR physiology, but may provide insights into therapeutic approaches which could be useful for augmenting the activity of mutant channels.

Interactions with coat proteins, adaptors, and vesicle fusion machinery regulate CFTR membrane trafficking

The mechanisms which regulate the trafficking of CFTR through the early secretory pathway are not well understood. CFTR, like other ER cargo destined for the Golgi, is concentrated at ER exit sites where it is packed into COP-II coated vesicles. Inhibition of COP-II coat assembly by the overexpression of dominant negative Sar1 GTPases causes CFTR to accumulate in the ER (Bannykh et al., 2000). Wang et al. suggested that the packaging of CFTR into COPII vesicles is mediated by a direct interaction between a di-acidic (D-A-D) motif found in CFTR NBD1 with two components of the COP-II coat (Sec23/Sec24) (Wang et al., 2004). The mutation of the second aspartic acid in the di-acidic motif to alanine (D567A) blocks the interaction with Sec23/Sec24 and prevents CFTR

maturation. However, these studies do not address whether the defects associated with the D567A mutation are specific to the COPII interaction or simply cause a global folding defect in CFTR.

Little is known about the trafficking of CFTR to the Golgi complex following ER export. Studies which follow CFTR maturation often rely on the observation that the core-glycosylated, ER form of CFTR (band B) migrates with an apparent molecular weight of 160 kD. As CFTR is trafficked through the cis and medial Golgi, the N-glycan is processed to a complex oligosaccharide, decreasing the electrophoretic mobility to 180 kD (band C) (Cheng et al., 1990). The maturation of wild-type CFTR is not affected by the expression of dominant negative Arf1, Rab1a, or Rab2 GTPases, which block conventional ER-to-Golgi trafficking (Yoo et al., 2002). However, the overexpression of syntaxin 13, a target SNARE expressed on late endosomes, blocked CFTR maturation. These data suggest that the exocytic trafficking of CFTR follows a non-conventional pathway which involves the sorting of CFTR from the ER to the late Golgi or endosomal compartments.

In the Golgi, CFTR interact with the PDZ domain-containing protein the “CFTR-associated ligand” (CAL) (Cheng et al., 2002). CAL is composed of two N-terminal coiled-coil repeats followed by a single PDZ domain (these domains are discussed in detail below). CAL is broadly expressed across tissues and predominantly localizes to the trans-Golgi network (TGN). Overexpression of CAL reduces the cell surface pool of CFTR by decreasing both the half-life of CFTR and the insertion of CFTR into the plasma membrane (Cheng et al., 2002). Subsequent studies have revealed that CAL overexpression targets mature CFTR for lysosomal degradation (Cheng et al., 2005). Furthermore, the expression of NHERF-1, a PDZ protein which binds to CFTR at or near the cell surface, restores CFTR

surface expression (Cheng et al., 2002). These data have led to a model whereby PDZ proteins “hand-off” CFTR in the Golgi or endosomal compartment to regulate the surface expression of CFTR. However, the importance of CAL in the regulation of CFTR has not yet been studied in relevant epithelial cells.

The surface expression of CFTR is also regulated by multiple soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) which are components of the vesicular fusion machinery. The V-SNARE syntaxin 1A directly binds the N-terminus of CFTR to regulate CFTR trafficking in *Xenopus* oocytes (Peters et al., 1999). Furthermore, this interaction also down regulates CFTR channel activity by reducing the open probability of CFTR at the apical cell surface (Naren et al., 2000; Naren et al., 1997; Naren et al., 1998). Syntaxin 1A may also cooperatively regulate CFTR with the t-SNARE SNAP-23 (synaptosome-associated protein of 23 kDa) (Cormet-Boyaka et al., 2002). Like syntaxin 1A, SNAP-23 negatively regulates channel gating. In addition, syntaxin 1a potentiates the SNAP-23-CFTR interaction, suggesting that these cognate t-SNARES may coordinately regulate CFTR as a heterodimeric complex. Moreover, the binding of Munc-18 to syntaxin 1A reverses the inhibitory effect on CFTR open probability, adding another layer of complexity to this regulatory interaction (Naren et al., 1997). Syntaxin 8 may also regulate CFTR function by inhibiting channel activity and by decreasing the amount of protein on the plasma membrane, but it is unclear whether the protein directly binds CFTR or functions in this capacity in polarized epithelial cells (Bilan et al., 2004).

Once CFTR reaches the cell surface it is rapidly internalized by clathrin-coated vesicles. The clathrin adaptor AP2 directly binds GST-CFTR C-terminal fusion proteins *in vitro* and mutagenesis studies suggest that the CFTR C-terminus contains multiple tyrosine-

based internalization motifs (Hu et al., 2001; Weixel and Bradbury, 2000). Once it is cleared from the cell surface, CFTR can be rapidly recycled back to the plasma membrane (Gentzsch et al., 2004; Picciano et al., 2003). Although the molecular mechanisms are poorly understood, especially in polarized cells, a number of proteins including RME1 (Picciano et al., 2003), Rab proteins (Gentzsch et al., 2004), myosin VI (Swiatecka-Urban et al., 2004), and PDZ proteins (Swiatecka-Urban et al., 2002) have been implicated at various stages of the retrieval and recycling process. As mentioned previously, there is a growing interest in the development of therapeutic agents that facilitate the ER-exit of $\Delta F508$ CFTR. Numerous studies have demonstrated that these rescued $\Delta F508$ proteins are trafficked to the cell surface and have chloride channel activity (Denning et al., 1992). However, more recently, several studies have shown that rescued $\Delta F508$ is rapidly internalized from the cell surface and inefficiently recycled (Gentzsch et al., 2004; Swiatecka-Urban et al., 2005a). Thus, impaired trafficking may complicate $\Delta F508$ rescue as a therapeutic approach. These observations highlight the importance of elucidating the mechanisms which regulate the cell surface stability and recycling of CFTR.

Cell surface CFTR is compartmentalized with the cAMP generating machinery and the cytoskeleton

At the apical cell surface, CFTR is compartmentalized with regulatory factors which modulate CFTR channel activity. Our previous work and the results of others are consistent with the membrane localization of the cAMP signaling machinery that regulates CFTR. In excised membrane patches, endogenous, membrane-associated PKA activity activates CFTR when exogenous cAMP is added in excess, or when membrane associated adenylate cyclase

is activated by receptors (Huang et al., 2001; Huang et al., 2000). Our lab previously used small peptides to specifically disrupt protein interactions in Calu-3 cells and further showed that PKAII is targeted to the plasma membrane by association with A-kinase anchoring proteins (AKAPs) (Huang et al., 2000), one of which may be the cytoskeletal-associated protein ezrin (Sun et al., 2000a). Our lab examined the activation of CFTR by adenosine using excised and cell-attached membrane patches from Calu-3 cells. The addition of adenosine to excised membrane patches potently stimulates CFTR channel activity (Huang et al., 2001). These data demonstrate that the adenosine receptor, G-proteins, adenylate cyclase, and PKA are all compartmentalized with CFTR within the patch preparations. Furthermore, in cell attached patches of Calu-3 cells, the addition of adenosine stimulated CFTR activity when it was added to the pipette solution (Huang et al., 2001). The local activation of CFTR by adenosine receptor signaling is mediated by phosphodiesterases (PDE), likely a member of the PDE4D family, which form a cAMP diffusion barrier around the channel (Barnes et al., 2005). In a yeast two-hybrid assay, Hallows et al. identified the AMP regulated kinase as a direct binding partner of the CFTR C-terminus (Hallows et al., 2000). AMP kinases phosphorylate CFTR *in vitro* and inhibit CFTR-mediated chloride conductance in *Xenopus* oocytes and Calu-3 cells.

At the apical cell surface, CFTR interacts with the highly related multi-PDZ proteins, NHERF-1 (EBP50), NHERF-2 (E3KARP), and NHERF-3 (PDZK1 or CAP-70) (Short et al., 1998; Sun et al., 2000b; Wang et al., 2000). Both NHERF-1 and -2 are composed of two tandem PDZ domains and a COOH-terminal Ezrin/Radixin/Moesin (ERM) binding domain (Reczek et al., 1997). CFTR binds to the first PDZ domain of NHERFs-1 and -2 which are tethered to the actin cytoskeleton via ezrin (Short et al., 1998). These interactions have been

proposed to stabilize CFTR at the apical cell surface. As such, GFP-CFTR lacking the PDZ binding motif exhibits a 60% increase in diffusional mobility compared to wild-type GFP-CFTR (Haggie et al., 2002; Haggie et al., 2004; Moyer et al., 1999; Moyer et al., 2000). Additionally, ezrin can interact with the regulatory subunit of PKA, scaffolding this kinase in close proximity to the channel (Sun et al., 2000a). Naren et al. demonstrated that NHERF-1 can exist in a macromolecular complex simultaneously with CFTR and the β 2 adrenergic receptor, an upstream activator of CFTR (Naren et al., 2003). Furthermore, Wang et al. demonstrated that PDZK1, facilitates CFTR dimerization by binding to two channels simultaneously, thereby potentiating CFTR activity (Wang et al., 2000).

More controversial is the role of PDZ proteins in the establishment of the apical polarity of CFTR. Using GFP-tagged CFTR constructs lacking the PDZ binding motif (CFTR- Δ TRL), Moyer et al. found that CFTR mis-localized to the lateral membranes (Moyer et al., 1999). More recent studies from this group suggest that CFTR is trafficked to the cell surface in an unpolarized fashion (Moyer et al., 2000). However, CFTR is concentrated at the apical cell surface via an efficient recycling mechanism which is dependent on binding to PDZ proteins. In contrast, three other groups have found that the apical trafficking and retention of CFTR was not dependent upon the PDZ binding motif (Benharouga et al., 2003; Milewski et al., 2001; Ostedgaard et al., 2003). These studies utilized either untagged or HA-tagged CFTR- Δ TRL expressed in epithelial cells. One interpretation of these data is that the presence of the large N-terminal GFP-tag, in combination with the deletion of the PDZ binding motif, contributes to the loss of apical polarity. However, these studies illustrate that the importance of CFTR binding to PDZ proteins is not fully understood.

Numerous regulatory factors have been identified for CFTR; however, it is not clear which ones are relevant in epithelial tissues or how they are organized. Using biochemical and proteomics techniques described in chapter 2, we identified two novel CFTR binding proteins, the B'ε subunit of the protein phosphatase PP2A and the cytoskeletal protein filamin. We find that the B'ε subunit interacts with the highly conserved C-terminus of CFTR, and this interaction targets a unique PP2A enzyme to CFTR. Furthermore, our data demonstrate that PP2A functionally regulates CFTR in airway epithelia and may be a relevant drug target for CF. In a similar screen, we find that filamins associate with the N-terminus of CFTR. The interaction with filamins regulates the cell surface stability and endocytic trafficking of CFTR. Taken together, our research highlights the power of using proteomics and mass spectrometry to identify functionally relevant protein-protein interactions.

Chapter 2: A single step approach to analyze affinity purified protein complexes by mass spectrometry

Introduction

The adaptation of mass spectrometry to the study of proteins has provided a powerful tool to analyze protein-protein interactions. In combination with classic biochemical approaches such as affinity purification or immunoprecipitation, MS provides a highly sensitive and unbiased method to identify the constituents of protein complexes. This basic approach has been extensively applied to studies ranging from the identification of individual protein interactions to the characterization of entire interactomes. However, relevant biological samples are often not compatible with MS analysis due to the presence of contaminating substances. These contaminants include salts and detergents, which are necessary for biochemical assays, but interfere with protein digestion and the quality of MS data (Parker et al., 2005). In addition, highly abundant proteins can mask and suppress the signals of less abundant proteins (Annesley, 2003). Depending on the method used, contaminants include the fusion proteins (such as GST) or antibodies used to purify protein complexes in pulldown or immunoprecipitation experiments. Furthermore, proteins which interact with fusion proteins or antibodies non-specifically increase the sample complexity, making it more difficult to identify the relevant proteins by MS.

To minimize sample contaminants, a purification step is often utilized prior to MS analysis. Most commonly, proteins are separated by SDS-PAGE, visualized by coomassie blue or other protein stains, excised and digested in-gel. The combination of SDS-PAGE and

in-gel digestion effectively eliminates many contaminants such as salts and detergents, while protein contaminants are resolved into discrete bands away from the proteins of interest. One drawback to SDS-PAGE separation is that proteins must be detected by coomassie blue or other detection methods, which may not be sensitive enough to detect less abundant proteins (Loiselle et al., 2005). Furthermore, peptide fragments are not fully recovered from the gel, often times less than 50%, resulting in lower sequence coverages (Alaiya et al., 2001; Oppermann et al., 2000). Alternatively, proteins or peptides can be purified in-solution using additional chromatography steps or dialysis to minimize high concentrations of salts and detergents, but not protein contaminants. Despite their effectiveness to “clean-up” sample contaminants, each of these methods results in significant sample loss, which is particularly problematic for studying low abundance proteins in the sample.

In this chapter, we detail the development of a new method to prepare and analyze protein complexes by mass spectrometry. Our goal was to develop a biochemical approach to study protein interactions which is MS compatible without requiring additional purification steps. We reasoned that this would allow us to achieve higher quality MS data and identify more proteins in our samples. To accomplish this we designed a procedure which allowed us to purify protein complexes while minimizing sample contamination. The combination of our approach with MS analysis provides a powerful means to analyze protein-protein interactions. In the subsequent chapters, we describe the regulation of CFTR by novel binding partners which were identified using the methods described here.

Experimental Procedures

Lysate preparation and protein purification

Mouse kidneys were homogenized in 10 volumes of binding buffer 150 (BB150: 50 mM Tris pH 7.6, 150mM NaCl, 0.2% CHAPS, 10mM EDTA, Roche complete protease inhibitor cocktail, and 1 mM PMSF) by physical disruption using a tissue homogenizer on ice. Lysates were tumbled at 4°C for 1 hour. Insoluble material was removed by ultracentrifugation at 100,000xg for 1 hour. Protein concentrations of cell lysates were determined by BCA assay (Pierce) and the final protein concentration was adjusted to 1 mg/ml. Lysate were pre-cleared of proteins endogenously coupled to biotin by tumbling with streptavidin agarose beads for 1 hour at 4°C at a ratio of 20 µl of settled beads per 1 ml lysate. The beads were removed by centrifugation at 3,000xg for 10min. Pre-cleared lysates were tumbled with the CFTR affinity matrices (20 nmoles of CFTR peptides immobilized to 100 µl of streptavidin agarose beads) for 4 hours at 4°C. Lysates were washed 5 times in 10 mls of BB150 without protease inhibitors for 5 minutes per wash. As a final wash step to remove excess salt and detergent, samples were washed briefly in a low salt buffer (10 mM sodium phosphate, pH 7.5 with 75 mM NaCl). Excess buffer was aspirated using a gel loading pipette tip.

Sample elution

To elute proteins associated with the CFTR affinity matrix, the beads were incubated in 1 volume (100µl) of 10% formic acid on ice for 5 minutes. ddH₂O was then added to a final volume of 1 ml per sample (adjusting the final concentration of formic acid to 1%). The addition of ddH₂O allows for the easy recovery of the supernatant from the beads while

decreasing the formic acid concentration so as not to introduce covalent modifications or hydrolyze the proteins. Finally, the beads were pelleted and the supernatant was collected, frozen, and lyophilized.

MS Analysis

The lyophilized samples were processed and analyzed by LC-MS/MS as described previously (Borchers et al., 1999). Briefly, the proteins were reduced, alkylated, and digested with modified trypsin. We analyzed the peptide mixtures using a Waters Q-ToF *micro*, hybrid quadrupole orthogonal acceleration time-flight mass spectrometer (Waters, Manchester, UK) with a Waters CapLC system configured with a PepMap™ C18 column. All data were acquired using Masslynx 4.0 software and then processed using the Proteinlynx module. The processed data was searched against updated NCBI nr and Sprot databases using Mascot search engine. Mascot probability based Mowse individual ions scores > 46 were accepted as indicating identity or extensive homology ($p < 0.05$). The MS/MS spectrum scores between 20-45 were examined individually with the acceptance criteria being that the parent and fragment ion masses were within the calibrated tolerance limits and that the spectrum contained an extended series of consecutive y- or b- ions.

Western blot analysis

Small scale experiments analyzed by western blot were performed similarly to large scale experiments with several exceptions. Lysates were generated from two 100mm dishes of Calu-3 cells and CFTR affinity columns were generated with 2 nmoles of CFTR peptide and 20 μ l of streptavidin agarose. Bound proteins were resolved by SDS-PAGE and

analyzed by western blot using anti-sera generated against NHERF-1 (Affinity bioreagents), NHERF-3 (provided by David Silver, Columbia University), Ezrin (Santa Cruz), and Sorting nexin 27 (provided by Wanjin Hong, Institute of Molecular and Cell Biology, Republic of Korea).

Results

Our goal was to develop a biochemical approach to study protein interactions which is MS compatible without requiring additional purification steps. To accomplish this, we designed a protocol with special attention to lysate preparation, choice of affinity ligand, and method of elution which allows us to maximally identify the proteins in our samples without further purification. Our basic approach involves using small, highly purified peptides instead of much larger fusion protein or antibodies to capture protein complexes. In addition, we developed a novel elution protocol using formic acid which disassociates protein bound to the beads, but does not add further contaminants to the sample. In this chapter we evaluate our method by comparing our results with a sample prepared in parallel, but analyzed by a more conventional approach utilizing SDS-PAGE separation and in-gel digestion (Figure 2.1A).

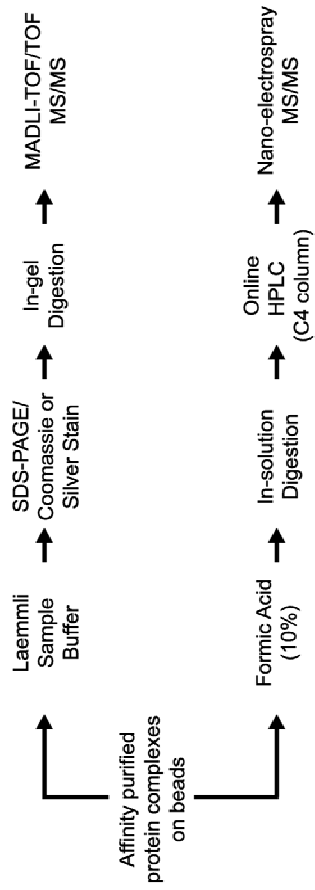
To test our method, we took advantage a well-studied protein interaction interface on the C-terminus of CFTR. CFTR is an epithelial specific chloride channel mutated in cystic fibrosis. The extreme C-terminus of CFTR contains a PDZ binding motif which terminates in the sequence D-T-R-L (Wang et al., 1998). Multiple PDZ proteins interact with CFTR to compartmentalize CFTR with regulatory factors and the cytoskeleton. Furthermore, PDZ proteins have been suggested to regulate CFTR membrane trafficking (Moyer et al., 1999).

Figure 2.1. Strategy to purify PDZ proteins associated with CFTR

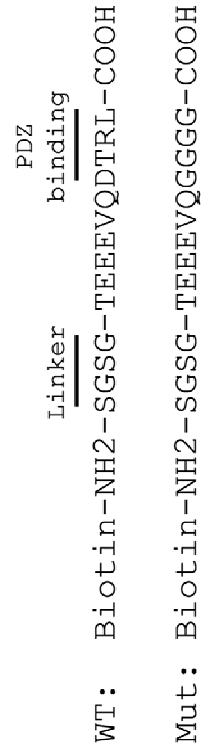
A. Flow chart outlining the techniques used for elution, separation, and MS analysis for conventional in-gel approach (top) compared to our in-solution approach (bottom). B. CFTR peptides used in this study. The peptides correspond to amino acids 1471-1480 of the CFTR C-terminus. To prevent steric hindrance between the peptide and the streptavidin beads, we separated the peptide sequence from the biotin moiety by using a serine-glycine-serine-glycine linker. C. Silver-stained gel of proteins which co-purified with wild-type CFTR[1471-1480] peptide or the 4G mutant control. All visible bands were excised, digested in-gel with trypsin, and analyzed by MALDI-TOF/TOF MS/MS. We successfully identified proteins from all bands as either NHERF-1 (*) or NHERF-3 (←).

A

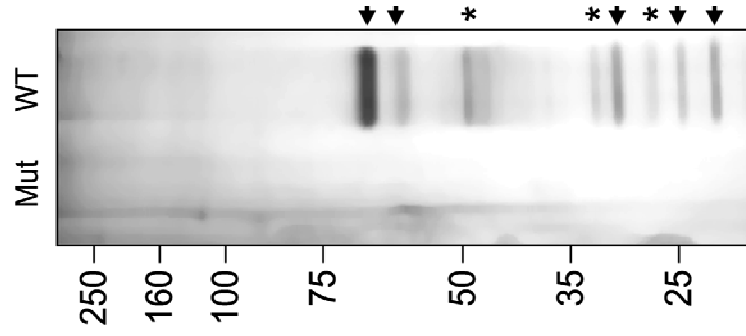
ELUTION **SEPARATION/DIGESTION** **MS ANALYSIS**



B



C



Work from our lab and others have described several PDZ proteins which associate with this motif including the sodium hydrogen exchange regulatory factor-1 (NHERF-1 or EBP50) (Short et al., 1998), NHERF-2 (E3KARP) (Sun et al., 2000b), NHERF-3 (PDZK1, CAP70, NaPi CAP-1) (Wang et al., 2000), and the CFTR-associated ligand (CAL) (Cheng et al., 2002). Studies of CFTR proteins lacking the PDZ binding motif have suggested that PDZ proteins regulate multiple aspects of CFTR biology. However, it is not clear whether additional, unidentified PDZ proteins may be involved in CFTR regulation. Thus, we sought to identify the entire complement of PDZ proteins which associate with the C-terminus of CFTR. To accomplish this, we probed mouse kidney cell lysates with a CFTR peptide affinity column and analyzed the bound proteins by LC-MS/MS. The affinity column was generated by coupling biotinylated, synthetic CFTR peptides, corresponding to the last 10 amino acids of CFTR (CFTR[1471-1480]), to streptavidin agarose beads. The peptides were N-terminally biotinylated which contain a serine-glycine-serine-glycine (SGSG) linker region followed by the CFTR sequence with a free C-terminal carboxylate group (Figure 2.1B). Control peptides were identical to CFTR[1471-1480] peptides, with the exception that the D-T-R-L PDZ binding motif was mutated to glycines (CFTR[1471-1480/4G]).

To isolate protein associated with the CFTR C-terminus, detergent soluble mouse kidney lysates were incubated with either CFTR[1471-1480] or CFTR[1471-1480/4G] affinity matrices. We chose mouse kidney lysates for these studies as they are a rich source of epithelia. The bound proteins were extensively washed, followed by a final wash in a low salt buffer. For our in-solution approach, bound proteins were eluted using formic acid, were subsequently diluted, lyophilized, and digested with trypsin in-solution (Figure 2.1A). We specifically chose to use formic acid for the elution because it is strong enough to

disassociate proteins bound to the beads and it is volatile and removed from the sample during lyophilization. Following digestion, the peptides were analyzed by LC-MS/MS using a Waters Q-ToF micro. As a comparison, we prepared duplicate samples in which we used a more conventional elution and analysis approach. We chose the following procedures as they follow the standard protocol for MS analysis of proteins from acrylamide gels at the UNC-Duke Michael Hooker proteomics center, which is similar to many other core proteomics facilities. After incubating the CFTR affinity column with mouse kidney lysates, the bound proteins were eluted with Laemmli sample buffer. The proteins were separated by SDS-PAGE electrophoresis and stained with coomassie blue or silver stain (Invitrogen). All visible bands were excised, digested in-gel with trypsin, and analyzed by MALDI-TOF/TOF MS/MS as previously described (Parker et al., 2005).

By comparison, we identified more proteins with higher sequence coverages using our in-solution method versus the conventional in-gel method. The silver stained, SDS PAGE separated sample revealed multiple bands which specifically co-purify with the CFTR[1471-1480] peptide, but not the CFTR[1471-1480/4G] mutant control (Figure 2.1C). We successfully identified proteins from all visible bands as either NHERF-1 or NHERF-3, both proteins known to associate directly with the CFTR COOH-terminus (Table 1). While we did identify NHERFs-1 and -3, two known direct binding partners of CFTR, by this conventional method, we did not identify NHERF-2, a previously identified binding partner. Furthermore, we did not identify any indirect or novel binding partners. We also identified NHERF-1 and NHERF-3 by our in-solution method (Table 1). Moreover, we find NHERF-2, as well as ezrin, which associates with CFTR indirectly through NHERF-1 and

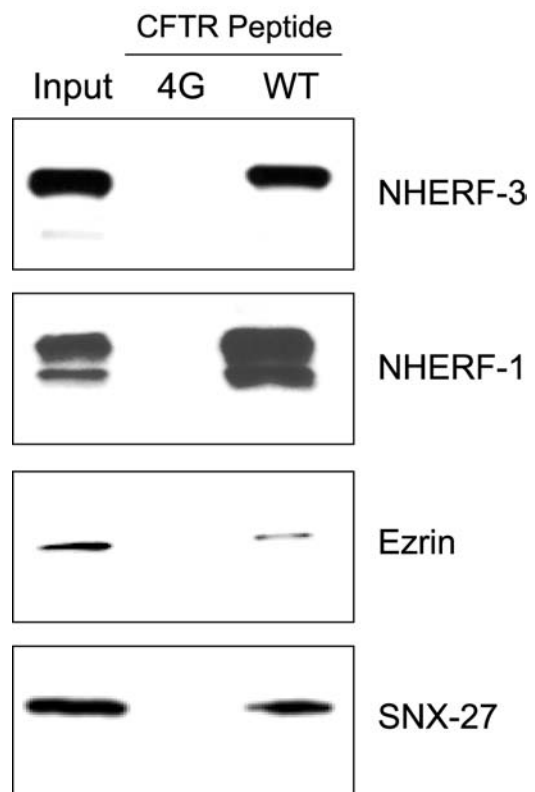
Table 1. A comparison of proteins identified by in-gel and in-solution approaches

A summary of the proteins identified by MALDI-TOF/TOF (In-gel approach) and by LC MS/MS (In-solution approach). In addition to achieving higher sequence coverages and more confident protein identification, we identified more proteins including indirect and novel CFTR binding proteins.

In-Gel MADLI-TOF/TOF		In-Solution LC MS/MS		Association
I.D.	% Coverage	I.D.	% Coverage	
NHERF-3	15%	NHERF-1	75%	Direct
NHERF-1	10%	NHERF-3	66%	
		NHERF-2	39%	Indirect
		Ezrin	16%	
		SNX27	21%	ND (Novel)
		PCC	3%	Background
		CFTR peptide	86%	

Figure 2.2. The PDZ proteins identified by MS specifically associate with the CFTR C-terminus

The affinity pulldown samples were analyzed by western blot using anti-sera generated against NHERF-3, NHERF-1, ezrin, and SNX-27. We find that all of these proteins specifically associate with the wild-type CFTR peptide but not the 4G mutant. These data suggest that SNX-27 may be a relevant CFTR binding partner which our lab is currently studying.



NHERF-2. Our in-solution method also resulted in the identification of a novel CFTR-associated protein, sorting nexin 27 (SNX27). Sorting nexins are proteins that regulate intracellular trafficking events. SNX27 contains a PDZ binding motif, a lipid binding PX domain, and a putative Ras-association/FERM domain. While the characterization of the CFTR-SNX27 interaction is not described in this dissertation, our data suggest that SNX27 regulates the endocytic trafficking of CFTR. We confirmed our MS protein identifications by western blot. Using antibodies specific to NHERF-1, NHERF-3, Ezrin, and SNX-27, we find that each of these proteins specifically co-purifies with the CFTR[1471-1480] wild-type peptide, but not the CFTR[1471-1480/4G] mutant (Figure 2.2).

Discussion

Here we describe a procedure which can be used to study protein-protein interactions by MS without costly additional purification steps. Our method relies on using highly purified peptides as affinity ligands, a low salt and detergent-free wash, and an acid elution. Based on our MS data, our approach significantly improved our ability to identify proteins associated with our affinity column.

The use of peptides in our approach is important for minimizing contaminants in the samples. By comparison, traditional affinity ligands such as GST-fusion proteins or antibodies are much larger and generate many more tryptic fragments in the sample. In addition, the small size of the peptides tends to minimize non-specific protein binding. Furthermore, peptides from commercial sources can be purchased at >95% purity, thus offering a nearly homogenous affinity reagent. One limitation to using peptides is that they may not recapitulate native protein structure and will not likely mimic large globular protein

domains. However, the peptide approach is well suited for studying protein-protein interactions associated with the cytosolic tails of transmembrane proteins. This is particularly useful in light of the observations that large transmembrane proteins are notoriously difficult to study by MS as they are often endogenously expressed at low levels and are only solubilized using high detergent concentrations which may disrupt protein interactions. In addition, this approach could also be used to study other protein interactions which involve linear sequences such as the SH3 or WW domains which interact with poly proline motifs.

While we did not detect any background proteins using the in-gel procedure, we only identified two direct binding proteins. In contrast, we identified more direct binding proteins, indirectly associated proteins, and previously uncharacterized binding proteins using the in-solution approach. We did detect a single background protein in the sample, propionyl coenzyme A carboxylase, co-purified with both the wild-type CFTR[1471-1480] peptide and the 4G mutant control. Propionyl coenzyme A carboxylase (PCC) is endogenously bound to biotin and thus, likely associates with the streptavidin beads independently of the peptides (Wood and Barden, 1977). It is also important to note that formic acid elution is stringent enough to disassociate the biotinylated CFTR peptides from the streptavidin agarose. While the peptide is separated from larger proteins by SDS-PAGE, the peptide was detected by MS using the in-solution method. However, because of the small size of the peptide (yielding only 2 tryptic fragments), the CFTR peptide is sufficiently separated from relevant peptides in the sample by HPLC and did not interfere with the MS analysis.

Using our approach we identified more proteins and achieved higher sequence coverages for all protein identified compared to the in-gel approach. Furthermore, we

identified a novel CFTR binding protein, SNX27. We predict that SNX27 interacts directly with CFTR via its PDZ domain. However, it may also interact indirectly through NHERFs-1, -2, or -3 via a PDZ or FERM domain interaction. Although, we do not describe the characterization of the CFTR-SNX27 interaction in this dissertation, we find that SNX-27 is highly concentrated in early endosomes where it likely regulates the endocytic sorting and recycling of CFTR. In addition to improving our ability to correctly identify proteins using MS data, the high sequence coverages improve the odds for the identification of post-translational modifications, such as phosphorylation. Indeed, we identified one phosphopeptide in our sample corresponding to a serine-phosphorylation at position 285 of NHERF-1 (data not shown). Previous studies have demonstrated that NHERF-1 is phosphorylated at multiple sites (Fouassier et al., 2005; Reczek et al., 1997). However, serine 285 has not been previously characterized.

In summary, we describe a novel approach for isolating and characterizing the components of protein complexes. First, the use of peptides as affinity ligands results in samples with low background due to proteins binding non-specific, especially compared to larger affinity ligands. Secondly, a brief wash in a low salt buffer helps reduce residual salts and detergents. Finally, the formic acid elution allows the purified proteins to be recovered from the affinity matrix, without adding additional contaminants. Taken together, this method provides a highly effective approach to the purification and preparation of samples for MS which does not require costly additional purification steps.

Chapter 3: CFTR is regulated by a direct interaction with the protein phosphatase PP2A

Introduction

Cystic Fibrosis (CF) is an autosomal lethal disease characterized by abnormal ion transport in epithelial tissues. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane chloride channel. The regulation of CFTR by the cAMP-dependent protein kinase (PKA) and other protein kinases has been extensively documented. PKA can phosphorylate the CFTR regulatory domain (R domain) on at least 11 serine residues (Chang et al., 1993; Cheng et al., 1991; Seibert et al., 1995). *In vivo*, PKA phosphorylation increases CFTR open probability and the number of channels in the plasma membrane (Ameen et al., 2003; Gadsby and Nairn, 1999). Work from our lab and others have demonstrated that PKA and other regulatory proteins are compartmentalized in close proximity to CFTR. The cellular machinery capable of generating cAMP, including the adenosine receptor and membrane bound adenylate cyclase are present with CFTR in apical membrane patches (Huang et al., 2001). A-kinase anchoring proteins (AKAPs) target PKA to protein complexes with CFTR (Huang et al., 2000; Sun et al., 2000a; Sun et al., 2000b) and the disruption of the PKA/AKAP interaction abolishes the ability of PKA to activate CFTR in response to physiologic stimuli (Huang et al., 2000). In addition, the phosphodiesterase PDE4D is also present with CFTR in patch preparations and forms a cAMP diffusion barrier at the apical plasma membrane (Barnes et al., 2005). Other CFTR regulatory molecules including protein kinase C (PKC) and the AMP-activated protein

kinase (AMPK) are found in protein complexes associated directly with CFTR (Hallows et al., 2000; Liedtke et al., 2002).

Less is known about the ability of serine/threonine phosphatases to regulate CFTR activity or how they are compartmentalized with CFTR. Work from many labs suggests that multiple phosphatases including PP2A, PP2B, PP2C, and alkaline phosphatase are involved in the deactivation of CFTR (Fischer et al., 1998; Hwang et al., 1993; Luo et al., 1998; Reddy and Quinton, 1996; Travis et al., 1997). *In vitro*, PP2A and PP2C are most effective in dephosphorylating purified CFTR and recombinant R domain. Furthermore, exogenous PP2A and PP2C inactivate CFTR in excised membrane patches (Berger et al., 1993; Luo et al., 1998). In human sweat ducts, cardiac myocytes, 3T3 fibroblasts, and Hi-5 insect cells, inhibitors of PP2A increase CFTR channel activity (Berger et al., 1993; Hwang et al., 1993; Reddy and Quinton, 1996; Yang et al., 1997). Likewise, PP2B inhibitors stimulate CFTR in NIH 3T3 fibroblasts (Fischer et al., 1998). However, the contribution of PP2A and PP2B to CFTR deactivation may vary in different cell types (Travis et al., 1997; Zhu et al., 1999). In baby hamster kidney cells (BHK), PP2C co-immunoprecipitates with exogenously expressed CFTR (Zhu et al., 1999). Additionally, PP2C overexpression in Fischer rat thyroid cells (FRT) decreases CFTR chloride conductance (Travis et al., 1997). However, the ability of endogenous PP2C to regulate CFTR in native epithelial tissues is unclear as no PP2C inhibitors have been identified. To date, no single phosphatase has been demonstrated to be both necessary and sufficient to completely down regulate CFTR channel activity, suggesting that CFTR is dephosphorylated by multiple phosphatases.

Here, we present evidence for a direct interaction between CFTR and the serine/threonine phosphatase PP2A. This interaction involves the COOH-terminus of CFTR

and the PP2A B'ε regulatory subunit. PP2A localizes to the apical cell surface where it negatively regulates CFTR channel activity in Calu-3 cells and intact mouse jejunum. Furthermore, inhibition of PP2A increases the airway surface liquid in primary human bronchial epithelial cells (HBE) by a mechanism requiring CFTR channel activity. We conclude that PP2A is indeed a relevant CFTR phosphatase in epithelial tissues.

Experimental Procedures

Cell culture and immunofluorescence

Calu-3 cells were maintained as described previously (Mohler et al., 1999). Human airway epithelial cells were obtained from freshly excised bronchial specimens from normal subjects by protease digestion, seeded directly as primary cultures on 12 mm Transwell Col membranes (T-Col; Costar, USA) in modified BEGM medium under ALI conditions, and studied when fully differentiated (3-5 weeks). Cultures with transepithelial resistances (R_t) > 300 Ω cm² were studied. Immunofluorescence and confocal microscopy were performed as described previously using anti-PP2A subunit antibodies (Kreda et al., 2005). Monoclonal anti-catalytic subunit, monoclonal anti-A regulatory subunit, and polyclonal anti-B' subunit were acquired from Upstate Biotechnology.

Affinity purification of CFTR-interacting proteins

Peptides corresponding to residues 1375-1401, 1411-1441, 1451-1476, and 1471-1480 of human CFTR, synthesized with a N-terminal biotin tag (Genemed Synthesis), were resuspended in 50 mM Tris-Cl (pH 7.4). 20 nmoles of the peptides were immobilized on 100 μ l of streptavidin agarose (Sigma Aldrich, St Louis, MO) and incubated with Calu-3 cell

lysates. Lysates were prepared by incubating twenty 100 mm dishes of Calu-3 cells in binding buffer (50 mM Tris-Cl, 150 mM NaCl, 0.2% CHAPS, and protease (Roche) and phosphatase (Sigma Aldrich) inhibitor cocktails) at 4°C for 1 hour. Following ultracentrifugation, the soluble fraction was pre-cleared over empty streptavidin beads and incubated with CFTR peptides conjugated to streptavidin beads for 1 hour. The bound fractions were washed extensively in binding buffer, eluted with 5% formic acid, and lyophilized. Prior to MS analysis, the protein samples were reduced, alkylated, and digested with proteomics grade trypsin (Sigma-Aldrich). The peptides were analyzed by LC-MS/MS on a Q-ToF *micro* (Waters, Manchester, UK). All data were acquired using Masslynx 4.0 software and then processed using Proteinlynx module. The processed data was searched against updated NCBI nr and Sprot databases using Mascot search engine. Mascot probability based Mowse individual ion scores > 46 were accepted as indicating identity or extensive homology ($p < 0.05$). The MS/MS spectrum scores between 20-45 were examined individually with the acceptance criteria being that the parent and fragment ion masses were within the calibrated tolerance limits and that the spectrum contained an extended series of consecutive y- or b- ions. Small scale experiments analyzed by western blot were performed using lysates from two 100mm dishes of Calu-3 cells and 2 nmoles of CFTR peptide.

***In vitro* binding assays**

Binding assays were performed as described previously, with several exceptions (Mohler et al., 1999; Scott et al., 2002). Briefly, the cDNA sequence of the human PP2A B'ε subunit was amplified by PCR and cloned into the BamHI and XhoI sites of pET-28C (Novagen, Madison, WI). Recombinant B'ε subunit was produced in either rabbit

reticulocyte lysates (Promega, Madison, WI) or in BL21 E. coli (Stratagene, La Jolla, CA). For reticulocyte lysates, the PP2A B'ε subunit was purified using Ni-NTA beads (Qiagen) in binding buffer (50mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Triton-X 100). To remove any reticulocyte proteins which co-purify with the B'ε subunit, the beads were washed 3X in binding buffer plus 2M urea followed by 3 washes in binding buffer. The B'ε subunit was eluted with binding buffer plus 10 mM EDTA. Bacterial expression of B'ε subunit was performed as previously described (Smith et al., 1999). Purified PP2A core dimer consisting of the catalytic and A regulatory subunit were obtained from Promega (Madison, WI).

Purified proteins were then incubated with indicated CFTR peptides immobilized to streptavidin beads in binding buffer plus 0.2% BSA for 1 hour at 4°C. Following extensive washing, the bound proteins were eluted, resolved by SDS-PAGE, and analyzed by western blot or Phosphorimager analysis.

Co-immunoprecipitation and Phosphatase Assays

Calu-3 cells were scraped into a hypotonic lysis buffer (150mM Tris-Cl, 10mM NaCl, and protease inhibitors) and physically disrupted by Dounce homogenization on ice. The lysates were centrifuged at 500xg for 10 minutes to remove nuclei and unbroken cells. The supernatant was subsequently centrifuged at 100,000xg for 1 hour to pellet cell membranes. Membranes were resuspended in binding buffer for 1 hour on ice. CFTR was immunoprecipitated using CFTR 596 antibodies (gift of Dr. J. Riordan, University of North Carolina at Chapel Hill) or isotype-matched control antibodies covalently conjugated to Protein G dynabeads. Bound proteins were washed extensively and analyzed by western blot

using specific antibodies for PP2A subunits or for phosphatase activity using a PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate Biotechnology).

CFTR currents in outside-out membrane patches of Calu-3 cells

The procedures were essentially as described previously (Huang et al., 2001). Briefly, CFTR Cl⁻ channel activity was recorded at a membrane potential of 30 mV with a 6-8 megaohm resistance for an open pipette. Both the pipette and the bath solutions were the same as described previously (Huang et al., 2001). CFTR channel activity was recorded digitally (PCLAMP software) for 300 seconds following patch excision. PP2A inhibitors or PKI, were included in the pipette solution as indicated.

CFTR currents in mouse jejunum

Details of this approach have been described previously (Grubb, 1995; Grubb, 2002). Briefly, sections of the midportion of mouse jejunum were studied under short circuit current (I_{sc}) conditions during a 90 minute recording. A constant voltage pulse (1-5 mV, 1 s duration) was applied to the tissue every minute (Physiologic Instruments, San Diego, CA). Potential difference (PD) and resistance were calculated by Ohm's law from the changes in I_{sc} in response to the voltage pulse. Tissues were treated with 100 μM endothall or vehicle.

Confocal microscopy measurement of PCL

The technique has been described in detail (Tarran et al., 2001). Briefly, PBS (20 μl) containing 2 mg/ml Texas Red-dextran (10 kDa; Molecular Probes, USA) and benzamil (10⁻⁴ M) was added to cultured bronchial epithelium and excess was aspirated to bring PCL height

to $\sim 7 \mu\text{m}$. The CFTR-specific inhibitor CFTR_{inh172} was also included where noted at a concentration of 10^{-5} M. To measure the average height of the PCL, 5 predetermined points, one central, four 2 mm from the edge of the culture were XZ scanned as previously described (Tarran et al., 2001). For all studies, perfluorocarbon (PFC) was added mucosally to prevent evaporation of the PCL and the culture placed on the stage of the confocal microscope over a serosal reservoir (80 μl TES-buffered Ringer). Okadaic acid was added to the apical surface as a dry powder in perfluorocarbon (5 μg Okadaic acid/25 ml PFC/ cm^2 culture). PFC has no effect on PCL height or ion transport as previously described (Tarran et al., 2001).

Results

PP2A physically associates with the COOH-terminus of CFTR

The COOH-terminus of CFTR mediates protein-protein interactions with PDZ proteins (Cheng et al., 2002; Short et al., 1998; Sun et al., 2000b; Wang et al., 2000), the adaptor protein AP-2 (Weixel and Bradbury, 2000), and AMP kinase (Hallows et al., 2000) to regulate cell surface stability, membrane trafficking, and channel activity. In the present study, we asked whether the highly conserved CFTR COOH-terminus can interact with additional proteins that regulate channel function. We chose to focus on the last 25 amino acids of CFTR (encompassing residues 1451-1476) which precede, but do not include, the PDZ binding motif. Previous studies suggest that this region is important for CFTR trafficking and channel activity (Ostedgaard et al., 2003); however, no protein interactions have been reported. We immobilized biotinylated CFTR[1451-1476] peptides on streptavidin agarose beads, incubated the peptides in cell lysates prepared from a Calu-3 cells, and eluted the bound proteins with formic acid. Using nano-LC MS/MS, we identified

the serine/threonine protein phosphatase PP2A associated with CFTR[1451-1476]. PP2A dephosphorylates CFTR *in vitro* and decreases channel activity in multiple cell systems (Berger et al., 1993; Hwang et al., 1993; Reddy and Quinton, 1996; Yang et al., 1997). Recently, Vastiau *et al.* also identified an interaction between the regulatory domain of CFTR and PP2A, suggesting that PP2A anchoring to CFTR may involve multiple contacts (Vastiau et al., 2005).

PP2A is a major cellular phosphatase which regulates many protein targets. PP2A functions as a heterotrimeric complex composed of a catalytic subunit and two regulatory subunits, A and B (Janssens and Goris, 2001). The specificity of PP2A is determined by the unique combination of regulatory subunits associated with the catalytic subunit. The A regulatory subunit is encoded by one of two genes α and β which are 86% identical. The A regulatory subunit is tightly associated with the catalytic subunit and functions as a scaffold to recruit the B regulatory subunit. The B regulatory subunit is highly divergent in comparison to the other PP2A subunits. The B regulatory subunit is divided into the B, B', B'', and B''' families which are encoded by at least 14 different genes, some of which produce as many as 5 splice variants. The diversity of regulatory subunits gives rise to over 75 distinct PP2A enzymes (Figure 3.1). By mass spectrometry, we observe peptides from all three PP2A subunits (Figure 3.2A). Importantly, the MS/MS spectra provide amino acid sequence information which allowed us to precisely identify the PP2A regulatory subunits associated with CFTR[1451-1476] as A α and B' ϵ (Figure 3.2B).

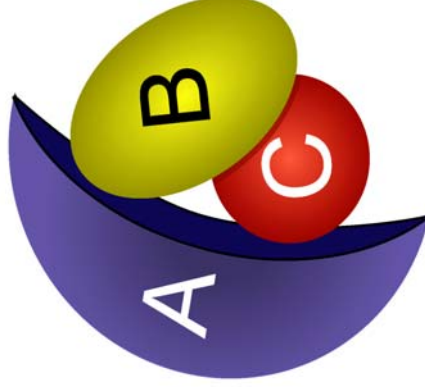
By western blot, we confirm that the PP2A catalytic, A regulatory, and B' regulatory subunits associate with CFTR[1451-1476], but not other CFTR C-terminal peptides (Figure 2.3A). Furthermore these PP2A subunits also co-precipitate with endogenous CFTR from

Figure 3.1. Diverse subunits give rise to the specificity of PP2A

PP2A functions as a heterotrimeric complex composed of a catalytic subunit and two regulatory subunits (A and B). The unique combination of regulatory subunits determines the substrate specificity and the subcellular localization of the catalytic subunit. Given the number of PP2A subunits identified to date, >75 unique combinations are possible.

A Regulatory Subunit

A α
A β



B Regulatory Subunit

B α
B β
B δ
B γ
B' α
B' β 1
B' β 2
B' γ 1
B' γ 2
B' γ 3

B' δ 1
B' δ 2
B' ϵ
PR48
PR59
PR72
PR93
PR110
PR130

Catalytic Subunit

C α
C β

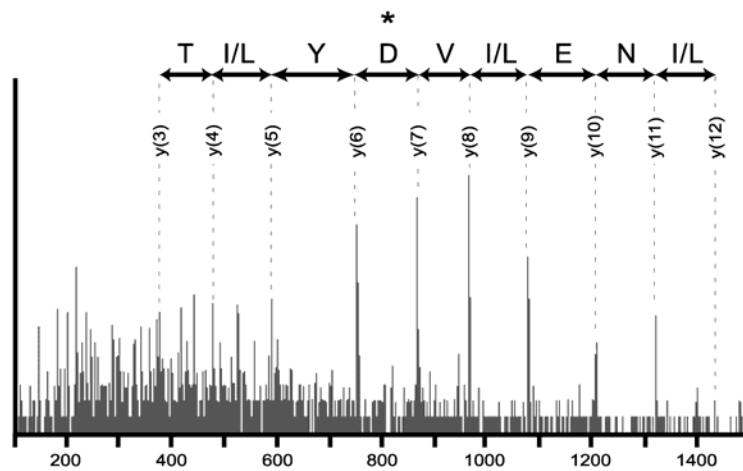
Figure 3.2. MS analysis of PP2A subunits associated with the CFTR COOH-terminus

A. Table of PP2A subunit peptides identified by MS/MS analysis of Calu-3 cell proteins associated with CFTR[1451-1476]. B. Example of an MS/MS spectrum used to specifically identify the B'ε subunit. Y ions and corresponding amino acids are labeled on the spectrum.

A

PP2A Subunit	Coverage	Mass	Sequence
Catalytic	3.6%	1339.66	YSFLQFDPAPR
A α (PR65 α)	7.1%	2193.16 1375.72 1108.53	IGPILDNSTLQSEVKPILEK TDLVPAFQNLTK LAGGDWFTSR
B' ϵ (PR61 ϵ)	5.8%	1622.85 1522.77	STLNELVDYITISR FLESQEFQPSIAK

B

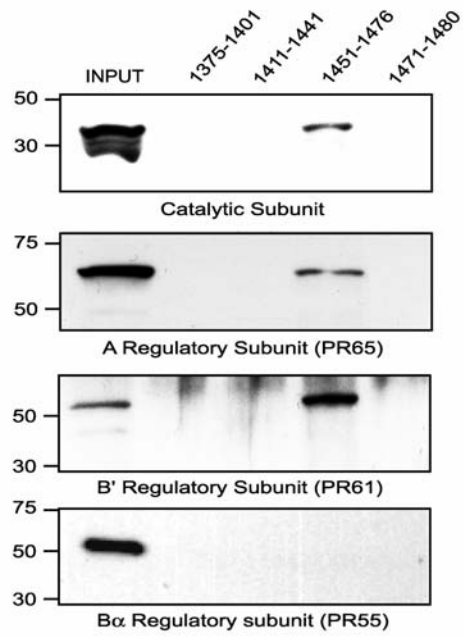
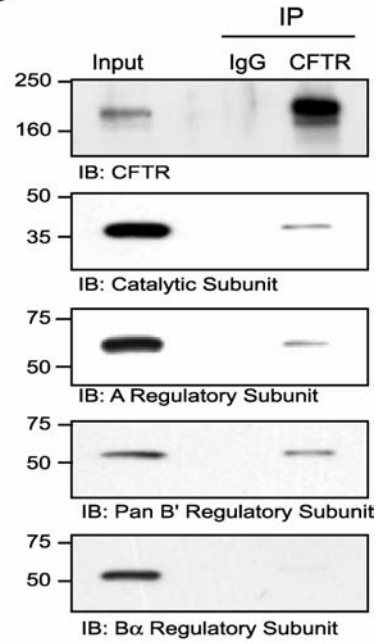
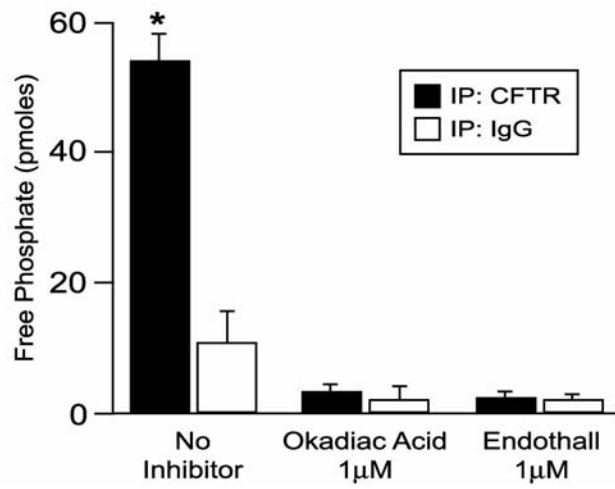


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B' α 112 RNTSVYEVLENLTA 99
 B' β 118 RTSGVCEVLENLAA 105
 B' γ 84 RNHTIYEVME SLAA 71
 B' δ 147 RSHTIYEV MENLGA 160
 B' ϵ 104 RSITIYDVLENLTS 91

Figure 3.3. The PP2A heterotrimer co-purifies with CFTR

A. Biotinylated CFTR peptides were immobilized on streptavidin beads and incubated in Calu-3 cell lysates. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using antibodies against the PP2A catalytic, A regulatory, B' regulatory, and B α regulatory subunits. B and C. Calu-3 cell lysates were incubated with either anti-CFTR monoclonal antibodies or isotype matched mouse IgG covalently coupled to protein G dynabeads. The beads were extensively washed and analyzed by (B) western blot using specific PP2A subunit antibodies or (C) assayed for serine/threonine phosphatase activity using an Upstate phosphatase assay kit. To determine the contribution of PP2A to the total phosphatase activity, the PP2A inhibitors Okadaic acid or endothall were added during the phosphatase assay (n=4 for each condition). The data represent mean values (n=5) + s.e.m. (*p < 0.0005).

A**B****C**

Calu-3 cell membranes (Figure 3.3B), consistent with an interaction *in vivo*. The PP2A B α subunit, which is structurally unrelated to the B' family, does not co-purify with CFTR[1451-1476] or co-immunoprecipitate with endogenous CFTR. In addition, we asked whether PP2A phosphatase activity purified with full-length CFTR. CFTR immunoprecipitates were assayed for PP2A using a colorimetric phosphatase assay. We find that a PP2A-like activity specifically co-precipitates with CFTR, but not an IgG control (Figure 3.3C). Characteristic of PP2A, the phosphatase activity was inhibited by 1 μ M okadaic acid or 1 μ M endothall, potent inhibitors of PP2A (IC₅₀ = 0.1nM and 90nM, respectively). Although okadaic acid and endothall can also inhibit PP1(IC₅₀ = 10nM and 5 μ M, respectively), previous studies have found no evidence to support the ability of PP1 to physically associate with CFTR, to dephosphorylate CFTR *in vitro*, or regulate channel activity (Berger et al., 1993; Luo et al., 1998; Zhu et al., 1999). Although PP2C has been shown to be associated with CFTR in BHK cells, the assay buffer does not contain Mg²⁺ which is required for PP2C activity. Taken, these data suggest that the phosphatase activity associated with CFTR is PP2A.

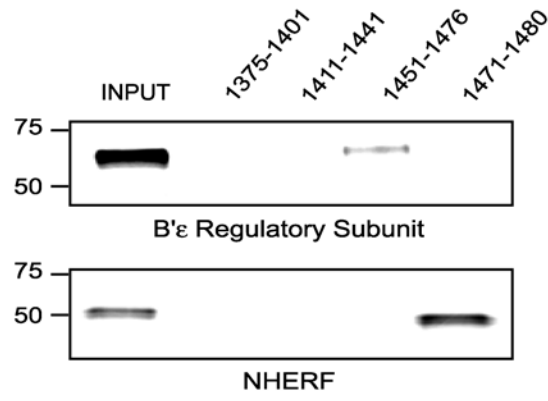
The PP2A B' ϵ subunit binds directly to the COOH-terminus of CFTR

It is clear that multiple phosphatases can regulate CFTR channel activity. However, the degree to which PP2A contributes to this regulation varies in different functional assays and cell systems. Given the diversity of PP2A enzymes, we were intrigued by the possibility that the B regulatory subunit may, in part, account for these differences. Therefore, we asked whether the PP2A B' ϵ subunit could interact directly with CFTR. We find that PP2A B' ϵ subunit expressed and purified from reticulocyte lysates binds to CFTR[1451-1476], but not other CFTR C-terminal peptides (Figure 3.4A). NHERF-1, previously shown to interact with

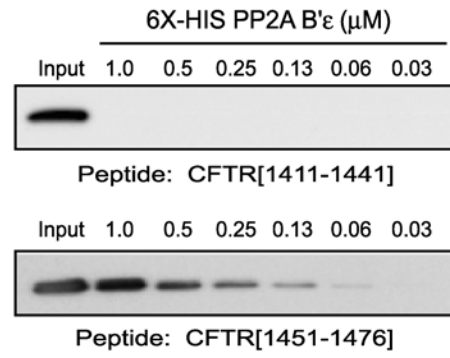
Figure 3.4. The B'ε subunit directly interacts with the CFTR C-terminus

A. The indicated CFTR COOH-terminal peptides were immobilized and incubated with radiolabeled B'ε subunit or NHERF-1. The bound fraction was separated by SDS-PAGE and analyzed by phosphorimage analysis. B. 2 μM CFTR[1411-1441] or CFTR[1451-1476] were immobilized and incubated with bacterially expressed B'ε subunit at the indicated concentrations. Bound proteins were washed and analyzed by western blot using a pan-B'ε antibody. C. Immobilized CFTR[1411-1441] or CFTR[1451-1476] were incubated with purified PP2A core dimer (0.05 U) with or without B'ε (1μg). Samples were analyzed by western blot using PP2A subunit antibodies.

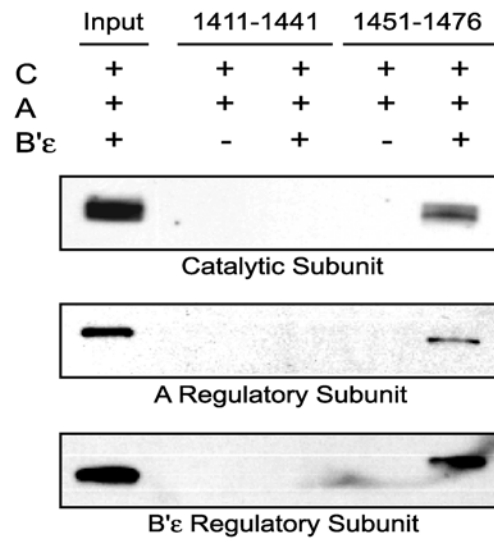
A



B



C



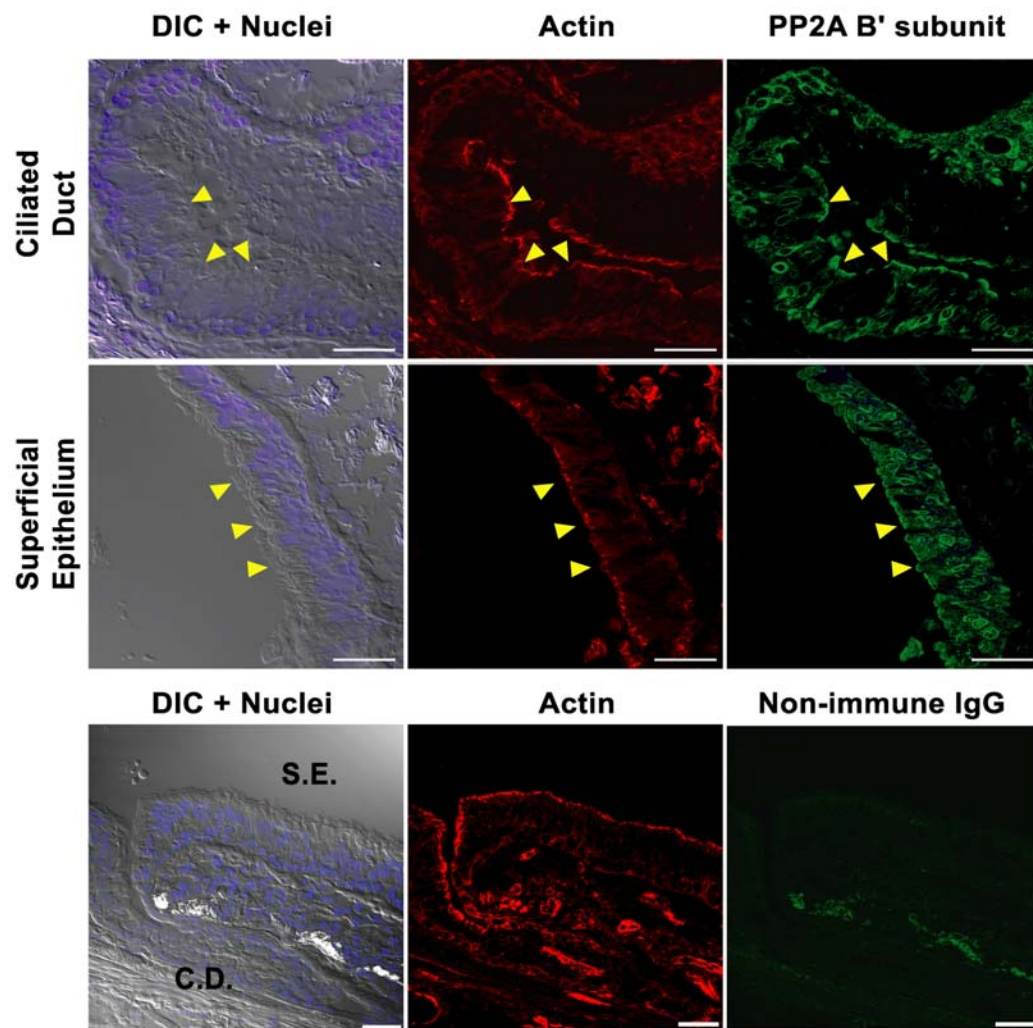
the extreme COOH-terminus of CFTR, is enriched by the CFTR[1471-1480] peptide, demonstrating the specificity of this assay (Figure 3.4A). Although we do not find the catalytic or A subunit associated with the purified B'ε subunit from reticulocyte lysates (data not shown), we cannot rule out that these subunits are present at low levels and may be influencing binding to CFTR[1451-1476]. Using bacterially-expressed, purified B'ε subunit we observe direct and dose-dependent binding to CFTR[1451-1476] (Figure 3.4B). Furthermore, we also find that the core PP2A dimer, composed of the catalytic and A regulatory subunit, do not bind to CFTR[1451-1476] unless the B'ε subunit is present (Figure 3.4C). While it is not clear if the B'ε subunit alone is necessary and sufficient for targeting PP2A to CFTR, these data suggest that the B'ε subunit may play a critical role in the binding specificity.

PP2A localizes to the apical cell surface of airway epithelia

If PP2A is a physiologically relevant CFTR phosphatase, we reasoned that its subcellular localization should partially overlap with CFTR. Given the large number of PP2A substrates, we expect the ubiquitously expressed catalytic and tightly coupled A regulatory subunits to have a broad cellular distributions. However, specific B regulatory subunits, which target the PP2A enzyme to distinct subcellular compartments, should have a more restricted localization. Using a pan B' antibody, which recognizes all five gene products (α , β , γ , δ , and ϵ), we examined the localization of PP2A in human airway. The B' subunits exhibit a broad subcellular distribution in human airway (Figure 3.5). We find that B' staining strongly localizes to the apical membrane of ciliated cells in superficial epithelia and gland ducts, consistent with the localization of CFTR in these tissues. Although we

Figure 3.5. The PP2A B' regulatory subunit localizes to the apical compartment of ciliated airway cells

Representative localization of the PP2A B' regulatory subunit in gland ducts and superficial epithelium of the human airway. Confocal images were acquired in four channels with independent laser sources: left, DIC and nuclear staining (DAPI, blue); center actin cytoskeleton (rhodamine phalloidin, red); right, PP2A B' subunit family (green). Arrowheads indicate regions of apical staining. The bottom panel shows IgG controls on sections of superficial epithelium (S.E.) and ciliated ducts (C.D.). *Scale bars* = 40 μm .



cannot unambiguously attribute the apical localization to the B' ϵ subunit, it is likely that this staining reflects the distribution of one or a combination of the cytosolic B' subunits, B' α , B' β , B' δ and B' ϵ (McCright et al., 1996). In addition, we observe staining of peri-nuclear membranes and nuclear speckles, which is consistent with the observation that the B' γ and B' δ localize to the nucleus (McCright et al., 1996; Tarran et al., 2005).

As we expected, the PP2A catalytic and A regulatory subunits are more broadly localized in airway epithelia (data not shown), but clearly localized to the apical cell surface of ciliated cells. The antibodies directed against the catalytic and A regulatory subunits, detect both gene products for each subunit. Thus, these antibodies label every PP2A molecule which we expect to give a broad distribution in all cell types. While the pan B' antibody detects five distinct gene products, this still only represents one out of four families of the highly divergent PP2A B regulatory subunit. Accordingly, the staining pattern for the B' subunit exhibits a more restricted distribution including the sub-apical plasma membrane. These localization data support our biochemical evidence for an *in vivo* interaction between CFTR/PP2A. Furthermore, the PP2A subunits are concentrated at the apical cell surface, suggesting a potential role in regulating the channel activity of CFTR at the plasma membrane.

PP2A inhibition prevents CFTR rundown in excised patches

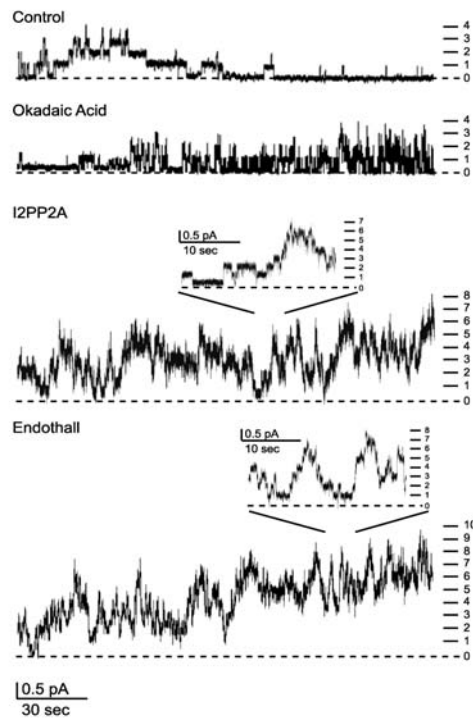
Our previous work and the results of others are consistent with the membrane localization of the cAMP signaling machinery that regulates CFTR. In excised membrane patches, endogenous PKA activity activates CFTR when exogenous cAMP is added in excess, or when membrane associated adenylate cyclase is activated by receptors (Huang et

al., 2001; Huang et al., 2000). In patch preparations, CFTR channel activity reflects the phosphorylation status of the channel which is regulated by a balance between PKA and endogenous phosphatases (Berger et al., 1993; Huang et al., 2001; Tabcharani et al., 1991). Consequently, CFTR channel gating is increased under conditions in which PKA activity is elevated, such as increased cAMP or the inhibition of phosphodiesterases (Barnes et al., 2005; Cheng et al., 1991). We hypothesize that “rundown” of CFTR activity following excision results from a shift in the balance between endogenous PKA and phosphatase activities. Based on our biochemical analyses demonstrating a physical association between CFTR and PP2A, we tested this hypothesis by examining CFTR rundown in excised patches under control conditions and in the presence of PP2A inhibitors. We compared the average pipette current during the first 30 seconds of outside out recordings with the average pipette current measured from 270 to 300 seconds, as performed in previous studies (Berger et al., 1991). Under control conditions, the pipette solution contained ATP and GTP, conditions which allow CFTR gating to reflect the activity of endogenous PKA (Huang et al., 2001). Furthermore, current flow through the patch is mediated by Cl⁻ traversing CFTR as no channel events are observed and pipette current is near zero with protein kinase A inhibitor (PKI, 100 nM) in the pipette (0.10 ± 0.10 at 0-30 seconds and 0.00 ± 0.00 at 270-300 seconds, $n=5$). Under these conditions CFTR activity declines variably over 5 minutes ($\Delta I = -0.66 \pm 0.21$, $n=18$) (Figure 3.6A and B). However, the presence of the PP2A inhibitors okadaic acid or endothall in the pipette prevented channel rundown, ($\Delta I = 0.51 \pm 0.24$ and $\Delta I = 0.81 \pm 0.88$, respectively, $n=10$ for each treatment) (Figure 3.6A and B). Although endothall and okadaic acid inhibit PP2A most potently, they can also inhibit other phosphatases such as PP1, PP4, and PP5 (Becker et al., 1994; Brewis et al., 1993).

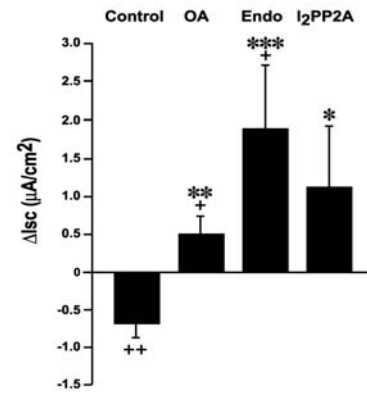
Figure 3.6. PP2A inhibitors functionally regulate CFTR

A and B. Basal CFTR currents were measured in apical patches were generated from Calu-3 cells in the outside-out configuration. Patches were recorded under control conditions as outlined in methods and materials. Where indicated patches were prepared in the presence of okadaic acid (3 nM), endothall (1 μ M), and I2PP2A (1.7 μ M). A. Representative traces for each condition are shown. For endothall and I2PP2A, traces were expanded on the X axis to clearly show discrete single channel events. B. Summary of the patch clamp data in Calu-3 cells. The Δ Isc for each treatment are compared to Δ Isc = 0 (T-test, $P < 0.05$; + = $P < 0.05$ and ++ = $P < 0.005$) and compared to Δ Isc of control samples (T-test, $P < 0.017$; Bonferroni's post-hoc relative to control, * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$). C. CFTR currents were measured in intact mouse jejunum in Ussing chambers as described previously. Tissues were treated with endothall (100 μ M) or vehicle for the duration of the recording. Δ Isc is represented as the change in CFTR chloride currents at time 0 versus compared to 90 minutes. The data represent mean values ($n=4$) \pm s.e.m. (* $p < 0.005$).

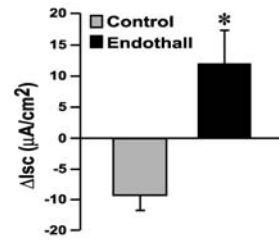
A



B



C



Therefore, we also tested the specific inhibitor I₂PP2A, which does not inhibit PP1, PP2B, or PP2C (Li et al., 1995a; Li et al., 1996). Similar to endothall and okadaic acid, I₂PP2A (1.7μM) also prevented CFTR channel rundown ($\Delta I = 1.13 \pm 0.85$, n=8) (Figure 3.6A and B).

PP2A inhibitors prevents CFTR rundown in an intact epithelium

Consistent with our observation that CFTR and PP2A physically interact, the effect of PP2A inhibitors on CFTR in excised patches suggests that PP2A is present in the patches and is, therefore, compartmentalized in close proximity to CFTR. Furthermore, the inhibitor data demonstrate that endogenous PP2A can regulate CFTR channel activity. These results strongly suggest that PP2A functions as a relevant CFTR phosphatase in epithelial tissues. We also tested whether PP2A inhibitors altered basal CFTR activity in an intact epithelium. We measured basal short-circuit chloride currents in freshly excised sections of mouse jejunum in Ussing chamber experiments. Previous studies demonstrated that CFTR constitutes the major chloride channel in these preparations, as jejunum from CFTR *-/-* mice do not exhibit significant chloride conductance (Clarke et al., 1992). We find that over the course of control experiments, basal CFTR activity declined over time ($\Delta I_{sc} = -9.2 \pm 2.6$, n=4) (Figure 3.6C). In agreement with our patch clamp data, we find that the PP2A inhibitor endothall (100μM) prevented rundown of CFTR activity, resulting in increased chloride conductance during the course of the 90 minute recording ($\Delta I_{sc} = 12.4 \pm 4.9$, n=4).

Inhibition of PP2A increases the airway surface liquid height

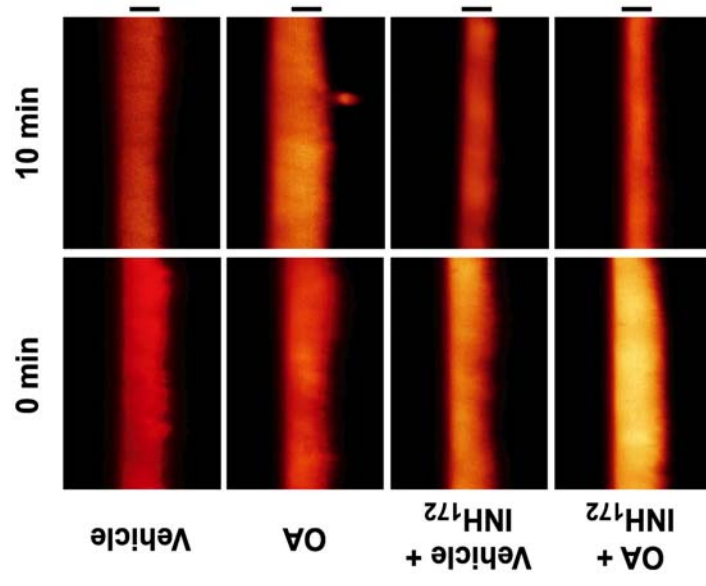
Airway epithelial cells regulate the composition of the thin layer of pericilliary liquid (PCL) critical for innate host defense. In CF lung disease, PCL volume is depleted,

reflecting an imbalance in solute transport across the epithelium (Guggino, 2001; Knowles and Boucher, 2002; Verkman, 2001; Wine, 1999). Ultimately, PCL depletion results in defective mucociliary clearance which supports bacterial colonization and exposes individuals to life-threatening infections (Guggino, 2001; Knowles and Boucher, 2002; Matsui et al., 1998b; Tarran et al., 2001; Verkman, 2001; Wine, 1999). Previous studies have demonstrated that CFTR mediates the majority of PCL secretion and plays a fundamental role in maintaining basal PCL volume (Tarran et al., 2005; Tarran et al., 2002). Therefore, we asked whether inhibition of PP2A would increase CFTR activity and thereby increase PCL levels in primary human bronchial epithelial (HBE) cultures. The PCL was labeled with fluorescent dextran as previously described (Tarran et al., 2001). PCL height was measured immediately before and 10 minutes after indicated treatments. Vehicle treatment did not significantly change PCL height (Figure 3.7). However, cultures treated with okadaic acid exhibited an ~30% increase in PCL height ($\Delta\text{PCL} = +2.61 \pm 0.7 \mu\text{m}$, $n=6$) (Figure 3.7). Because PP2A inhibition is likely to influence the activities of many cellular proteins, we asked whether CFTR was required for the increase in PCL. Consistent with previous data demonstrating the importance of CFTR in PCL secretion, we find that the CFTR inhibitor, CFTR_{inh172}, causes PCL height to decrease by ~30% ($\Delta\text{PCL} = -3.21 \pm 0.9 \mu\text{m}$) (Ma et al., 2002). Furthermore, in the presence of CFTR_{inh172}, okadaic acid was similar to the CFTR_{inh172} alone ($\Delta\text{PCL} = -2.3 \pm 1.1 \mu\text{m}$). Thus, the inhibition of PP2A with okadaic acid increases PCL height by a mechanism which requires CFTR. These results are consistent with our biochemical and electrophysiological studies demonstrating that CFTR is physically and functionally associated with PP2A in epithelial tissues.

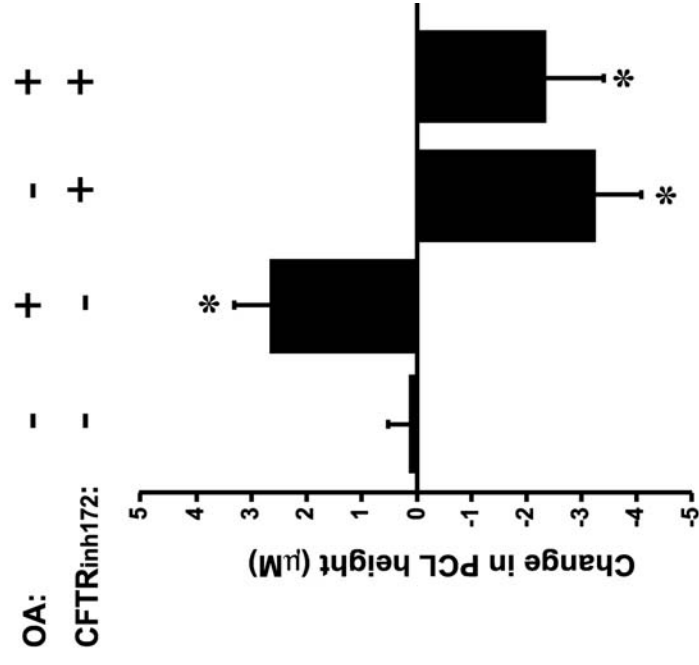
Figure 3.7. PP2A inhibition increases PCL height in primary human airway cultures

A. PCL height was assessed in primary cultures of human bronchial epithelia cells treated with vehicle (PBS) or okadaic acid (5 μ g/25ml PFC/cm² culture) in the presence or absence of CFTR inhibitor 172 (10⁻⁵M) for 10 minutes. The PCL was labeled with Texas red dextran and the height was measured by confocal microscopy. Representative images taken at time 0 or 10 minutes for each condition are shown. *Scale bar = 7 μ m.* B. Summary of confocal data represented as percent change in PCL height at time 0 versus 10 minutes of the indicated treatments. The data represent mean values (n=6 for all conditions with the exception of n=7 for okadaic acid) \pm s.e.m. (Treatments were compared to controls by Mann Whitney U-test, *p < 0.02).

A



B



Discussion

The compartmentalization of signaling machinery with downstream effectors ensures the specificity and fidelity of signal transduction in response to extracellular stimuli. Previously, we demonstrated that CFTR is localized in close proximity with regulatory proteins *in vivo* including the adenosine receptor, adenylate cyclases, PKA, and phosphodiesterases (Barnes et al., 2005; Huang et al., 2001; Huang et al., 2000). In addition, many signaling proteins which regulate CFTR activity, including PKA (Huang et al., 2000; Sun et al., 2000a; Sun et al., 2000b), PKC (Liedtke et al., 2002), PP2C (Zhu et al., 1999), and AMP kinase (Hallows et al., 2000) have been shown to be organized in multi-protein complexes directly associated with CFTR. The data in the present study add to this model by demonstrating that the phosphatase PP2A is both physically and functionally linked to CFTR.

The regulation of CFTR by phosphatases has been extensively studied. While it is clear from the work of many labs that multiple phosphatases can affect CFTR channel activity, the specificity of this regulation is poorly understood. We identified PP2A in a biochemical screen for proteins that associate with residues 1451-1476 of the CFTR COOH-terminus. The power of our approach is illustrated by the fact that we unambiguously identified the exact PP2A regulatory subunits associated with CFTR by mass spectrometry as A α and B' ϵ . To date, at least 21 distinct PP2A regulatory subunits have been identified (2 A regulatory and 19 B regulatory subunits) (review in (Janssens and Goris, 2001)). Importantly, it is the unique combination of regulatory subunits that governs the enzymatic activity, substrate specificity, and localization of PP2A enzymes (McCright et al., 1996;

Tehrani et al., 1996). Thus, the identification of the exact subunits associated with CFTR provides insight into the specificity of CFTR channel regulation by phosphatases.

Using purified proteins, we find that the divergent B' ϵ subunit interacts directly with CFTR[1451-1476] and is required for the binding of the catalytic and A regulatory subunits. These data suggest that the B' ϵ subunit may target the PP2A enzyme to CFTR. It is intriguing to speculate that the expression of the B' ϵ subunit may account for the differences in the ability of PP2A to regulate CFTR channel activity in different cell-types and tissues. Many of the CFTR phosphatase studies have utilized heterologous expression systems such as Chinese hamster ovary (CHO) cells (Luo et al., 1998), baby hamster kidney (BHK) cells (Zhu et al., 1999), and Hi-5 insect cells (Yang et al., 1997), which may or may not express relevant CFTR phosphatases and subunits (Luo et al., 1998; Yang et al., 1997; Zhu et al., 1999). We are unable to ask whether the PP2A B' ϵ subunit is expressed in these cell types by RT-PCR, as there is insufficient sequence information available at this time. However, the expression of specific PP2A subunits in these cells should be an important consideration for future experiments. Recently, Vastiau *et al.* reported an interaction between CFTR and PP2A, which involves the direct binding of the ubiquitously expressed PP2A A α regulatory subunit to the CFTR regulatory domain (Vastiau et al., 2005). To demonstrate the specificity of the CFTR/A α interaction, Vastiau *et al.* show that the over-expressed heat repeats 1-10 caused a decrease in CFTR rundown, presumably due to the disruption of the A α /CFTR interaction. However, the PP2A B subunits also bind to heat repeats 1-10 of the A subunit and would have been dislodged from the phosphatase complex. Thus, in these assays the observed decrease in CFTR rundown could be attributed to the disruption of the interaction between CFTR and both the PP2A A and B regulatory subunits. Nonetheless, the anchoring

of PP2A to CFTR chloride channel may involve multiple sites of interaction mediated by the PP2A A α and B' ϵ subunits.

Previous studies have demonstrated a clear role for PP2A in the deactivation or rundown of CFTR in native sweat ducts, cardiac myocytes, BHK cells, 3T3 fibroblasts, and Hi-5 cells (Berger et al., 1993; Hwang et al., 1993; Reddy and Quinton, 1996; Yang et al., 1997). However, the importance of PP2A in CFTR regulation in epithelial tissues seems variable. Thus, we chose to study the physical and functional association of CFTR and PP2A in airway epithelia. Indeed, we find that CFTR and PP2A interact in Calu-3 cells as the PP2A catalytic, A regulatory, and B' regulatory subunits specifically co-precipitate with endogenous CFTR (Figure 3.3B). Additionally, we examined the localization of PP2A subunits in human airway. If in fact PP2A regulates CFTR channel activity in epithelial tissues, we would expect the phosphatase to be present at the apical cell surface consistent with the localization of CFTR. The ubiquitously expressed PP2A catalytic subunit and A regulatory subunit have a diffuse staining pattern including the apical membrane of the superficial epithelium (data not shown). Alternatively, staining with a pan-B' regulatory subunit antibody shows a more restricted distribution, including strong staining at the apical plasma membrane (Figure 3.5). Thus, the PP2A subunits associated with CFTR are present at the apical cell surface where CFTR functions. Since the available B' antibodies detect all family members, we cannot rule out the possibility that other B' subunits other than B' ϵ may also associate with CFTR or localize to the apical surface of airway epithelia. Dozier *et al.* identified an interaction between Chk2 and the PP2A B' γ subunit and found that the other highly similar B' family members, B' α , B' β , B' δ , and B' ϵ were also able to bind to Chk2 *in vitro* (Dozier et al., 2004). Consequently, other PP2A B' regulatory subunits may interact

with CFTR. However, we do not find an association between CFTR and a structurally unrelated B regulatory family member, B α (PR55a) by pulldown (Figure 3.3A) or immunoprecipitation (Figure 3.3B). Thus, the PP2A molecules associated with CFTR at least appear to be specific for the B' family.

We find that CFTR and PP2A are functionally associated in airway and intestinal epithelial cells. In the presence of PP2A inhibitors, CFTR rundown was blocked in outside-out membrane patches from Calu-3 cells and intact mouse jejunum (Figure 3.6). Consistent with our biochemical observations, the presence of PP2A in the membrane patches containing CFTR strongly suggest that these proteins are closely compartmentalized. Furthermore, our data suggest that PP2A is a relevant CFTR phosphatase in epithelial tissues, in agreement with Vastiau *et al.* who find that the addition of okadaic acid in Caco-2 epithelial cells delayed channel rundown following forskolin treatment (Vastiau et al., 2005). In T84 and airway epithelial cells, PP2A inhibitors did not block the rundown of CFTR following maximal activation by forskolin (Travis et al., 1997; Zhu et al., 1999). A major difference between our data compared to other studies is that we choose to measure the effect of PP2A inhibitors on basal CFTR currents as opposed to CFTR currents following maximal activation with forskolin or PKA. It is possible that endogenous PP2A plays a role in regulating basal CFTR currents, but that other phosphatases contribute to the deactivation of hyper-phosphorylated CFTR. Future studies are needed to address the relative contribution of different phosphatases to the regulation of CFTR channel activity in epithelial tissues.

In the airways, the mucus layer provides a defensive barrier which traps and clears bacteria and noxious particles from the airways (Guggino, 2001; Knowles and Boucher, 2002; Verkman, 2001; Wine, 1999). The underlying PCL provides a low viscosity fluid

which facilitates ciliary beating and mucus clearance (Gimeno-Alcaniz and Sanz, 2003; Hallows et al., 2000; Matsui et al., 1998a). Based on inhibitor studies, CFTR has been demonstrated to play a central role in the maintenance of basal PCL height (Tarran et al., 2005; Tarran et al., 2002). Furthermore, the loss of CFTR function results in decreased PCL height and increased viscosity. If in fact PP2A is a relevant CFTR phosphatase in airway epithelia, we reasoned that the inhibition of PP2A should increase CFTR chloride conductance and ultimately increase PCL height in primary HBE cultures. We find that the inhibition of PP2A by the addition of okadaic acid to primary HBE cultures produces a 30% increase in PCL height, consistent with an increase in CFTR activity (Figure 3.7). Additionally, when CFTR channel activity is blocked by CFTR_{inh172}, okadaic acid does not have a significant effect on the PCL. Thus, okadaic acid treatment increases PCL height through a CFTR dependent mechanism, consistent with our hypothesis that CFTR is compartmentalized with and regulated by PP2A in epithelial tissues.

In addition to regulating channel activity by dephosphorylating CFTR, PP2A may also be important for mediating other signaling and trafficking events for CFTR. For example, AMPK, which directly binds to CFTR and negatively regulates channel activity, can also directly interact with PP2A via the A regulatory subunit (Gimeno-Alcaniz and Sanz, 2003; Hallows et al., 2000). Moreover, the direct interaction between CFTR and AMPK has been mapped to CFTR residues 1420-1457, adjacent to the residues where we observe for PP2A, 1451-1476 (Hallows et al., 2000). It is interesting to speculate that PP2A and AMPK may function to stabilize each others interactions with CFTR or may compete for binding to the CFTR COOH-terminus.

In summary, we establish that CFTR is physically and functionally associated with PP2A. Our data demonstrate that PP2A directly interacts with CFTR via the B'ε regulatory subunit. Furthermore, we provide the first evidence that PP2A regulates CFTR in airway epithelia, the major tissue affected in CF. Finally, we demonstrate that the inhibition of PP2A in well differentiated HBE cultures increases PCL height. Because, decreased PCL underlies the basic defect in airway clearance observed in CF patients, our data suggest that PP2A may be a useful therapeutic target for CF.

Chapter 4: Filamins regulate the cell surface stability and endocytic sorting of CFTR

Introduction

In this chapter, we examine the importance of the 80 amino acid, cytosolic N-terminus of CFTR. By engaging in inter- and intra-molecular interactions, the N-terminus of CFTR regulates channel biosynthesis, trafficking, and gating. CFTR proteins lacking the N-terminus do not exit the ER, demonstrating that the N-terminus is absolutely required for normal CFTR biosynthesis (Cormet-Boyaka et al., 2004). In studies by Zhang et al, the J-domain containing chaperone, cysteine string protein (CSP), was found to associate with both the CFTR N-terminus and R domain (Zhang et al., 2002). While the physiological importance of this interaction is not clear, overexpression of CSPs in *Xenopus* oocytes and HEK-293 cells suggest a role in the ER-retention or folding of CFTR. In addition, the CFTR N-terminus also interacts with multiple soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Cormet-Boyaka et al., 2002; Ganeshan et al., 2003; Naren et al., 2000; Naren et al., 1997; Naren et al., 1998). Naren et al. initially demonstrated that the cytosolic domain of syntaxin 1A directly binds to CFTR, thereby reducing the open probability of the channel (Naren et al., 1997). Moreover, the binding of Munc-18 to syntaxin 1a reverses the inhibitory effect on CFTR, adding an additional layer of complexity to this regulatory interaction. The t-SNARE SNAP-23 (synaptosome-associated protein of 23 kDa) also interacts with CFTR and, like syntaxin 1A, SNAP-23 negatively regulates channel gating (Cormet-Boyaka et al., 2002). In addition, syntaxin 1a potentiates the SNAP-23 interaction with CFTR suggesting that these cognate t-SNARES may coordinately regulate CFTR as a heterodimeric complex. Interestingly, an intra-molecular interaction

between the CFTR N-terminus and the regulatory domain also regulates channel gating (Fu et al., 2001). Residues 46-63 of the CFTR N-terminus form an alpha helix which interacts with the R-domain via several negatively charged amino acids. Furthermore, disruption of this interaction decreases the open probability of CFTR. It is intriguing to speculate that SNARE proteins regulate CFTR gating by competitively inhibiting the N-terminus/R-domain interaction. However, the validity of this model has not yet been tested.

While the regulation of CFTR by SNARE proteins has predominantly focused on CFTR channel activity, SNARE proteins also regulate CFTR trafficking. Using CFTR with an extra-cellular epitope-tag, Peters et al. show that overexpression of syntaxin 1a specifically decreases the cell surface expression of CFTR (Peters et al., 1999). More recently, syntaxin 8 was also identified as a putative binding partner for the CFTR N-terminus (Bilan et al., 2004). In heterologous systems, the over-expression of syntaxin 8 alters the localization of CFTR from the plasma membrane to the recycling compartment, suggesting a role in endocytic trafficking. Taken together, our current understanding of the CFTR N-terminus suggests that this domain interacts with multiple proteins to specifically regulate channel biosynthesis, trafficking, and gating.

The extreme N-terminus of CFTR is highly conserved and is the site of multiple disease causing mutations. However, this region of the N-terminus has not been implicated in any regulatory function or interaction. Using the biochemical approaches described in the previous chapters, we specifically asked whether the extreme N-terminus of CFTR is an interface for protein-protein interactions. Indeed, we find that the CFTR N-terminus directly interacts with filamin-A (FLN-A) and filamin-B (FLN-B). Filamins are classically known to function as actin cross-linking proteins. However, filamins also bind to ion channels,

receptors, and soluble signaling proteins and may organize signaling complexes. In addition, filamins regulate cell surface expression, endocytosis, and recycling of a variety of plasma membrane proteins including the von Willebrand's receptor subunit glycoprotein Iba ($\text{GPIb}\alpha$) (Feng et al., 2005; Feng et al., 2003; Williamson et al., 2002; Xu et al., 1998), $\beta 2$ - and $\beta 3$ -integrins (Glogauer et al., 1998; Loo et al., 1998a; Pfaff et al., 1998; Sharma et al., 1995), the inward rectifying potassium channel 2.1 (Kir2.1) (Sampson et al., 2003), and the hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) (Gravante et al., 2004). By studying disease-causing mutations in the CFTR N-terminus, we identified a missense mutation, serine 13 to phenylalanine (S13F), disrupts the interaction with filamin. In the absence of filamin binding, the surface pool of S13F CFTR is significantly decreased and CFTR is prematurely delivered to lysosomes where it is degraded. Our data highlight a previously unrecognized regulatory interaction between filamins and the CFTR N-terminus and provide a mechanistic explanation linking the S13F mutation to human disease.

Experimental Procedures

Plasmids

CFTR mammalian expression constructs were prepared in pcDNA3.1(+). All CFTR point mutants used in this study were generated by site directed mutagenesis using the QuickChangeXL kit (Stratagene) and sequenced at the UNC DNA sequencing facility. Exotope CFTR (herein referred to as HA-CFTR), generated by introducing an HA-tag into a modified second extra-cellular loop of CFTR, was provided by Dr. Martina Gentsch (University of North Carolina at Chapel Hill) and described previously (Gentsch et al., 2004). Full-length human FLN-A in the pCMV mammalian expression vector and the

bacterial expression constructs used to express GST-FLN-A fusion proteins were provided by Dr. Mike Schaller (University of North Carolina at Chapel Hill).

Cell culture

Human embryonic kidney cells (HEK-293), HeLa cells, and baby Hamster kidney (BHK) cells were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. M2 and A7 cells, provided by Dr. Gary Thomas (Oregon Health Sciences University), were cultured in DMEM supplemented with 2% FBS and 8% normal calf serum (NCS). BHK cells stably expressing HA-CFTR, provided by Dr. Martina Gentzch (University of North Carolina at Chapel Hill) was grown in selection media supplemented with 500 µg/ml methotrexate. Calu-3 cells were maintained as described previously (Thelin et al., 2005). Well-differentiated human primary airway epithelial cells (WD-PAE) were isolated from freshly excised bronchial specimens from normal subjects and cultured as described previously (Thelin et al., 2005). Transient transfections were performed using Effectene for HEK-293 (Qiagen) or Lipofectamine 2000 (Invitrogen) for HeLa and BHK cells. Assays were carried out 24-48 hours post-transfection. HA-CFTR was expressed in M2 and A7 cells using adenovirus, provided by Dr. Wanda O'Neil (University of North Carolina at Chapel Hill), at an MOI of 20.

Antibodies and other reagents

CFTR monoclonal antibodies 293 and 570 (used for western blots) and CFTR-570 cross-linked to protein G dynabeads (used for immunoprecipitation) were provided by Dr.

Jack Riordan (University of North Carolina at Chapel Hill). Full-length FLN-A was analyzed using monoclonal antibodies 1678 and 1680 (Chemicon), while GST-FLN-A truncations were detected using monoclonal anti-GST antibodies (Santa Cruz). Cell surface labeling of HA-CFTR was performed using HA monoclonal antibody HA.11 (Covance).

Affinity purification of CFTR-interacting proteins

Peptides corresponding to residues 1-25 of CFTR (wild-type and various mutants) were synthesized followed by a serine-glycine-serine-glycine (SGSG) linker region and a C-terminal lysine residue coupled to biotin (Tufts University Peptide Core Facility). Other CFTR peptides corresponding to residues 11-35, 27-45, 38-68, and 63-80 of the CFTR N-terminus contained the SGSG linker and biotin at the N-terminus. All peptides were resuspended in 50 mM Tris-Cl (pH 7.4) and used in affinity purification experiments analyzed by mass spectrometry (MS) or western blot as described in Thelin et al (Thelin et al., 2005). Bound proteins were eluted with 100 μ l CFTR disaggregation buffer (50 mM Tris, pH 6.8, with H₃PO₄, containing 2% SDS, 15% glycerol, 2% β -mercaptoethanol, 1 mM EDTA, and 0.02% bromophenol blue (Sarkadi et al., 1992)) and resolved by SDS-PAGE. For MS experiments, gels were stained with Coomassie blue per UNC-Duke Michael Hooker Proteomics Center standard procedures (<http://proteomics.unc.edu>). Visible bands were excised and prepared for MS analysis by MALDI TOF/TOF MS/MS previously described (Loiselle et al., 2005).

Bacterial expression of GST-FLN-A fusion proteins and domain mapping

FLN-A fusion proteins including the calponin homology domains and filamin repeats 1-4, 5-7, 7-11, 12-15, 14-20, 20-23, and 23-24 were expressed in DH5 α bacteria as GST-fusion proteins. Briefly, 5 mls of overnight culture was inoculated into a 50 ml culture and grown at 37 °C for 2 hours. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μ M and the bacteria were incubated for an additional hour. GST-fusion proteins were purified as described previously (Smith et al., 1999). All fusion proteins were dialyzed into PBS and concentrated using 5,000 MWCO vivaspin columns (Vivascience) to a final concentration of 10 μ M.

The GST-filamin fusion proteins or GST alone were diluted in PBS with 0.1% Triton-X 100 to a final concentration of 100 nM. CFTR peptides, CFTR[1-25] or CFTR[S13F], were immobilized on streptavidin agarose (1 μ g peptide to 20 μ ls beads) and incubated with 200 μ l of the GST-FLN-A suspensions. The samples were tumbled at 4 C for 1 hour, washed 3 times for 5 minutes per wash in PBS with 0.1% Triton-X 100, and eluted with Laemmler sample buffer. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using anti-GST antibodies.

Surface plasmon resonance

The interaction between the CFTR N-terminus and FLN-A was monitored using surface plasmon resonance with a BIAcore3000 (Biacore) at 25°C, with flow rates of 20–25 μ l/min, using HEPES-buffered saline. Biotinylated CFTR N-terminal peptides (1000–15,000 response units per peptide) were immobilized on streptavidin sensor chips. GST-FLN repeats 1-4, 12-14, or GST alone were injected over the sensor surfaces using injections (KINJECT

command) of 20–35 μ l, with buffer flowed over the sensor chip to promote dissociation of the FLN-A fusion proteins with dissociation times extending to 1000 s. The FLN-A fusion proteins routinely dissociated from each sensor surface completely as judged by the stable response unit levels maintained for each flow cell over the course of the experiments. Between injections, the chip surface was regenerated by injecting 50 mM NaOH (pH 3.0) over the sensor chips. The signals from a blank flow cell in which no peptide was immobilized were used to normalize all sensorgrams. For kinetic evaluations, sensorgrams from each set of titrations were aligned, and the normalized data were globally fit to a 1:1 binding model (RI = 0) with BIAevaluation 3.2 software (Biacore). Resulting thermodynamic constants are the result of three independent sets of titrations.

CFTR immunoprecipitation

Co-immunoprecipitation experiments were performed as described in Thelin et al. Briefly, CFTR was immunoprecipitated from crude membranes solubilized in binding buffer (50mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Triton-X 100) prepared from Calu-3, M1, or HEK-293 cells. CFTR was immunoprecipitated using the CFTR 570 antibody covalently bound to protein-G dynabeads. Bound proteins were washed 4 times for 5 minutes per wash in binding buffer. Bound proteins were eluted with CFTR disaggregation buffer, resolved by SDS-PAGE, and analyzed by western blot.

Immunohistochemistry

Calu-3 cells were grown on permeable supports and studied after achieving transepithelial resistance (R_t) > 300 Ω cm². Calu-3 cells were fixed with ice-cold methanol

for 2 minutes and blocked for 2 hours in PBS with 2mg/ml BSA, 4% non-fat milk, 1% fish gelatin, and 0.1% Triton-X 100 at room temperature. Primary antibodies (CFTR 570 or filamin 1678) were diluted into blocking buffer and incubated on cells overnight at 4°C, washed 3 times for 10 minutes per wash in blocking buffer without milk. Secondary antibodies (anti-mouse antibodies conjugated with Alexa-488) were incubated with cultures in blocking buffer for 1 hour. Rhodamine phalloidin was included with the secondary antibody to label the actin cytoskeleton. Excess antibodies and phalloidin were removed by washing 3 times for 10 minutes per wash in PBS. Transwells were mounted on slides with Vectasheild (Vector Laboratories) mounting media. The preparation of primary WD-PAE cultures and frozen sections of human sweat duct were prepared as previously described (Kreda et al., 2005). Confocal microscopy was performed as described previously (Kreda et al., 2005).

Pulse-Chase Analysis

Wild-type CFTR, S13F CFTR (no interaction with filamins), S13A CFTR (interacts with filamins similarly to wild-type), and Δ F508 (folding mutant which does not exit the ER) were transiently expressed in HEK-293 cells. After 24 hours post-transfection, the cells were starved in cysteine/methionine free DMEM for 30 minutes. 250 μ Ci/ml 35 S-promix (GE Healthcare) was incubated with the cells for an additional 30 minutes. The labeling media was removed and the cells were chased with cold DMEM supplemented with 10% FBS and incubated at 37°C for the indicated chase periods. Cells were recovered by incubating with 15 mM ice-cold sodium citrate and pelleted by centrifugation. CFTR was immunoprecipitated from whole cell lysates as described above. Immunoprecipitates were

resolved by SDS-PAGE, the gels were dried and imaged by Phosphorimager analysis. For lysosomal inhibitor studies, 10 $\mu\text{g/ml}$ leupeptin was included in the culture media.

CFTR surface labeling and ELISAs

Wild-type, S13F, S13A, and ΔF508 HA-CFTR constructs were transiently expressed in HeLa or BHK cells grown on glass cover slips (immunofluorescence) or 24-well plates (ELISAs). Cells were fixed with 4% PFA, blocked for 2 hours in blocking buffer (PBS with 2mg/ml BSA, 4% non-fat milk, and 1% fish gelatin), labeled in anti-HA antibodies in blocking buffer for 2 hours, washed 3 times for 10 minutes per wash, and labeled with either Alexa-488 (immunofluorescence) or HRP conjugated (ELISAs) secondary antibodies. Surface CFTR was analyzed in the absence of detergents, while 0.1% Triton-X 100 was added for total CFTR analysis. For surface ELISAs, labeled cells were incubated with the HRP substrate ABTS (Pierce) for 30 minutes at room temperature and analyzed by a microplate reader at 405 nm.

Single-particle tracking (SPT) assays

SPT experiments were performed collaboratively with Yun Chen and Ken Jacobson (University of North Carolina at Chapel Hill). Anti-biotin IgG gold (BBInternational, UK) was dissolved in Ham's F12 nutrition mixture (Life Technologies/Gibco-BRL) supplemented with 25 mM HEPES and 15% serum (HHS), sedimented by centrifugation for 15 min at $11,000 \times g$ at 4°C and resuspended in HHS. Cells on the cover slips were first incubated with biotinylated primary HA antibodies for 10 minutes and washed 3 times with 1 ml of HH per wash. The coverslips were mounted to form an open chamber on the slide with spacers on

two edges of the coverslip. 100 μ l of the solution containing anti-biotin gold was injected into the chamber and incubated with cells for 10 minutes at 37°C. Unbound gold particles were then washed away by 100 μ l of HH. A final washing step with 100- μ l-injected HHS was performed to remove unbound gold and the sample chamber was sealed with wax and mounted on the microscope; particle trajectories were recorded for the following 30 min at 37°C, maintained by an air curtain incubator.

Computer-enhanced video microscopy, described earlier (Lee et al., 1991), was used to image colloidal gold bound the plasma membrane. Briefly, the CFTR proteins labeled with bound gold were imaged in brightfield mode and recorded with a video camera (Newvicon Hamamatsu). After online background subtraction and contrast enhancement with an image processing unit (Argus 20, Hamamatsu, Japan), video frames were recorded in time lapse mode (1800 frames with 30 frames/s) on the hard disk of a computer (O2, Silicon Graphics, Mountain View, CA). Recorded movies were analyzed by the commercial software package ISEE (Inovision Corp., Durham, NC) that identifies relative changes of gold particle positions on the cell lamella with a precision of ± 20 nm. All trajectories were visually inspected to ensure correct tracking of gold particles.

CFTR internalization assays

To track the internalization of CFTR, HeLa and BHK cells expressing wild-type or S13F HA-CFTR were cultured on glass cover slips. Live cells were chilled to 4°C on ice and incubated with HA antibodies diluted in phenol-red and serum free DMEM at a concentration of 0.05 μ g/ μ l for 10 minutes. Unbound antibodies were washed off and the cells were returned to 37°C for various times to allow the CFTR/antibody complexes to

internalize. Cells were fixed in 4% PFA, incubated in blocking solution with 0.1% Triton-X 100, and labeled with Alexa-488 conjugated anti-mouse antibodies. Lysosomes were labeled by incubating cells in 50nM lysotracker red dye (Molecular Probes) for 30 minutes.

Peptide transfection

As an alternative to using the S13F mutation to study CFTR surface expression in the absence of filamin binding, we introduced competitive peptides into cells using a cationic lipid delivery system. Specifically, non-biotinylated CFTR[1-25] or CFTR[1-25/S13F] peptides were introduced into BHK cells stably expressing HA-CFTR using the Pro-Ject cationic lipid delivery system (Pierce). For each individual well of a 12-well plate, the Pro-Ject complexes were formed using 5 μ l of Pro-Ject reagent plus 5 μ g of CFTR peptide in a total volume of 40 μ l of HEPES buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.0). Since the peptides cannot be visualized, a tracer, Alexa-488 conjugated Fab' fragments were mixed with the peptides at a molar ratio of 4:1 (peptide-to-Fab' fragment). The surface expression of HA-CFTR was analyzed by immunofluorescence 8 hours after the addition of the peptide-lipid complexes as described above.

Results

The CFTR N-terminus is highly conserved

To identify additional regions of the CFTR N-terminus important for channel function, we compared the sequences of CFTR across species to the analogous region of the highly related ABC-transporters, the multi-drug resistance-associated proteins (MRPs). A region encompassing the first 25 amino acids of CFTR shares the highest degree of homology with the MRP proteins (Figure 4.4.1A). Although no functional significance has

been assigned to residues 1-25 of CFTR or the MRPs, the amino acid conservation suggests an important role for these proteins. Using the cystic fibrosis mutation database (www.genet.sickkids.on.ca/cftr), a repository for CFTR mutations identified in patients with clinical symptoms of CF, we identified three missense mutations in amino acids 1-25, proline 5 to leucine (P5L), serine 13 to phenylalanine (S13F), and tryptophan 19 to cysteine (W19C). Strikingly, these mutations occur on CFTR residues which are absolutely conserved across species and the MRP proteins. Although P5L, S13F, and W19C are rare CFTR mutations, we speculated that they could provide functional insights into the importance of the extreme N-terminus of CFTR. To that end, we examined the basic molecular defect in CFTR biogenesis caused by the P5L, S13F, or W19C mutations. Each mutation, incorporated into the full-length, untagged CFTR protein was transiently expressed in HEK-293 cells. Wild-type or mutant CFTR proteins were immunoprecipitated from Triton X-100 soluble membranes and analyzed by western blot. The core-glycosylated, ER form of CFTR (band B) migrates with an apparent molecular weight of 160 kD. As CFTR is trafficked through the cis and medial Golgi, the N-glycan is processed to a complex oligosaccharide, decreasing the electrophoretic mobility to 180 kD (band C) (Cheng et al., 1990). Thus, monitoring the glycosylation state of CFTR provides as useful method to study CFTR maturation and trafficking. At steady state, wild-type CFTR is predominantly present as the maturely glycosylated band C (Figure 4.1B). Conversely, P5L and W19C are found only as the ER, core-glycosylated band B protein (Figure 4.1B). This is not surprising in light of previous observations that missense mutations in many regions of CFTR, including the N-terminus, cytosolic loops, nucleotide binding domains, and COOH-terminus disrupt CFTR biosynthesis

Figure 4.1. The N-terminus of CFTR is required for normal biogenesis and function

A. A primary sequence alignment of N-terminal amino acids of CFTR and MRP proteins. B. Wild-type CFTR or the indicated mutants were transiently expressed in HEK-293 cells and analyzed by western blot. C. Wild-type CFTR or the indicated mutants were transiently expressed in 16HBE14o-, HEK-293, or HeLa cells and analyzed by western blot. D. Western blot data for CFTR proteins expressed in HEK-293 cells was quantitated using densitometry. For all CFTR proteins, the level of mature band C CFTR was normalized to the level of band B CFTR. Paired t-tests were performed to compare levels of mature CFTR protein to wild-type CFTR (** is $p < 0.005$, *** is $p < 0.0005$) or to zero (+ is $p < 0.01$, ++ is $p < 0.001$) for an $n = 3$.

and therefore do not exit the ER (Cheng et al., 1990; Seibert et al., 1997; Seibert et al., 1996). We were particularly interested in S13F CFTR which clearly exhibits a significant pool of mature band C protein. However, the S13F band B:C ratio is clearly different from wild-type CFTR reflecting a 3.6-fold decrease relative to wild-type (Figure 4.1B and C). In contrast, mutation of serine 13 to alanine, S13A, has a band B:C ratio similar to wild-type. This suggests that the decrease in mature CFTR associated with the S13F mutation likely reflects the presence of a bulky, hydrophobic phenylalanine residue, as opposed to an absolute requirement for a serine at position 13. We observe similar decreases in the band C protein for S13F CFTR in both HeLa cells and an airway epithelial cell line, 16HBE14o-, demonstrating that the defect in S13F maturation is not cell type specific and is recapitulated in airway epithelial cells where CFTR is expressed (Figure 4.1D). Thus, the N-terminus can play a role in CFTR biogenesis or in the trafficking and stabilization of the mature protein.

The N-terminus of CFTR interacts directly with filamins

Our mutagenesis studies demonstrate that the extreme N-terminus of CFTR is necessary for channel biosynthesis. Furthermore, the decrease in band C CFTR associated with the S13F mutation suggests that the N-terminus may additionally regulate CFTR trafficking or protein half-life. Thus, we speculated that the highly conserved N-terminus may mediate a protein-protein interaction which is affected by the S13F mutation. To test this hypothesis, we created CFTR affinity columns using immobilized CFTR peptides corresponding to residues 1-25 of the N-terminus, CFTR[1-25]. The CFTR columns were incubated with 1 mg of total protein solubilized from Calu-3 airway epithelial cells and washed extensively. Bound proteins were eluted, resolved by SDS-PAGE, and visualized

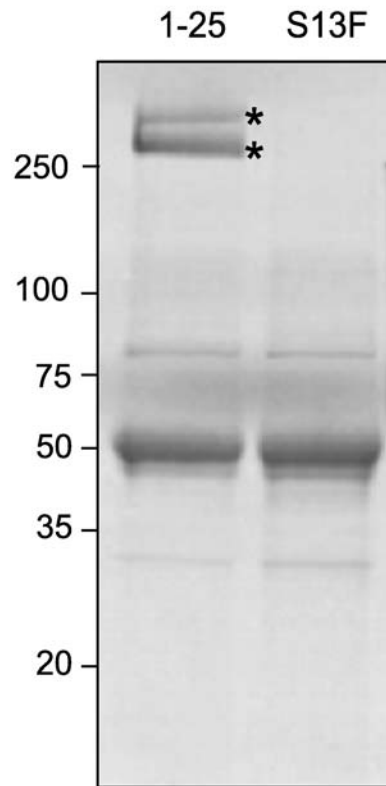
with coomassie blue. We detected several distinct bands associated with both CFTR[1-25] and CFTR[1-25/S13F]. However, two high molecular weight bands (>250 kD) specifically and abundantly co-purify with wild-type CFTR[1-25], but not peptides which incorporate the S13F mutation, CFTR[1-25/S13F] (Figure 4.2A). These bands were excised, subjected to in-gel digestion with trypsin, and analyzed by mass spectrometry (MS). By MALDI TOF/TOF MS/MS, we identified the high molecular weight doublet as human filamin A (FLN-A or ABP-280) and filamin B (FLN-B or ABP-278) (Figure 4.2B). FLN-A and FLN-B are composed of two actin binding calponin homology domains, 24 Ig-like filamin repeats, and a COOH-terminal dimerization domain (Figure 4.3) (Stossel et al., 2001). There are three FLN genes that share 60-80% sequence identity but differ in the number of FLN repeats and in their tissue distribution. FLN-A and -B are broadly expressed while FLN-C is restricted to skeletal and heart muscle. Initially identified as proteins able to cross-link actin filaments to form orthogonal networks (Wang and Singer, 1977), filamins are now known to bind ion channels, receptors, and soluble signaling molecules, suggesting that filamins can facilitate the activation of local signaling events (Table 2). Additionally, filamins regulate protein trafficking, in the biosynthetic and endosomal systems, and helps stabilize ion channels and cell surface receptors at the plasma membrane (Table 2). Filamins have overlapping and unique functions and can form homo- or heterodimers, further increasing the complexity of FLN-containing protein complexes (Stossel et al., 2001).

To validate that filamin is a relevant CFTR binding protein, we further characterized the interaction in biochemical assays. In agreement with the MS data, FLN-A co-purifies with the CFTR[1-25] peptide from Calu-3 lysates by western blot (Figure 4.4A). Moreover, purified, full-length FLN-A interacts with CFTR[1-25], demonstrating that the interaction is

Figure 4.2. Filamins interact with the N-terminus of CFTR.

A. The biotinylated peptides corresponding to residues 1-25 or 1-25 (S13F) of CFTR were immobilized on streptavidin agarose and incubated with Calu-3 cell lysates. Bound proteins were resolved by SDS-PAGE and visualized with coomassie blue. Asterisks indicate the bands which were excised and processed for MS analysis. B. Summary of MALDI-TOF/TOF MS data used to identify excised bands as filamin-A and filamin-B.

A



B

I.D.	% Coverage	Peptides
Filamin-A	34%	81
Filamin-B	47%	102

Figure 4.3. Filamins integrate cell signaling and mechanics.

Filamins are composed of two N-terminal actin binding domains (ABD) and a variable number of filamin repeats, the most C-terminal which mediates dimerization (D). The filamin repeats function as protein-protein interaction surfaces that bind to soluble signaling molecules, receptors, and ion channels tethering them to the cytoskeleton.

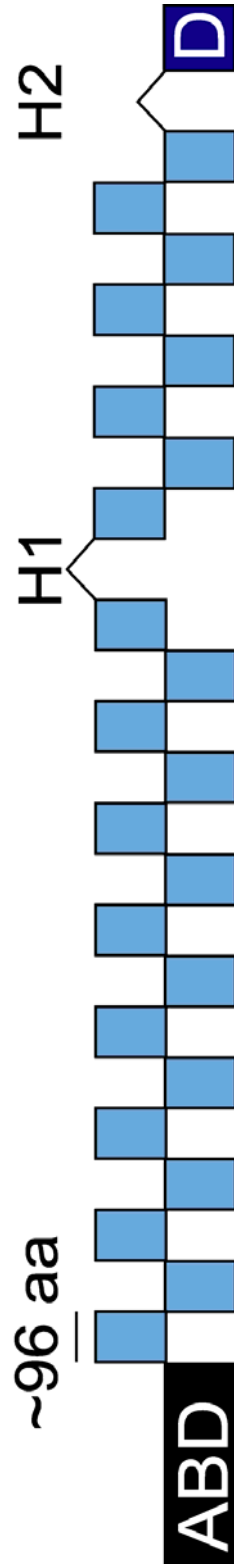


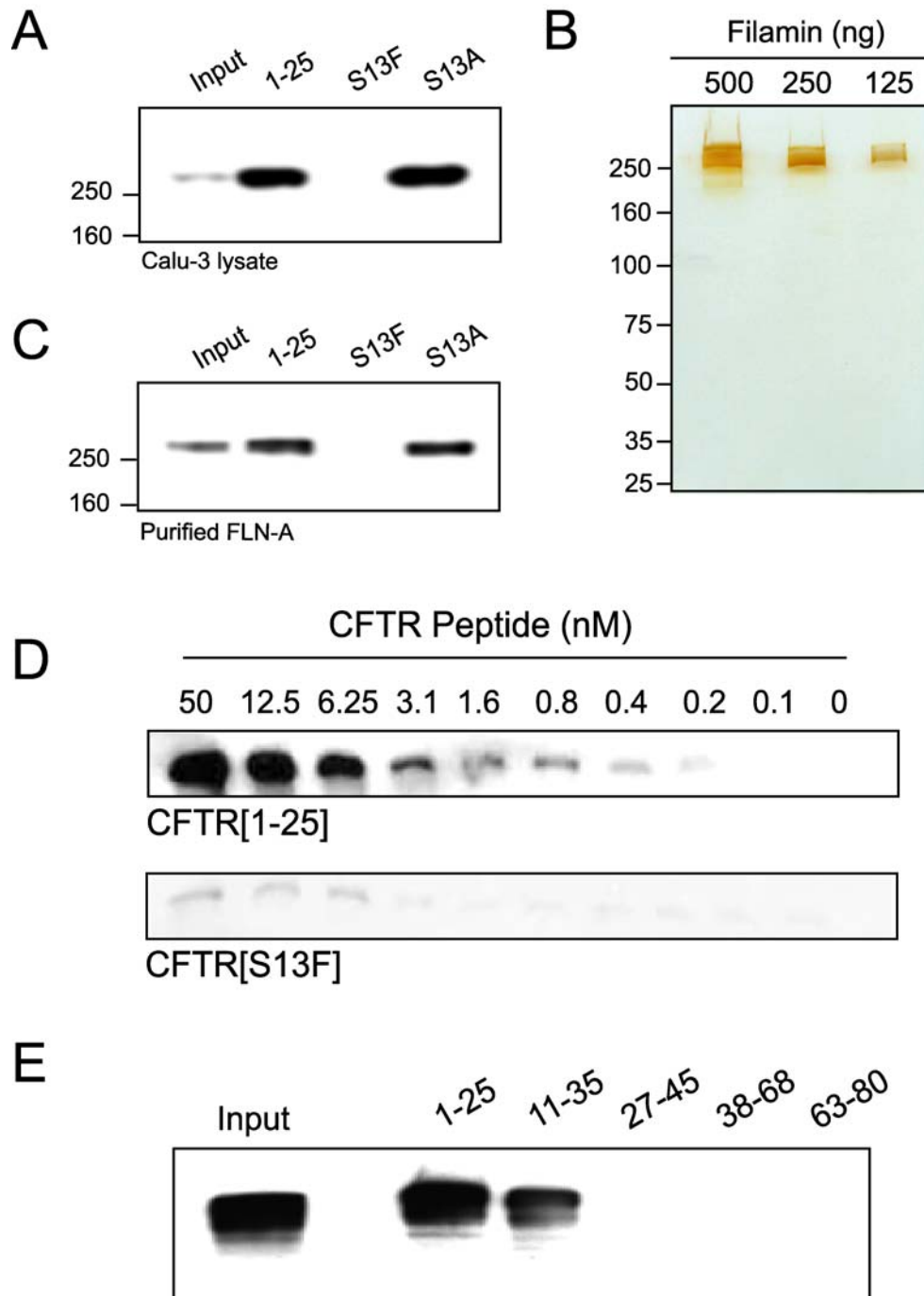
Table 2. Filamins regulates the biosynthesis, trafficking, and activity of numerous transmembrane proteins

Filamins interact with a wide variety of transmembrane proteins including G-protein coupled receptors, ion channels, and adhesion receptors. References: (Arthur et al., 2005; Christodoulides et al., 2001; Cranmer et al., 2005; Dubois et al., 2003; Dyson et al., 2003; Englund et al., 2001; Enz, 2002; Enz and Croci, 2003; Feng et al., 2005; Feng et al., 2003; Glogauer et al., 1998; Gravante et al., 2004; Gu et al., 1999; He et al., 2003; Kanaji et al., 2002; Kovacsovics and Hartwig, 1996; Li et al., 2000; Loo et al., 1998a; Meyer et al., 1997; Nakamura et al., 2005b; Ohta et al., 1991; Onoprishvili et al., 2003; Petrecca et al., 2000; Pfaff et al., 1998; Pi et al., 2002; Sampson et al., 2003; Seck et al., 2003; Sharma et al., 1995; Takafuta et al., 1998; Tu et al., 2003; Williamson et al., 2002; Xu et al., 1998).

FLN Interactors	Function
Beta (1, 2) Integrin	Mechanoprotection
Calcitonin Receptor	Endocytic recycling
Calcium-sensing Receptor	Scaffolding and regulation of downstream signaling
Dopamine Receptor (D2, D3)	Trafficking and compartmentalization with regulatory proteins
Glycoprotein Iba	Biosynthesis and stabilization of platelet adhesion
Furin	Endocytic trafficking
Insulin Receptor	Compartmentalization of signaling molecules
Kir2.1	PM localization
Kv2.4	Increase PM current density
Metabotropic Glutamate Receptor 7	Not determined
Mu Opioid Receptor	Internalization and desensitization
Pacemaker Channel (HCN1)	PM localization
Prostate Specific Membrane Antigen	Internalization and enzymatic activity

Figure 4.4. The S13F mutation abolishes the interaction between full-length CFTR and filamin

A. The indicated biotinylated CFTR peptides were immobilized on streptavidin agarose and incubated with Calu-3 cell lysates. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using α -FLN-A antibodies. B Silver stained gel of human FLN-A purified from human platelets. C. The indicated biotinylated CFTR peptides were immobilized on streptavidin agarose and incubated purified FLN-A. D. Purified FLN-A (50 nM) was incubated with the indicated concentrations of immobilized CFTR peptides. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using α -FLN-A antibodies. E. The indicated biotinylated CFTR peptides were immobilized on streptavidin agarose and incubated with Calu-3 cell lysates. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using α -FLN-A antibodies.



direct (Figure 4.4B and C) and concentration-dependent (Figure 4.4D). Importantly, the incorporation of the S13F mutation abolishes the interaction with FLN-A (Figure 4.4A, B, and D). In addition, using overlapping CFTR peptides that cover the entire N-terminus, we find that filamin interacts with both CFTR[1-25] and CFTR[11-35] (Figure 4.4E). Thus, the overlapping sequence, CFTR residues 11-25, defines the filamin binding motif. Conversely, we mapped the CFTR binding site on FLN-A using a series of FLN-A fusion proteins. Immobilized CFTR[1-25] or CFTR[1-25/S13F] were incubated with purified FLN-A fusion proteins corresponding to the calponin homology domains or filamin repeats 1-4, 5-7, 7-11, 12-15, 14-20, 20-23, or 23-24. We find that FLN-A repeats 1-4 bind to the CFTR N-terminus with the highest affinity (Figure 4.5A). We used the FLN-A repeats 1-4 protein in surface plasmon resonance experiments to determine the affinity of this interaction. We find that FLN-A repeats 1-4 specifically interact with the CFTR[1-25] peptide with a disassociation constant of 320 nM (Figure 4.5B). In preliminary experiments, FLN-B also directly associates with CFTR (data not shown). While our lab is currently characterizing the CFTR-FLN-B interactions, the work described here focuses specifically on FLN-A.

CFTR and FLN-A interact *in vivo*

Using *in vitro* assays, we clearly demonstrate that FLN-A directly interacts with the CFTR N-terminus. Furthermore, the S13F mutation disrupts filamin binding and may provide a mechanistic explanation for why the levels of mature S13F CFTR are reduced compared to wild-type CFTR. However, to associate within a biological context, CFTR and FLN-A must be co-expressed in the same cells and at least partially co-localize to the same subcellular compartment. Therefore, we examined this interaction in the context of the full-

length proteins by immunoprecipitation, and asked whether CFTR and FLN-A co-localize in relevant tissues. FLN-A co-immunoprecipitates with endogenous CFTR from Calu-3 cell lysates, but not an IgG control (Figure 4.6A). While these data strongly suggest that CFTR and filamin interact in the cell, we cannot rule out that that these proteins associate post-lysis. Thus, we performed a mixing experiment to test whether CFTR and filamin must be expressed in the same cells to interact by co-immunoprecipitation. CFTR and human FLN-A were expressed in mouse M1 cells as diagrammed in Figure 4.6B. Taking advantage of a FLN-A monoclonal antibody which detects human but not mouse FLN-A, we detect hFLN-A in CFTR immunoprecipitates when the proteins were expressed in the same cells (Figure 4.6C). However, we do not observe an interaction between CFTR and FLN-A when the proteins were expressed independently and mixed post-lysis. Thus, the CFTR-FLN-A interaction takes place in intact cells.

We next studied the localization of FLN-A in polarized epithelia known to express CFTR. In polarized Calu-3 cells, FLN-A is associated with intracellular vesicular structures and the cell cortex where it is highly concentrated at the sub-apical compartment (Figure 4.7A). Likewise, CFTR is observed primarily at the apical cell surface in Calu-3 cells. We observe a similar localization of FLN-A in well differentiated primary airway epithelial cells (WD-PAE) and human sweat duct (Figure 4.7B and C). The strong apical localization of FLN-A in polarized epithelia suggest that CFTR and FLN-A may interact at or near the cell surface; however, do not preclude an interaction in an intracellular compartment.

Figure 4.5. The N-terminus of CFTR associates with FLN repeats 1-4.

A. Filamin domains were expressed as GST fusion proteins and incubated with immobilized CFTR peptides. Bound proteins were resolved by SDS-PAGE and analyzed by western blot using a GST antibody. B, C. Surface plasmon resonance (SPR) was used to determine the affinity of the CFTR/FLN interaction. CFTR[1-25] was immobilized on a streptavidin SPR chip and the binding of FLN fusion proteins was analyzed. B. GST FLN-A repeats 1-4 (10^{-6} M), but not GST repeats 12-14 or GST alone, bind to CFTR[1-25]. C. GST FLN repeats 1-4 interact with CFTR[1-25] with an apparent K_d of 320 nM.

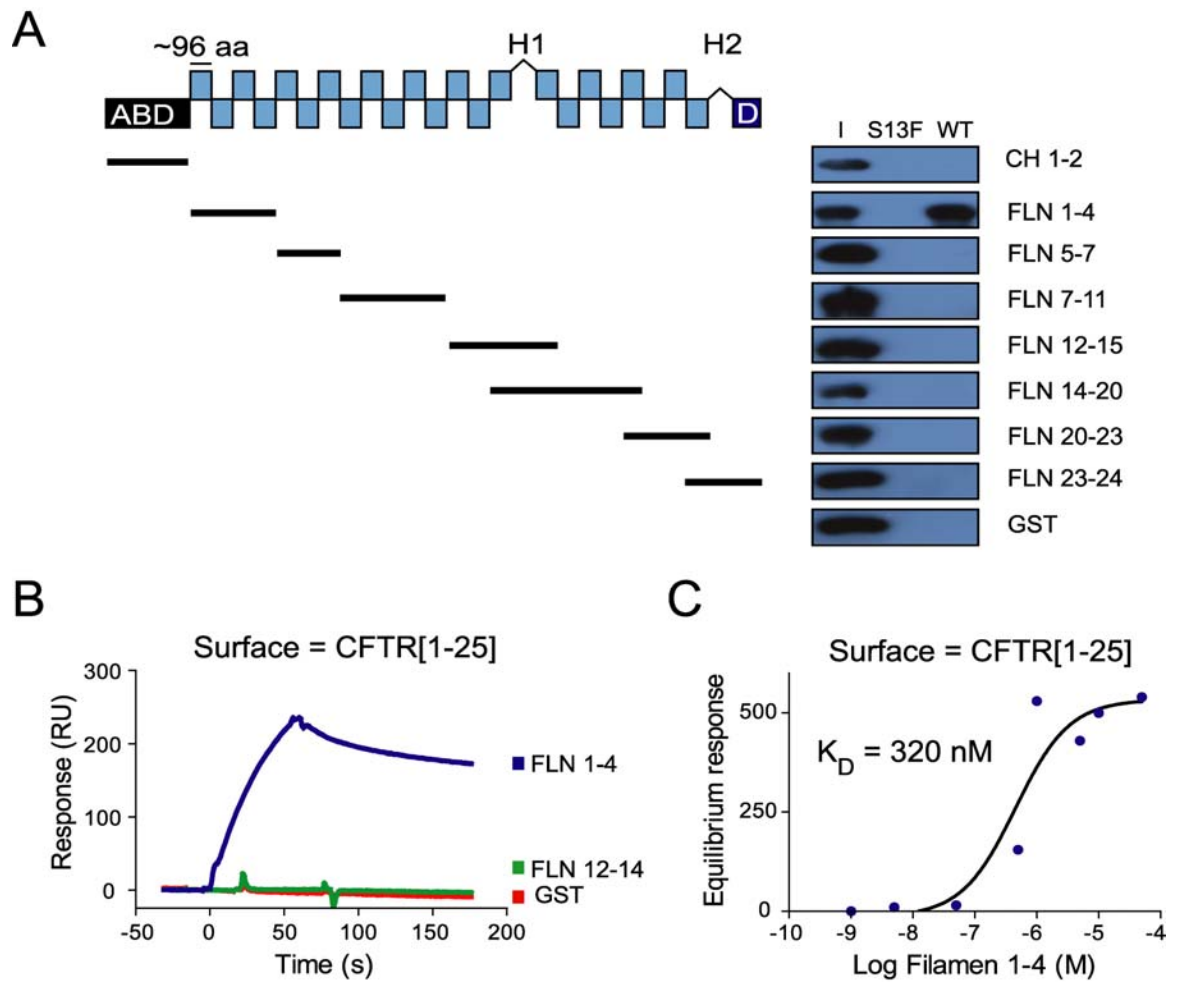


Figure 4.6. FLN-A co-precipitates with endogenous CFTR

A. Triton-X 100 soluble membranes from Calu-3 cell were incubated with α -CFTR monoclonal antibodies covalently bound to protein G dynabeads. Bound proteins were analyzed by western blot using α -CFTR and α -FLN-A monoclonal antibodies. Inputs represent 5% of the total lysates. B. Schematic of mixing experiment. Human CFTR was expressed in mouse M1 cells. Human filamin was expressed in the same cells as CFTR (co-expressed) or in a separated dish of M1 cells (mixed). For “mixed” samples, lysates prepared from cells expressing CFTR alone and human filamin alone were mixed immediately after lysis. C. The analysis of the mixing experiment. CFTR was immunoprecipitated from lysates and analyzed by western blot using α -CFTR and α -FLN-A monoclonal antibodies.

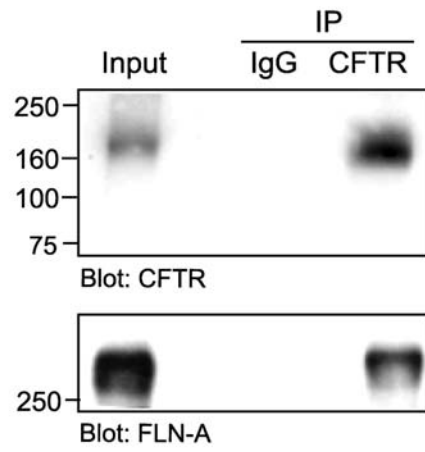
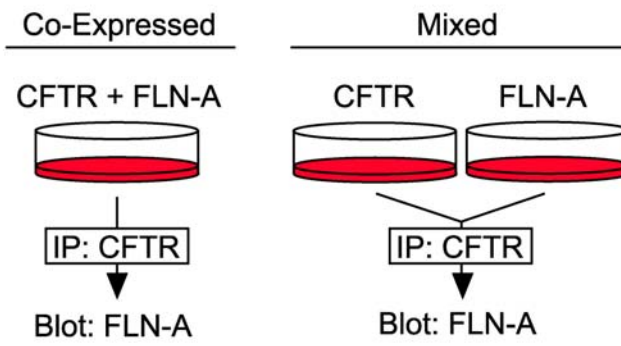
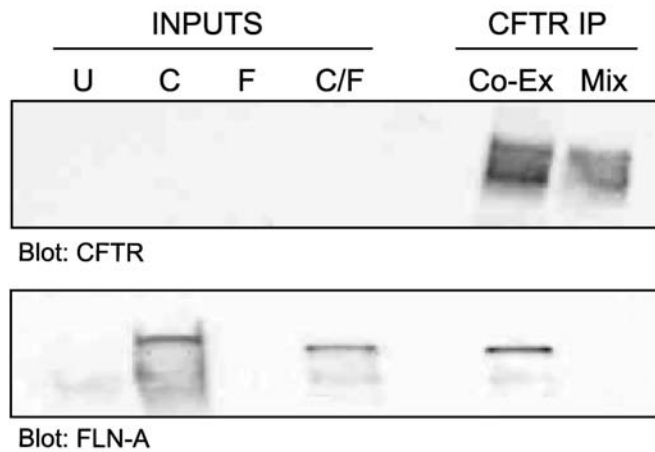
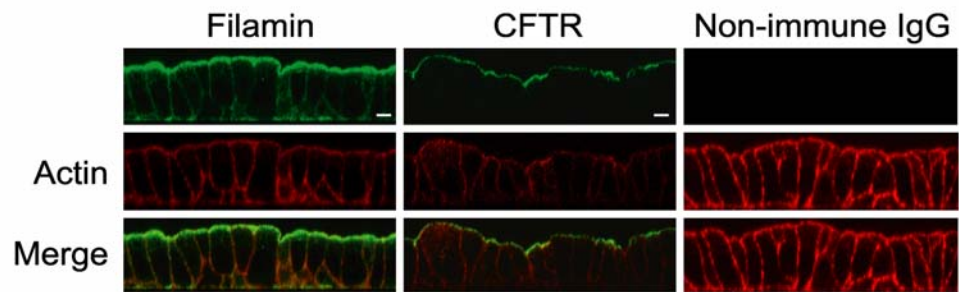
A**B****C**

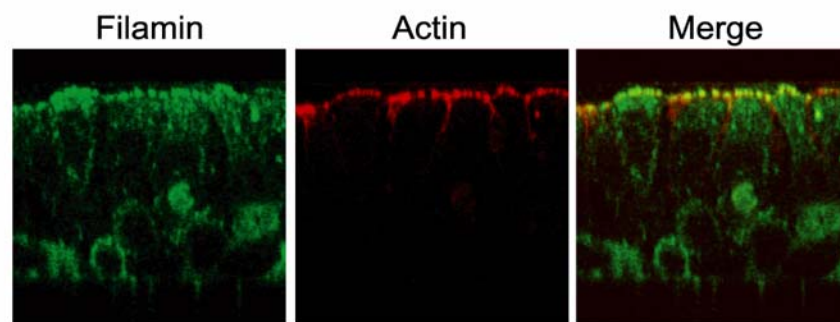
Figure 4.7. FLN-A localizes to the sub-apical compartment of polarized epithelia

A and B. Polarized Calu-3 cells (A) or WD-PAE cells (B) were grown on permeable supports and stained with FLN-A or CFTR antisera (shown in green). No specific staining was observed using the secondary antibody alone. Cell morphology (shown in red) was determined using rhodamine phalloidin to label actin. Images are XZ scans from a Leica confocal microscope. C. Frozen sections of human sweat ducts were stained with FLN-A as indicated. Cell morphology was determined using rhodamine phalloidin to label actin. Images are XY scans from a Leica confocal microscope. *Scale bars = 5 μm.*

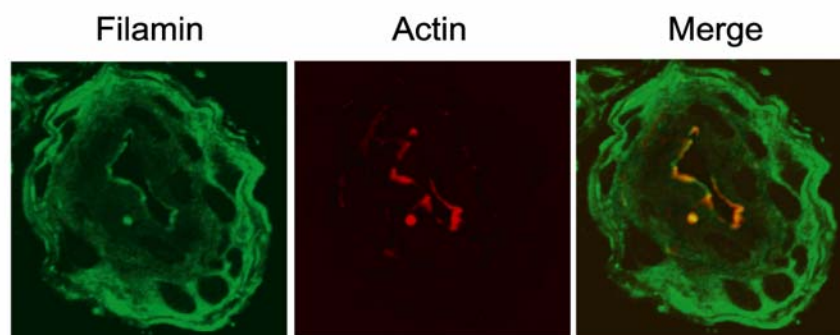
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B



C



The importance of CFTR residue 13 for FLN binding and CFTR biogenesis

We next sought to study the functional significance of the CFTR-filamin interaction in cells. The S13F mutation abolishes filamin binding when incorporated into the CFTR[1-25] peptide, suggesting that this mutation provides a powerful tool to study FLN-A interaction in the context of the full-length channel. To validate that the S13F mutation disrupts filamin binding in a cellular context, wild-type or S13F CFTR were immunoprecipitated from transiently transfected HEK-293 cells and analyzed by western blot. While FLN-A clearly co-precipitates with wild-type CFTR, FLN-A is not detected in the CFTR/S13F immunoprecipitates (Figure 4.8A). We also compared the effects of mutating serine 13 to other amino acids to further understand the importance of amino acid charge and size on the CFTR-FLN-A interaction. For many of the amino acid substitutions including S13A, S13N, S13Q, S13D, S13E, and S13H, the band B:C ratio of CFTR is indistinguishable from wild-type CFTR (Figure 4.8A). Furthermore, FLN-A binding is also similar to wild-type for these mutants demonstrating that a serine at position 13 is not absolutely required for the interaction. Because S13A CFTR behaves similarly to wild-type CFTR with respect to the steady state distribution (Figure 4.1C) and FLN-A binding in both peptide pulldowns (Figure 4.4A and 4C) and co-immunoprecipitation experiments (Figure 4.8A), we have chosen to use it as a control in all other studies.

By comparison, the substitution of S13 with an acidic amino acid, glutamic or aspartic acid, appears to significantly diminish, but not abolish filamin binding. While it is not clear whether S13 can be phosphorylated *in vivo*, these data suggest a potential regulatory mechanism. Furthermore, the S13I and S13R mutants are only observed in the core-glycosylated state indicating that they disrupt folding and are not competent to exit the ER.

FLN-A binding is not observed for S13I or S13R (Figure 4.8A). While it is not clear whether the S13I and S13R mutations disrupt FLN-A binding, an intriguing possibility is that the CFTR-FLN-A interaction takes place in a post-ER compartment. This prediction is further supported by the observation that ER-retained Δ F508 CFTR does not interact with FLN. Furthermore, our immunohistochemistry data suggest that CFTR and FLN-A may interact near the cell surface consistent with the accumulation of both proteins near the apical membrane in polarized epithelia. To test this hypothesis, HEK-293 cells were grown at a permissive temperature, 28°C, which allow Δ F508 to traffic out of the ER. Under these conditions, we find that Δ F508 CFTR partially matures as indicated by the appearance of band C (Figure 4.8B). By immunoprecipitation, FLN-A co-purifies with rescued Δ F508, but not Δ F508 from cells grown at 37°C. Thus, we speculate that CFTR and FLN-A may interact in a post-ER compartment. Taken together, these mutagenesis studies further suggest that FLN-A interaction is relatively resilient to changes in amino acid size and charge at position 13 of the CFTR N-terminus. However, substitutions of S13 to large hydrophobic or highly charged residues can decrease the FLN-A interaction with CFTR, as well as affect CFTR biogenesis. Another important finding from these studies is that CFTR and FLN-A likely interact in a post-ER compartment.

Filamin binding regulates the turnover of CFTR

Our data demonstrate that the N-terminus of CFTR mediates a protein-protein interaction with filamins which is disrupted by the disease-causing S13F mutation. To characterize the mechanism underlying the decreased amount of mature CFTR in the band B:C ratio observed for S13F, we analyzed CFTR maturation and degradation by pulse-chase

over a 24 hour period (Figure 4.9A). Either wild-type, S13F, or S13A CFTR were transiently expressed in HEK-293 cells, labeled for the indicated times, immunoprecipitated, and analyzed by autoradiography. As additional control, we included the Δ F508 CFTR in these studies since this mutant represents a biosynthetically defective CFTR protein which fails to mature. After four hours of chase, wild-type and S13A CFTR have almost completely matured as indicated by the conversion of band B:C, while Δ F508 exhibits no maturation (Figure 4.9A-C). Furthermore, during the two and four hour time points, S13F CFTR does not significantly differ from wild-type and S13A CFTR. Thus, these data suggest that filamins do not regulate CFTR maturation, consistent with our observation that CFTR and FLN-A do not likely interact in the ER. Given that S13F CFTR clearly exits the ER, it is unlikely that this mutation causes a global folding defect in CFTR. Moreover, we do not observe a change in the band B:C ratio for S13F CFTR in cells grown at 28°C, conditions which permit partial Δ F508 export from the ER (data not shown). Conversely, mature S13F CFTR is significantly less stable than mature wild-type or S13A CFTR during the 24 hour chase. Specifically, the half-life of band C S13F CFTR ($t_{1/2}$ = 14.1h) is reduced by nearly 40% relative to wild-type and S13A ($t_{1/2}$ = 22.5 h, and 21.6h respectively) (Figure 4.9A and D). Thus, the reduction of steady state complex-glycosylated S13F CFTR primarily reflects an increase in the turnover of the mature CFTR protein.

Filamins regulate the stability and dynamics of CFTR at the plasma membrane

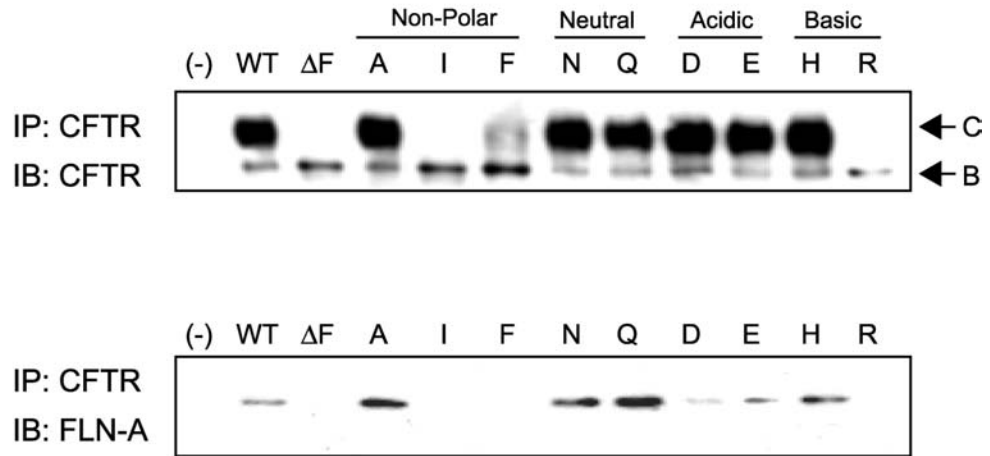
We find that the turnover of mature CFTR, rather than CFTR biosynthesis, is most significantly affected by the loss of filamin binding. These results are consistent with numerous studies which demonstrate a role for filamin in the regulation of plasma membrane

Figure 4.8. CFTR mutations affect filamin binding

A. Serine 13 of CFTR was mutated to additional amino acids. Wild-type CFTR or the indicated mutants were transiently expressed in HEK-293 cells. CFTR was immunoprecipitated from whole cell lysates as described in the experimental procedures. Immunoprecipitates were analyzed by western blot using α -CFTR and α -FLN-A antibodies.

B. Wild-type, Δ F508, S13F, or S13A CFTR were transiently expressed in HEK-293 cells. For rescued Δ F508 (r Δ F508), cells were grown at 28°C, conditions which permit the partial ER-exit of Δ F508. CFTR was immunoprecipitated and bound proteins were analyzed by western blot using α -CFTR and α -FLN-A monoclonal antibodies.

A



B

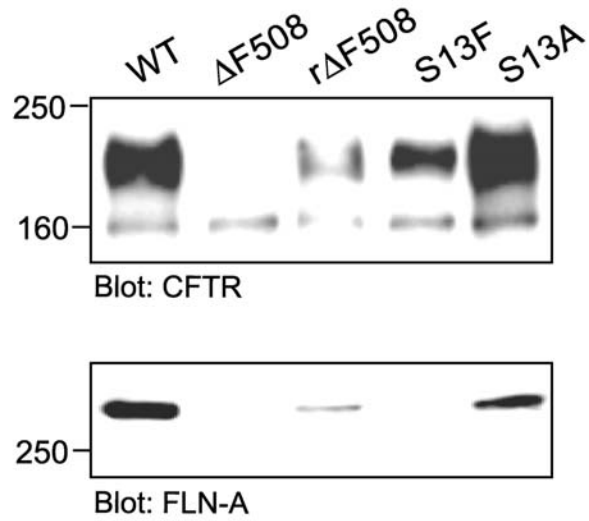
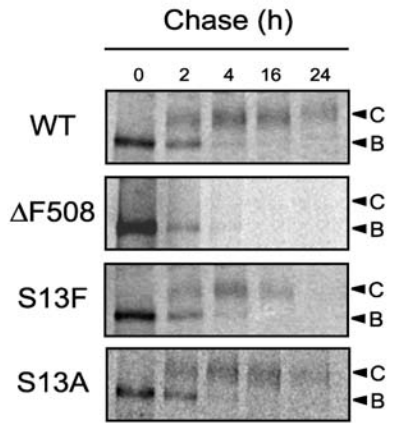


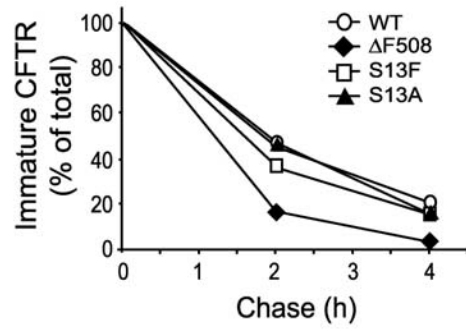
Figure 4.9. The S13F mutation decreases the half-life of mature CFTR.

A. HEK-293 cells expressing wild-type, S13F, S13A, or Δ F508 CFTR were labeled with ^{35}S -methionine/cysteine for 30 minutes and chased with excess cold methionine/cysteine for the indicated times. CFTR was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. B. The loss of band B CFTR was tracked at the 0, 2, and 4 hour chase times. The band B CFTR signal from each time point was quantitated and represented as the percentage of total CFTR at the zero time point. C. CFTR maturation was monitored at the 0, 2, and 4 hour chase times. The band C CFTR signal from each time point is represented as the percentage of total CFTR at the zero time point. The data in A, B, and C are representative of 4 different experiments. D. The half-life of CFTR was calculated by following the decrease of band C CFTR during the 4, 16, and 24 hour chase times. The band C CFTR signal from each time point is represented as the percentage of total CFTR at the 4 hour time point. We chose the 4 hour time point as it is the earliest time where all labeled band B CFTR has been chased to mature band C. The data summaries results from 4 different experiments. The half-life was determined by time in which the total amount of CFTR at 4 hours was reduced by 50% (indicated by dashed line).

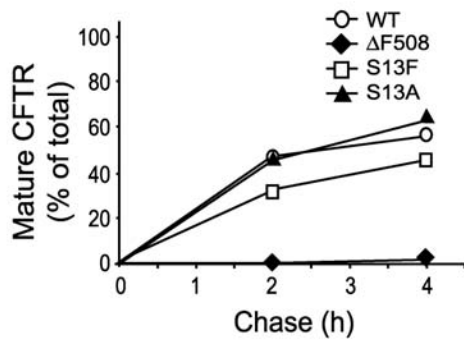
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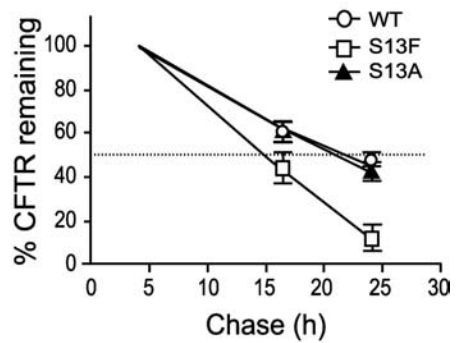
B



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D

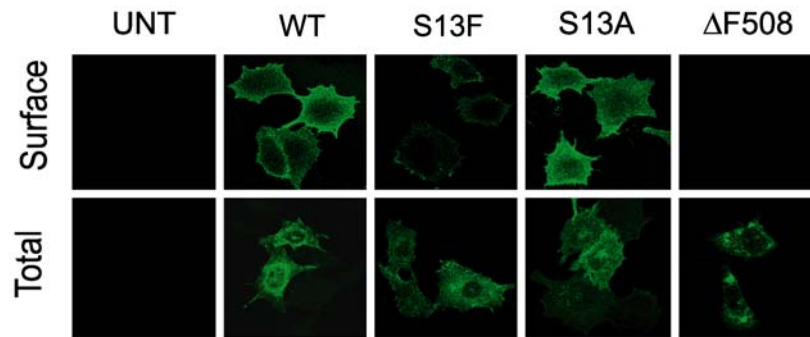


stability of transmembrane proteins. Because filamins couple directly to actin, they have been proposed to anchor their transmembrane binding partners to the cytoskeleton. Furthermore, the cell surface expression is significantly decreased for proteins such as GPI β , β 1 and β 2 integrins, the inward rectifying potassium channel Kir2.1, and the cardiac pacemaker channel HCN1 (Table 2) in cells lacking FLN-A. To examine whether filamins regulate the plasma membrane pool of CFTR, we fluorescently labeled cell surface CFTR proteins which contain an extracellular HA-epitope (HA-CFTR) in unpermeabilized cells. HeLa cells were transiently transfected with either wild-type, S13A, S13F, or Δ F508 HA-CFTR constructs, fixed in the absence of detergents, and labeled with anti-HA antibodies followed by fluorescent secondary antibodies. We observe significant amounts of wild-type and S13A CFTR at the cell surface, while no signal is observed for Δ F508 (Figure 4.10A). The lack of Δ F508 CFTR staining reflects intracellular-retention, as reticular staining consistent with the ER is clearly observed in cells permeabilized with Triton-X 100. Unlike Δ F508 CFTR, we observe surface staining for S13F CFTR; however, the staining is greatly reduced compared to wild-type and S13A CFTR. To quantitatively analyze these differences, we used cell surface ELISAs to measure the plasma membrane pool of CFTR relative to the total cellular CFTR. In agreement with our observations by immunofluorescence, a substantial pool of wild-type and S13A CFTR localize to the cell surface (41.2% and 37.8%, respectively), while almost no Δ F508 is observed (Figure 4.10B). Furthermore, the surface pool of S13F CFTR (8.7%) is significantly less than wild-type and S13A CFTR; however it is significantly greater than Δ F508. Thus, in the absence of filamin binding, S13F CFTR localizes to the cell surface, albeit at a level 78.8% reduced relative to wild-type CFTR.

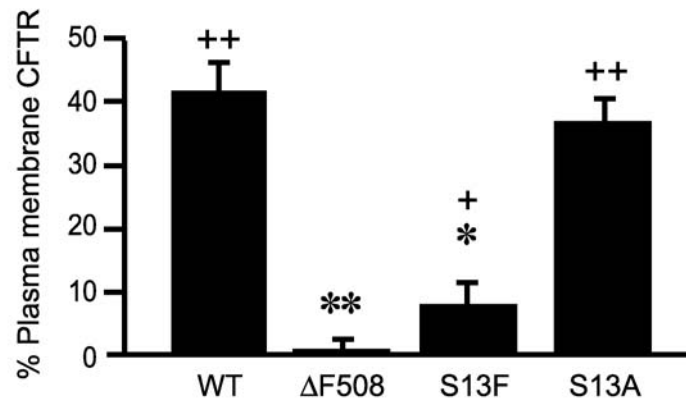
Figure 4.10. S13F CFTR is expressed on the cell surface, but to a lesser extent than wild-type

HeLa cells expressing the indicated HA-CFTR exotope constructs were fixed in 4% PFA, labeled with a mouse anti-HA antibody and a goat anti-mouse secondary antibody conjugated with (A) Alexa-488 or (B) horseradish peroxidase. Surface labeling of CFTR was carried out in the absence of detergents, while cells were permeabilized with 0.1% Triton-X 100 for total CFTR. A. Surface or total CFTR was observed by indirect immunofluorescence using a Leica confocal microscope. *Scale bars* = 10 μm . B. The plasma membrane pool of CFTR was quantitated by ELISA. Labeled cells were incubated in ABTS for 30 minutes at 37°C and read at 405nm. Plasma membrane CFTR is expressed as a percentage of total CFTR.

A



B

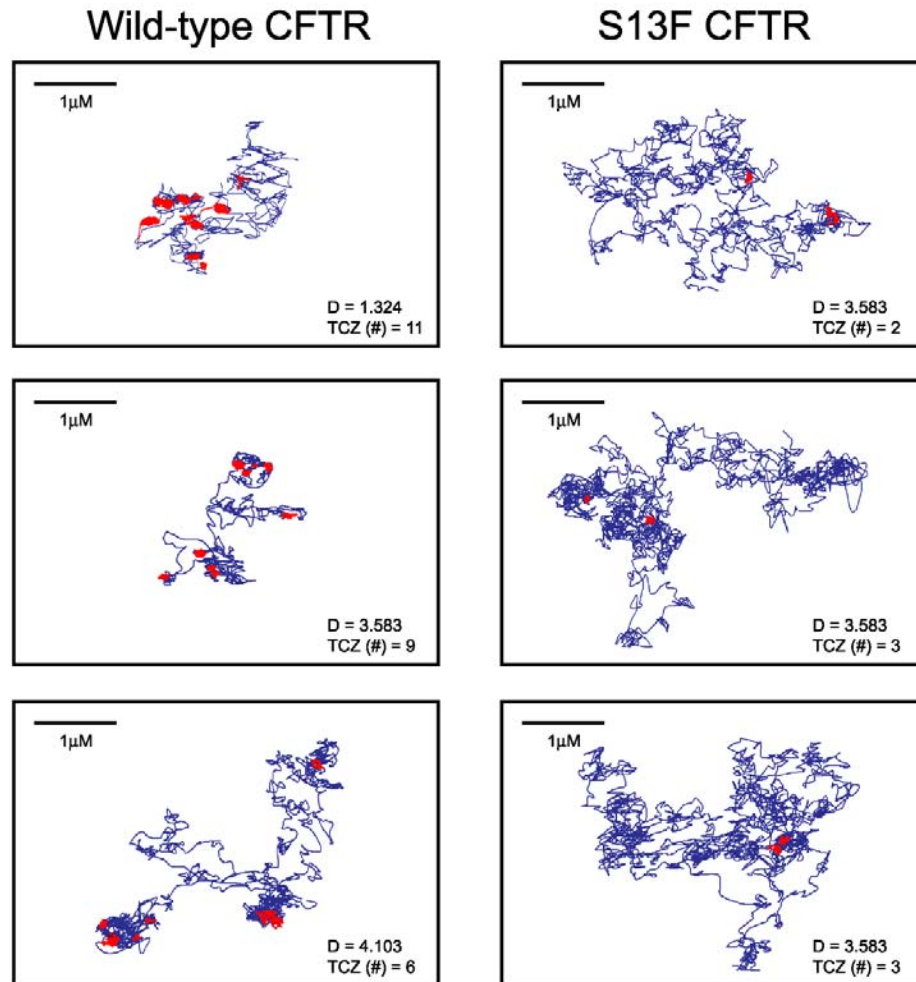


Cytoskeletal interactions regulate the surface expression and spatial organization of transmembrane proteins on the plasma membrane. We previously showed that filamins regulate the surface expression of CFTR as the surface levels of the filamin binding mutant, S13F, is significantly reduced. We next used single particle tracking (SPT) experiments to further analyze the effects of filamin binding on plasma membrane CFTR. SPT allows us to track the mobility of plasma membrane CFTR in real-time. Previous studies have used this technique to observe the effects of lipid raft compartmentalization, membrane confinement, and cytoskeletal anchorage (Kusumi and Sako, 1996; Lambert et al., 2002; Sako et al., 1998; Sheets et al., 1997). Thus, we reasoned that SPT would allow us to observe changes in the membrane dynamics of CFTR associated with filamin-mediated cytoskeletal anchorage. Cell surface HA-CFTR proteins were labeled in live HeLa cells using anti-HA antibodies followed by colloidal gold-conjugated secondary antibodies. We then tracked the trajectories of single gold particles were measured using computer enhanced video microscopy. We analyzed and compared the trajectories of at least 50 different gold-particles from cells expressing wild-type or S13F CFTR. By SPT, wild-type CFTR diffused randomly across the plasma membrane followed by short periods of transient confinement (Figure 4.11A). On average, wild-type CFTR spent 9% of the total recording time in transient confinement zones (TCZs) ranging in size from 100-300 nm in diameter (Figure 4.11B). While the exact nature of these confinement zones is not clear, they may represent interactions with the cytoskeleton or compartmentalization in lipid rafts. To determine whether the association with filamins is important for the transient confinement of CFTR, we examined S13F CFTR by SPT. In contrast to wild-type, we find that S13F CFTR exhibits significantly less transient confinement. The confinement of S13F CFTR is reduced >50% relative to wild-type, which

Figure 4.11. S13F reduces the plasma membrane confinement of CFTR

Single particle tracking experiments were performed as described in the experimental procedures. Wild-type or S13F HA-CFTR were transiently expressed in HeLa cells. Cell surface CFTR proteins were labeled with gold particles conjugated to antibodies. A. An example of a 60 second trajectory for wild-type or S13F CFTR. The entire trajectory is shown in blue while statistically defined confinement zones are shown in red. B. Summary of parameters measured during SPT experiments. Data represent n = 40 for each condition.

A



B

CFTR	D (10^{-10} cm ² /s)	RCT (%)	TCZs (#)	TCZ Dwell (s)
Wild-type	3.45 ± 0.41	8.41 ± 2.50	6.28 ± 1.03	1.00 ± 0.32
S13F	2.73 ± 0.71	4.45 ± 1.16 **	2.28 ± 0.33 **	0.30 ± 0.03 **

reflects a decrease in both the number of confinements and the time spent in a single TCZ (Figure 4.11A and B). Interestingly, the diffusion coefficient of wild-type and S13F were not greatly different (3.22 ± 0.53 versus $2.22 \pm 0.40 \times 10^{-10} \text{cm}^2/\text{sec}$), suggesting that, for CFTR, incorporation into TCZ does not alter the rate of diffusion in the membrane. Qualitatively, we frequently observed labeled S13F proteins which were not mobile on the cell surface and appeared to be engulfed by surrounding membranes. Because these molecules are not mobile they are not included in the SPT analysis. However, this behavior is consistent with internalization. Taken together our surface labeling and SPT experiments demonstrate that filamins modulate both the expression and the confinement of CFTR at the plasma membrane. Furthermore, these data support the hypothesis that filamins play a role in the cell surface stability and spatial organization of CFTR on the cell surface by coupling the channel to the actin cytoskeleton.

S13F CFTR is prematurely targeted to lysosomes

In addition to regulating the stability of cell surface proteins at the plasma membrane, filamins are key regulators of endosomal trafficking. In cells lacking FLN-A, the normal sorting pathways of proteins such as furin (Liu et al., 1997), PSMA (Anilkumar et al., 2003), and the calcitonin receptor (Seck et al., 2003) are altered, resulting in the mis-localization of these proteins to various endosomal compartments. At the plasma membrane, CFTR is rapidly internalized into clathrin-coated vesicles and efficiently returned to the cell surface via endocytic recycling (Bradbury et al., 1999; Bradbury et al., 1994; Lukacs et al., 1997; Peter et al., 2002; Prince et al., 1999; Prince et al., 1994). If the normal recycling of CFTR was impaired in the absence of filamin binding, we predict that this would account for the

decreased cell surface levels. To that end, we followed the endocytic trafficking of internalized CFTR proteins. HeLa cells transiently expressing either wild-type or S13F HA-CFTR were chilled to 4°C to block internalization and labeled with anti-HA antibodies. After removing excess antibodies, the cells were warmed to 37°C for 2, 4, or 8 hours after which the cells were fixed, permeabilized and labeled with fluorescent secondary antibodies. Using the same HA-CFTR construct stably expressed in BHK cells, Gentzsch et al. found that internalized CFTR is predominantly localized to early endosomes by 1 to 2 hours, late endosomes by 4 hours, the TGN by 20 hours, and lysosomes by 24 hours (Gentzsch et al., 2004). In our experiments both wild-type and S13F CFTR exhibit partial overlap with the early endosome marker EEA1 and internalized transferrin at the 2 hour time point (data not shown). However, we observe the most striking difference between wild-type and S13F CFTR at the later 4 and 8 hour time points (Figure 4.12A). At both 4 and 8 hours, we observe a combination of cell surface and vesicular staining for wild-type CFTR. Consistent with the findings of Gentzsch et al, we observe no co-localization of wild-type CFTR with lysosomes by 8 hours (Gentzsch et al., 2004). In contrast, we see a stronger punctuate pattern for S13F CFTR at 4 and 8 hours, with no visible surface staining. Furthermore, at 8 hours, the distribution of S13F CFTR exhibits significant overlap with lysosomes labeled with lysotracker red (Figure 4.12A). Thus, these data suggest that filamins play a role in the endocytic recycling of CFTR.

In our initial studies of the CFTR N-terminal mutations, we found that the steady-state distribution of S13F CFTR displays a decrease in the mature band C protein, which reflects increased degradation. Additionally, S13F is prematurely sorted to lysosomes, which may explain why mature S13F CFTR is degraded more rapidly than the wild-type protein.

Therefore, we examined the half-life of wild-type, S13F, or S13A CFTR by pulse chase in the presence of the lysosomal protease inhibitor leupeptin. Strikingly, leupeptin significantly increased the half-life of S13F CFTR from 13.4 hours to 18.3 hours (Figure 4.12B). Although leupeptin did not fully rescue S13F to the half-life of wild-type CFTR ($t_{1/2} = 21.9$ hours), it suggests that lysosomal mediated degradation accounts for the majority of S13F turnover.

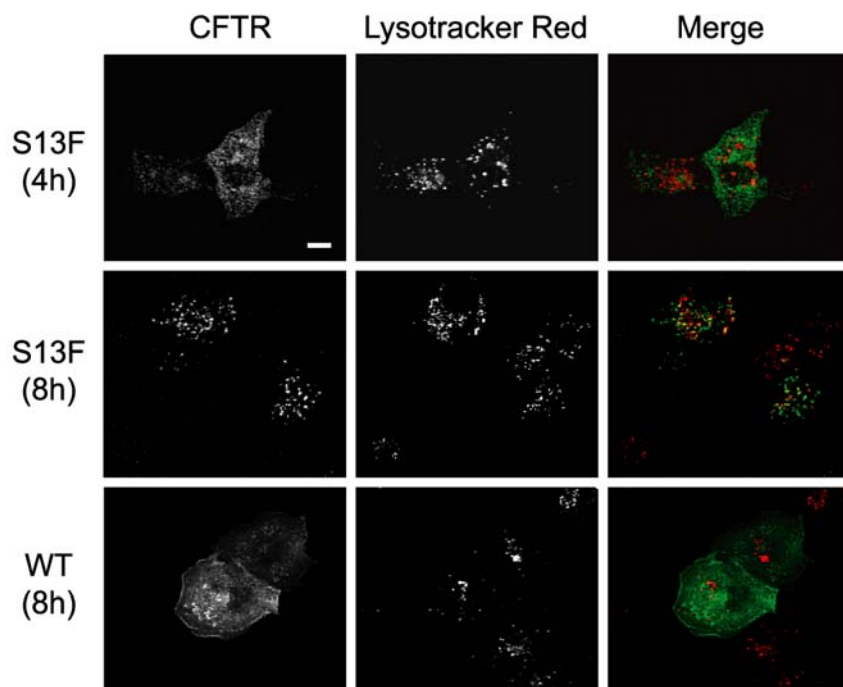
The S13F defects reflect the loss of filamin binding

Our data suggest that filamin binding affects the cell surface stability, endocytic trafficking, and half-life of mature CFTR. However, we were concerned that some of the defects observed for S13F CFTR may be independent of filamin binding. To address this issue, we asked whether we could recapitulate the decrease in CFTR surface expression by using other means to abolish the CFTR-filamin interaction. First, we took advantage of a well characterized melanoma cell line which lacks endogenous FLN-A (M2) and a paired cell line stably re-expressing FLN-A (A7). By immunofluorescence, we clearly observe a decrease in the cell surface pool of CFTR in the FLN-A null M2 cells compared to the A7 cells (Figure 4.13A). Again, using the cell surface ELISA, we find that the percent of CFTR on the cell surface is reduced by 67% in the M2 cells relative to the A7 cells (Figure 4.13B). This decrease is not as statistically significant as the 80% reduction of cell surface levels observed for S13F CFTR compared to wild-type CFTR. However, we speculate that wild-type CFTR can interact with FLN-B in M2 cells while S13F CFTR reflects a loss of binding to both FLN-A and FLN-B. In peptide pulldown experiments using M2 and A7 cells, we isolated high molecular weight bands with the CFTR[1-25] peptide which were absent with the CFTR[1-25/S13F] peptide (data not shown). By MS, we identified these bands as FLN-

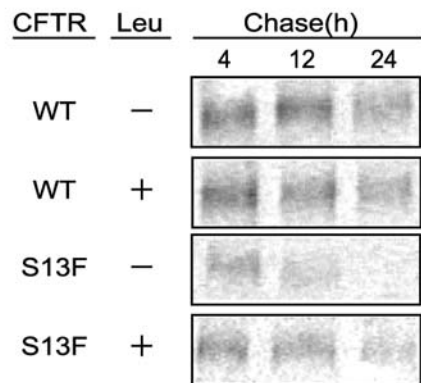
Figure 4.12. S13F is prematurely trafficked to lysosomes where it is degraded

A. Wild-type or S13F HA-CFTR were transiently expressed in HeLa cells. The cells were chilled to 4°C on ice, incubated with α -HA antibodies, washed extensively, and were then re-warmed for the indicated chase times. The cells were subsequently fixed with 4% PFA, permeabilized with 0.1% Triton-X 100, and internalized CFTR was labeled with Alexa-488 anti-mouse antibodies. To label lysosomes, lysotracker red (50 nM) was incubated with the cells for 1 hour prior to fixation. CFTR was observed by indirect immunofluorescence using a Leica confocal microscope. B. HEK-293 cells expressing wild-type or S13F CFTR were labeled with ^{35}S -methionine/cysteine for 30 minutes and chased with excess cold methionine/cysteine for the indicated times in the presence or absence of the lysosomal inhibitor leupeptin (10 $\mu\text{g}/\text{ml}$). CFTR was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. C. The half-life of CFTR was calculated by following the decrease of band C CFTR during the 4, 12, and 24 hour chase times. The data summaries results from 3 different experiments. The half-life was determined by time in which the total amount of CFTR at 4 hours was reduced by 50% (indicated by dashed line).

A



B



C

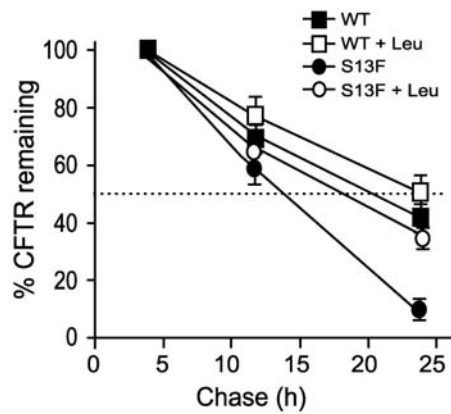
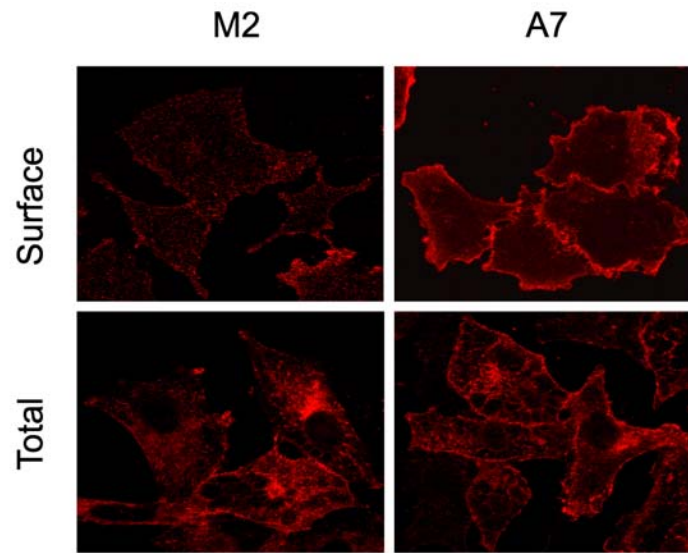


Figure 4.13. CFTR surface expression is reduced in cells lacking FLN-A

A. HA-CFTR was expressed using adenovirus in FLN-A null M2 cells or FLN-A re-expressing A7 cells. The cell surface pool of CFTR was examined by indirect immunofluorescence (A) or surface ELISA (B) as described previously. For ELISAs, GFP adenovirus infected cells were used as a negative control.

A



B

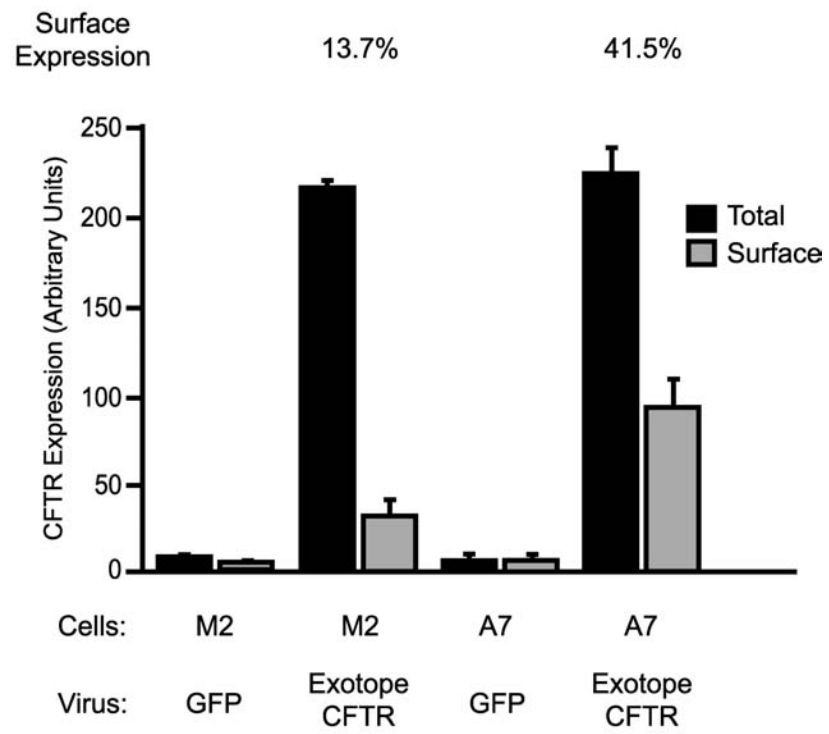
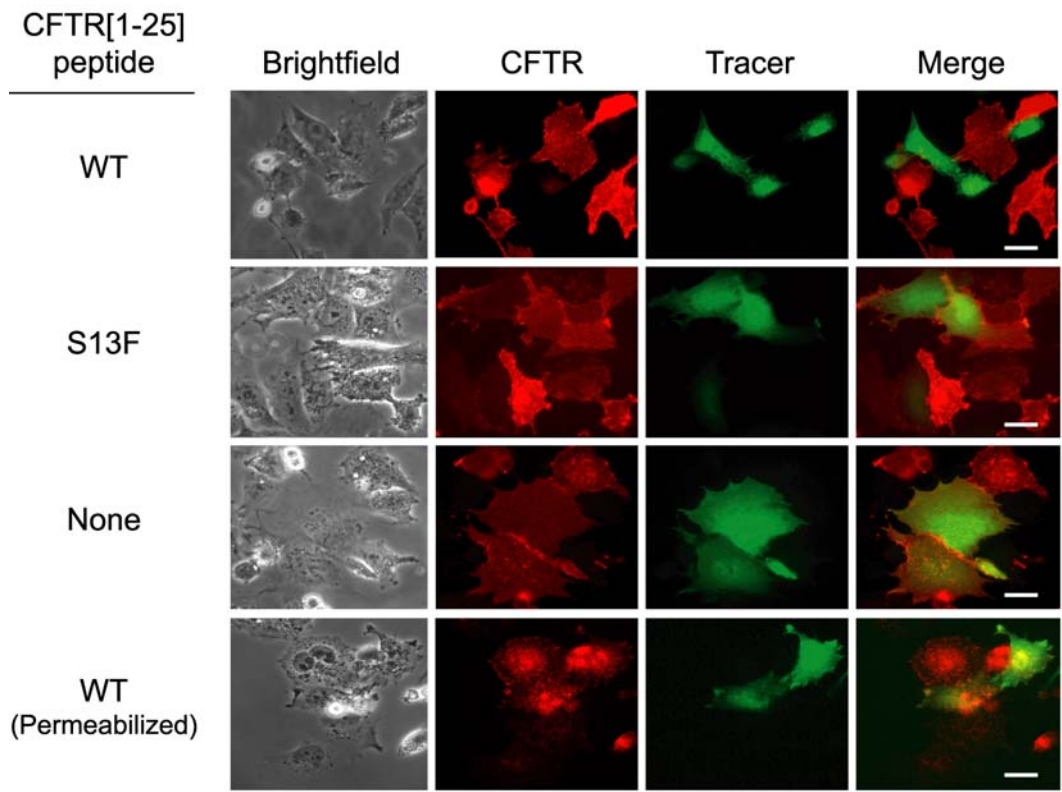


Figure 4.14. Competitive peptides decrease CFTR surface expression

CFTR[1-25] or CFTR[1-25/S13F] peptides were introduced into BHK cells which stably express HA-CFTR using the ProJect cationic lipid delivery system. Because the peptides cannot be visualized, fluorescently labeled Fab' fragment was mixed with the peptides prior to transfection. Cell surface CFTR was labeled with fluorescent antibodies in unpermeabilized cells 8 hours post-transfection. Total CFTR was also examined in 0.1% Triton-X 100 permeabilized cells to ensure that the CFTR[1-25] peptide did not affect CFTR expression. The cells were imaged using a Zeiss fluorescent microscope. *Scale bars = 5 μm.*



A and FLN-B from A7 cells, however, only FLN-B was identified from the M2 cells. This result was confirmed by western blot using FLN-A antibodies (data not shown). In summary, we observe a decrease in the plasma membrane pool of CFTR in FLN-A null cells, which is consistent with our observations for S13F CFTR.

In a second approach, we used a cationic lipid transfection system to introduce CFTR peptides into cells to disrupt the CFTR-Filamin interaction *in situ*. Using this approach, we successfully delivered several fluorescent probes into cells such as FITC-labeled antibodies, Fab' fragments, and streptavidin. While these probes appeared to be abundantly delivered to cells, the number of transfected cells was low (~10%). The low transfection efficiency precludes using assays such as a cell surface ELISA. Therefore, we chose to analyze the effects of the peptide transfection using single cell assays. We examined the surface expression of CFTR in BHK cells stably expressing exotope CFTR and transfected with the non-biotinylated CFTR[1-25] peptide co-transfected with FITC-labeled Fab' fragments as a tracer. Unlike the untransfected cells, we find that the transfected cells exhibit little to no CFTR surface expression (Figure 4.14A). Importantly, this effect was not observed when the S13F peptide or the tracer alone were transfected. Thus, using several different approaches to manipulate the CFTR-filamin interaction, we consistently find that filamin binding is required for the normal surface expression of CFTR. These data strongly support our hypothesis that the defects associated with the S13F mutation reflect a loss of filamin binding and provides a mechanistic explanation for why this missense mutation causes cystic fibrosis.

Discussion

Filamins are structural proteins which regulate cellular organization. Composed of two N-terminal actin binding domains and 24 Ig-like filamin repeats (the most C-terminal which mediates dimerization), filamin dimers are able to crosslink actin filaments (Gorlin et al., 1990). Filamins were originally identified as gelation factors capable of organizing orthogonal actin networks critical for structures such as the cell cortex (Glogauer et al., 1998; Wang and Singer, 1977). Filamins are now known to bind to more than 30 different proteins ranging from ion channels, matrix receptors, G-protein coupled receptors, and soluble signaling molecules (Stossel et al., 2001). Like their role in cytoskeletal organization, filamins generally regulate structural and organizational aspects of their binding proteins. Based largely on studies using the FLN-A null M2 cells and the FLN-A re-expressing A7 cells, investigators have suggested that the association with filamins modulates the plasma membrane expression, endocytic trafficking, and protein complex formation for numerous cell surface proteins. We find that filamins associate with the highly conserved N-terminus of CFTR and that the disease-causing missense mutation in CFTR, S13F, abolishes the interaction (Figure 4.4). Consistently, we find that in the absence of filamin binding, the cell surface expression (Figures 4.10, 4.13, and 4.14), endocytic trafficking (Figure 4.12), and half-life of CFTR (Figure 4.9) is impaired. Thus, filamins regulate multiple aspects of CFTR biology.

Thus far, we have studied the function of the CFTR-filamin interaction using over-expression systems including HEK-293, HeLa, and BHK cells. The ability to transiently transfect these cells make them a convenient model system for initial studies. While heterologous systems are commonly used to study CFTR, they may not accurately reflect the

regulation of CFTR in epithelial tissues. Therefore, in future studies it will be critical to examine the CFTR-filamin interaction in relevant cell types such as airway epithelial cell models including WD-PAE, 16HBE14o-, and Calu-3 cells; as well as intestinal epithelial models such as T84 and Caco-2 cells. Importantly, we have already shown that the pool of mature S13F CFTR is decreased in 16HBE14o- cells similar to our findings in fibroblasts (Figure 4.1C). Thus, we expect that the interaction with filamins will be important for CFTR in epithelial cells. We are currently generating reagents such as adenovirus and retrovirus, which will be used to express CFTR proteins in epithelia. Since filamins are so critical to the maintenance of the actin cytoskeleton, we rationalized that disrupting the normal function of FLN-A or FLN-B would severely impair epithelial cell polarity. Thus, we will take advantage of our observation that the S13F mutation disrupts filamin binding to study the functional significance of this interaction in epithelial cells.

However, while it is attractive to attribute defects such as plasma membrane stability and impaired endocytic sorting to the lack of filamin binding, it is possible that the S13F mutation causes additional phenotypes independent of filamin. Benharouga et al. reported that CFTR lacking the last 70 amino acids ($\Delta 70$ CFTR) is normally trafficked to the cell surface (Benharouga et al., 2001; Haardt et al., 1999). However, $\Delta 70$ CFTR is not stable and is thereby ubiquitinated and degraded via the proteasome. The net effect of the $\Delta 70$ CFTR defect is that this protein displays nearly normal maturation and increased degradation of the mature protein. It is possible that mature S13F CFTR, like $\Delta 70$ CFTR, degraded more rapidly because it results in CFTR mis-folding which destabilizes the protein. Our data argue that this is not the case and that S13F CFTR is functionally distinct from $\Delta 70$ CFTR based on several lines of evidence. First, maneuvers which rescue CFTR defects, such as growing

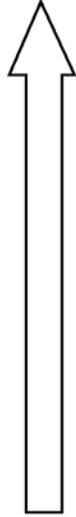
cells at reduced temperatures, do not increase the mature pool of S13F, but do rescue $\Delta 70$ CFTR (data not shown). In addition, $\Delta 70$ CFTR is more heavily ubiquitinated than wild-type CFTR (Benharouga et al., 2001). Conversely, we do not find any difference between the ubiquitination status of S13F and wild-type CFTR (data not shown). Furthermore, the decreased surface expression of S13F CFTR is recapitulated by introducing competitive peptides into cells expressing wild-type CFTR or by expressing wild-type CFTR in the FLN-A null M2 cells (Figure 4.13). The cell surface defects observed for the S13F CFTR are more severe than wild-type CFTR expressed in M2 cells (4-fold versus 3-fold reduction in surface expression). However, S13F CFTR reflects a loss of FLN-A and FLN-B binding (Figure 4.4), while M2 cells express FLN-B which also interacts with CFTR. Thus, it is likely that FLN-B is also a relevant CFTR interacting protein (discussed in greater detail in Chapter 5). Finally, the introduction of competitive CFTR NH₂-peptides into cells disrupts the cell surface expression of CFTR (Figure 4.14). This effect is specific to the wild-type peptide as the S13F peptide has no effect in this assay. Altogether, our data strongly argue that the S13F defects are specific to the loss of filamin binding.

In future experiments, we will utilize additional mutations in CFTR residues 11-25 to study the interaction with filamins. The identification of additional CFTR mutations which disrupt filamin binding will further validate the link between the S13F phenotypes and CFTR regulation by filamins, as well as, provide valuable information regarding the nature of this interaction. The recent crystal structure of GPIIb α C-terminus and repeat 17 of FLN-A provides important insights into the CFTR/filamin interaction which will use to design other CFTR mutants incapable of interacting with filamins (Nakamura et al., 2005b). FLN repeats are composed of an Ig-like β -sandwich fold. In the crystal structure, residues 562-572 of the

Figure 4.15. The filamin binding motif of CFTR is similar to other filamin binding proteins

Primary sequence alignment of the filamin binding motif of CFTR, GPIb α , and the β 7-integrin. Identical amino acids are shown in black while conserved amino acids are shown in gray. The arrow indicates the experimentally determined β -sheet structure for GPIb α which is predicted for CFTR based on multiple secondary structure predictions (analyzed using PELE at <http://workbench.sdsc.edu>).

CFTR		A	S	V	S	K	L	F	S	W	T	R	P	I	22	
GPIbα	9	L	P	T	F	R	S	S	L	E	L	W	V	R	P	574
β7Integrin	560	N	P	L	Y	K	S	A	I	T	T	I	N	P	R	789
	775															



GPIb α COOH-terminus adopt a β -sheet conformation when inserted between beta sheets C and D of FLN-A repeat 17. This interaction relies most heavily on a stretch of hydrophobic residues in GPIb α which make contacts with the filamin repeat. A bioinformatics analysis of the CFTR NH₂-terminus reveals that residues 11-21 are predicted to form a β -sheet. Furthermore, there are several residues conserved between CFTR and GPIb α which are critical for the GPIb α /FLN-A interaction (Figure 4.15). These include several large hydrophobic residues, as well as, a proline which terminates the β -sheet. Similarly, the filamin binding domain of the β 2-integrin C-terminus also shares this β -sheet conformation and spacing of hydrophobic residues. Amino acid differences in the filamin binding domains of CFTR, GPIb α , and β 7-integrins, likely account for their preferences for different filamin repeats. However, the overall similarities suggest a common mode of binding. We predict that mutating the other residues in this putative β -sheet in the CFTR N-terminus, such as L15, F16, F17, S18, and W19 may also disrupt the CFTR/filamin interaction. Tryptophan 19 is also highly conserved in the CFTR and NH₂-terminus and the mutation to cysteine (W19C) is associated with human disease. We find that the W19C is a folding mutant which does not exit the ER. However, in peptide binding experiments W19C interacts with filamin (Figure 4.1 and data not shown), although it is not clear whether the binding affinity is altered. In future studies, we will take advantage of this structural information to design additional CFTR mutations to further characterize the interaction with filamins.

We are excited to continue our studies of the CFTR-filamin interaction in epithelial cells as our lab has had a long-standing interest in understanding how CFTR and other ion channels are regulated by cytoskeletal attachments and the compartmentalization with regulatory factors. Work from our lab and others have shown that PDZ proteins, like

filamins, serve as scaffolds to organize membrane proteins into regulatory complexes with signaling molecules and the cytoskeleton. The association of CFTR with plasma membrane PDZ proteins may serve multiple functions in polarized cells; these functions likely differ based on the cell type and the specific constellation of PDZ proteins and their binding partners that are expressed. From studies examining CFTR proteins lacking the final three amino acids (CFTR/ Δ TRL), investigators have suggested that the association with PDZ proteins modulates CFTR surface expression (Cheng et al., 2002; Moyer et al., 1999; Moyer et al., 2000) and diffusional mobility (Haggie et al., 2004), links CFTR to upstream regulators and downstream effectors (Naren et al., 2003; Puchelle et al., 2002), potentiates channel function (Raghuram et al., 2003; Raghuram et al., 2001; Wang et al., 2000) and plays a role in CFTR-mediated regulation of other cellular processes (Raghuram et al., 2003; Raghuram et al., 2001; Wang et al., 2000). In addition, PDZ proteins accumulated on intracellular compartments, including the Golgi-associated proteins CAL (Cheng et al., 2002) and the endosomal associated SNX27 (unpublished data), may modulate various aspects of CFTR protein trafficking.

Initial studies in epithelial cells suggested that PDZ proteins play a role in the apical polarization of CFTR. In MDCK cells, Moyer et al. found that GFP-CFTR preferentially accumulates on the apical plasma membrane, while GFP-CFTR/ Δ TRL is unpolarized (Moyer et al., 1999). These data led Moyer et al. to conclude that the CFTR PDZ binding motif is an apical targeting signal. Furthermore, it was suggested that CFTR is initially delivered to both the apical and basolateral membranes and that the apical accumulation results from both increased apical delivery of CFTR from the endosomes and the interaction with actin-associated apical PDZ proteins (Moyer et al., 1999). However, three other groups have since

presented contradictory evidence which report that the PDZ binding motif is not required for the apical localization of CFTR. In studies by Benharouga et al., Ostedgaard et al., and Milewski et al., the apical polarity of CFTR proteins with an extracellular HA-tag or untagged CFTR was maintained despite C-terminal truncations which delete the PDZ binding motif (Benharouga et al., 2003; Milewski et al., 2001; Ostedgaard et al., 2003). Our data suggesting that CFTR is linked to the actin cytoskeleton via both the N- and C-terminus may help resolve this controversy. In preliminary experiments, we find that GFP-CFTR does not efficiently interact with FLN-A, providing a potential explanation why GFP-CFTR/ Δ TRL is equally distributed on the apical and basolateral membranes (Figure 4.16). Thus, we predict that the apical expression of CFTR requires an interaction with either filamins or PDZ proteins.

If filamins do indeed play a role in the polarized expression of CFTR, they are likely to do so by regulating trafficking events and the stability of CFTR at the plasma membrane. Moyer et al. suggest that efficient recycling at the apical, but not basolateral membranes, significantly contributes to the apical accumulation of CFTR (Moyer et al., 2000). In unpolarized cells, we find that internalized S13F CFTR accumulates in lysosomes 3-times faster than wild-type CFTR (8 hours versus 24 hours). These data are consistent with a role for filamins in the endocytic trafficking and recycling of CFTR. In addition, we find that filamins regulate the surface expression of CFTR. The plasma membrane pool of S13F CFTR is significantly less than that of wild-type CFTR. Moreover, the surface expression of wild-type CFTR is likewise reduced in M2 cells which lack FLN-A or in the presence of peptides which compete for filamin binding. Taken together, our data suggest that filamins, like PDZ proteins, may regulate processes important for the retention of CFTR at the apical

membrane including efficient endocytic recycling and cell surface stability. In future experiments we will test the requirement of filamins and PDZ proteins for the apical localization of CFTR. We will express CFTR mutants which cannot bind to filamins (S13F CFTR), PDZ proteins (Δ TRL CFTR), or both filamins and PDZ proteins (S13F/ Δ TRL CFTR). We predict that disrupting the interaction between CFTR and filamins or PDZ proteins will not alter the apical distribution of CFTR, while the S13F/ Δ TRL CFTR double mutant will be unpolarized (Figure 4.16).

In addition to a potential role in the apical retention of CFTR, filamins may also participate in the regulation of CFTR activity. For example, filamins may indirectly regulate CFTR activity by competing with other N-terminal interactions involved in channel gating. It is not clear how filamin binding to the CFTR affects the interaction with syntaxins or the intra-molecular interaction between the CFTR N-terminus and R-domain. However, competing with either interaction would be predicted to alter channel gating. Therefore, we will monitor CFTR channel activity in patch clamp experiments in which we compare the gating of wild-type and S13F CFTR expressed in epithelial cells.

Filamins may also regulate the CFTR activity by clustering the channel in the plasma membrane. By indirect immunofluorescence, Kir 2.1 (Sampson et al., 2003) and HCN1 (Gravante et al., 2004; Li et al., 2000) were observed to be clustered into “hotspots” at the cell surface in FLN-A expressing A7 cells but not FLN-A null M2 cells. Furthermore, mutations which disrupt FLN-A binding in the D2-dopamine receptor impair dopamine-mediated inhibition of adenylyl cyclase, which may result from decreased receptor clustering (Li et al., 2000). Numerous studies have addressed whether CFTR functions as a monomer

Figure 4.16. An N-terminal GFP fusion blocks filamin binding to CFTR.

Untagged or GFP-tagged CFTR was immunoprecipitated from HEK293 cells. Bound proteins were separated by SDS-PAGE and analyzed by western blot using FLN-A antisera. Although equal amounts of WT and GFP-tagged CFTR were immunoprecipitated (Top left panel), the addition of the GFP tag to CFTR clearly decreases the ability of filamin to associate with the channel (Top right panel).

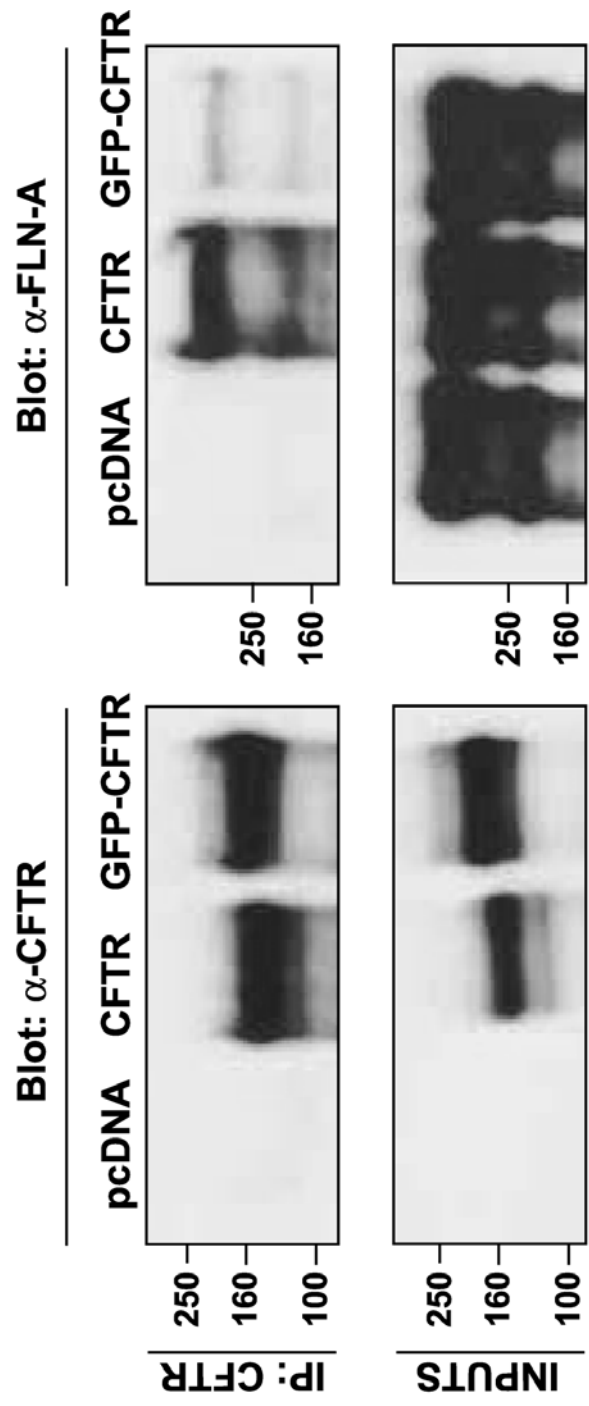
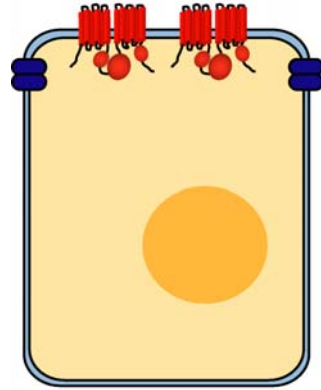


Figure 4.17. A model of CFTR polarized trafficking

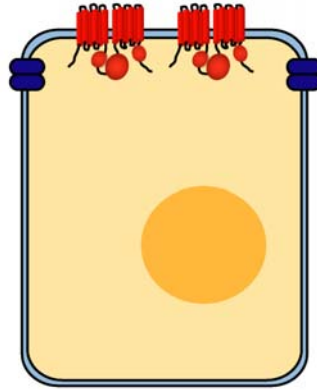
The interaction with PDZ proteins at the CFTR C-terminus has been predicted to regulate the apical sorting of the channel in studies where CFTR is N-terminally tagged with GFP. However, this model is controversial as studies using untagged proteins find that CFTR does not require the PDZ binding motif for its apical polarity. We hypothesize that the N-terminal interaction with filamin may reconcile this controversy. We propose that filamins and PDZ proteins may coordinately regulate the apical sorting of CFTR. Based on previous studies, we predict that mutations that disrupt filamin binding (S13F) or PDZ binding (Δ TRL) will not individually alter the apical polarity of CFTR. However, the double mutant (S13F/ Δ TRL) will be unpolarized.

Wild-type
CFTR



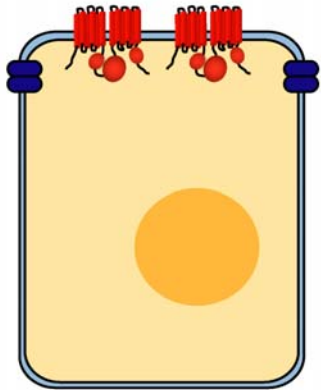
Apical

S13F
CFTR



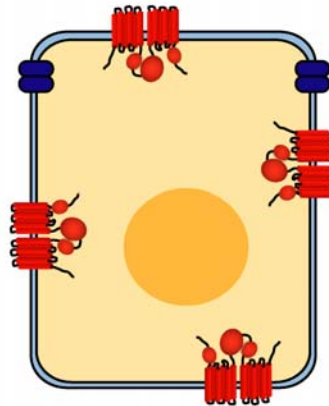
Apical

Δ TRL
CFTR



Apical

S13F/ Δ TRL
CFTR



Unpolarized

or dimer *in vivo*, however, this remains a controversial topic. Studies using electrophysiological, biochemical, and biophysical approaches have provided evidence for CFTR dimerization *in vivo*. In patch-clamp experiments, the presence of multiple CFTR proteins in a single patch potentiates CFTR chloride channel activity (Fischer and Machen, 1994; Pasyk and Foskett, 1995). Furthermore, this cooperative gating has been observed to result in the simultaneous opening of CFTR channel pairs. Ramjeesingh et al. combined the use of cell-impermeable cross-linkers with SDS-PAGE electrophoresis and size-exclusion chromatography to show that a pool of CFTR purified from intestinal epithelial cells migrates with a molecular weight consistent with a CFTR dimer (Ramjeesingh et al., 2003). Furthermore, (Eskandari et al., 1998) et al. examined CFTR proteins expressed in the plasma membrane of *Xenopus* oocytes by freeze fracture electron microscopy . In these studies, the diameter of CFTR was consistent for a transmembrane protein with 24 transmembrane domains, suggesting that CFTR, which has 12 transmembrane domains, forms dimers *in vivo*. The PDZ protein NHERF-3 (PDZK1 or Cap70) has been proposed to facilitate dimerization by simultaneously binding to two CFTR proteins using PDZ domains 3 and 4 (Wang et al., 2000). While exogenous NHERF-3 can potentiate CFTR channel activity, it is not clear whether it can mediate dimerization between two full length CFTR proteins. Filamins are also excellent candidates to regulate CFTR dimerization. Filamins are known to function as dimers *in vivo*, and as such, may facilitate CFTR dimerization by compartmentalizing two channels in close proximity (Weihsing, 1988). In addition to organizing oligomeric CFTR complexes, filamins may also compartmentalize CFTR with upstream activators and downstream effectors. In summary, filamins may also play a previously unrecognized role in regulating CFTR channel activity which may include directly regulating channel gating,

competing for an interaction with another regulatory factor, or compartmentalizing signaling molecules with CFTR. In the following chapter we explore these topics in more depth and present preliminary data which suggest that filamins are required for efficient receptor-mediated activation of CFTR.

Chapter 5: Future directions

This dissertation describes the development of methods used to characterize protein-protein interactions by mass spectrometry. Using these methods we identified two novel CFTR binding proteins, the B'ε subunit of the serine/threonine phosphatase PP2A and the cytoskeletal protein filamin. While many regulatory factors have been identified for CFTR, it is not clear which ones are relevant in epithelial tissues or how they are organized. Our work demonstrates that PP2A is a relevant CFTR phosphatase in airway and intestinal epithelia. Furthermore, we provide a mechanism by which PP2A, via the B'ε regulatory subunit is compartmentalized with CFTR. Our studies on filamins suggest that these proteins anchor CFTR to the cytoskeleton and regulate the plasma membrane stability and endocytic trafficking of CFTR. In the following discussion, we explore the implications of these interactions in terms of cell signaling specificity and channel regulation.

CFTR channel gating reflects a balance between the activities of endogenous kinases and phosphatases. The phosphorylation of the CFTR R-domain on multiple serine residues increases channel gating. While PKA is the dominant activator of CFTR (Chang et al., 1993; Cheng et al., 1991; Seibert et al., 1995), it is clear that multiple kinases including PKC (Berger et al., 1993; Chappe et al., 2003; Fischer et al., 1998; Fouassier et al., 2005; Liedtke et al., 2002) and cGMP-dependent kinase II (Berger et al., 1993) stimulates channel activity; while the AMP-dependent kinase has been shown to negatively regulate CFTR (Hallows et al., 2000). Still less is known regarding the dephosphorylation of CFTR in epithelia. Multiple phosphatases including PP2A, PP2B, and PP2C dephosphorylate and functionally

regulate CFTR (Fischer et al., 1998; Hwang et al., 1993; Luo et al., 1998; Reddy and Quinton, 1996; Travis et al., 1997).

In the future it will be important to understand how individual phosphorylation sites on CFTR are regulated by specific kinases and phosphatases and how these sites affect channel activity. The recent development of several phospho-specific CFTR antibodies by the laboratory of Jack Riordan provides important reagents for studying the functional significance of individual phosphorylation sites on CFTR. These antibodies recognize epitopes in the CFTR R-domain which are lost when serine residues in the epitope are phosphorylated. In preliminary experiments, the dephosphorylation of maximally activated CFTR was monitored in BHK cells. The dephosphorylation of serine residues 737 and 813 is blocked by the presence of PP2A inhibitors; however, the kinetics of dephosphorylation of other serine residues are not affected. Interestingly, CFTR proteins lacking the last 40 amino acids (CFTR Δ 40), which includes the PP2A binding site we identified, is dephosphorylated similarly to CFTR in the presence of PP2A inhibitors. That is, serine residues 737 and 813 are not dephosphorylated on the CFTR Δ 40 protein (Tamas Hedges, personal communication). In addition to supporting our observation that PP2A is associated with the CFTR C-terminus, these data suggest that specific phosphatases will mediate the dephosphorylation of specific residues on CFTR.

In addition to regulating channel function, phosphorylation/dephosphorylation may regulate other aspects of CFTR biology. Studies with other transmembrane proteins demonstrate that phosphorylation can regulate protein-protein interactions, trafficking events, and target proteins for degradation. For example, the IL-6 receptor subunit, gp130, is degraded in response to phosphorylation at serine 782 (Mitsuhashi et al., 2005). PP2A

antagonizes the phosphorylation dependent degradation, as okadaic acid, a PP2A inhibitor accelerates the turnover of gp130. Additionally, a number of transmembrane proteins including TRPP2 (Kottgen et al., 2005), human cytomegalovirus glycoprotein B (Crump et al., 2003), and PC6B (Xiang et al., 2000) bind to the adaptor proteins PACS-1 or 2 which regulate protein trafficking in the TGN and endosomal compartments. The binding of PACS proteins to transmembrane cargo is regulated by casein kinase II phosphorylation of both PACS and the cargo molecule (Molloy et al., 1998; Scott et al., 2003). Furthermore, PP2A can disrupt the PACS/cargo interaction via dephosphorylation to regulate these trafficking events. Although not previously demonstrated for CFTR, the interaction between CFTR and PP2A may be important for regulating a previously unrecognized aspect of CFTR biology.

The phosphorylation of CFTR may also regulate its interaction with filamins. We find that the substitution of serine 13 to acidic residues, S13D and S13E, commonly used to mimic serine phosphorylation, showed reduced binding to FLN-A by immunoprecipitation (Figure 4.8A). It is intriguing to speculate that this interaction is regulated by phosphorylation, although serine 13 is not contained within an obvious phosphorylation motif. For other filamin binding proteins including GPIIb α (Meyer et al., 1997), β -integrins (Sharma et al., 1995), and the high affinity immunoglobulin receptor (Ohta et al., 1991), ligand engagement results in the disassociation of filamin binding. One possibility is that, at the plasma membrane, the activation state of CFTR may regulate filamin binding. In future experiments, we will examine filamin binding in the presence of factors that stimulate CFTR activity, such as adenosine and forskolin. Alternatively, the CFTR-filamin interaction may be regulated by calcium signaling. To date, calcium is the best characterized regulator of filamins. Increases in intracellular calcium cause the disassociation of filamin from actin in a

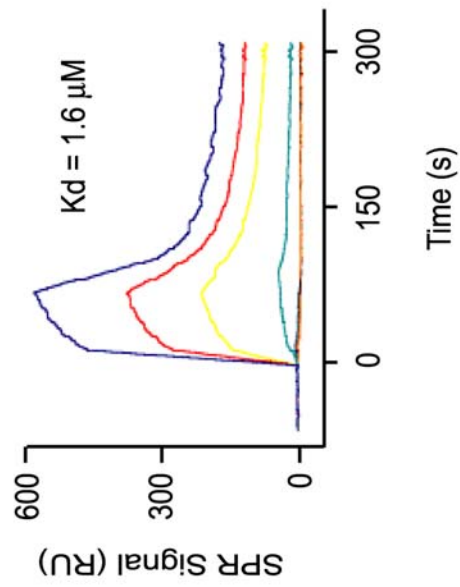
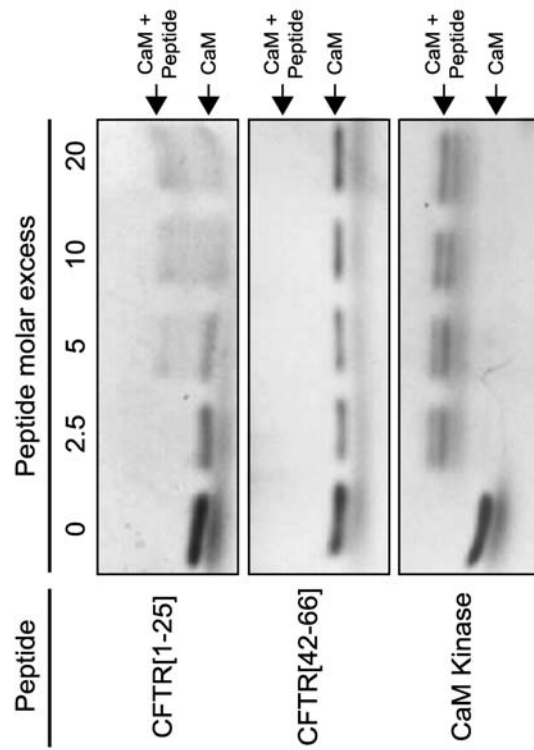
calmodulin dependent manner (Nakamura et al., 2005a). Furthermore, the hinge regions of filamin are sensitive to cleavage by the calcium dependent protease, calpain (Azam et al., 2001; Kwak et al., 1993; Onji et al., 1987). In preliminary studies, we find that calmodulin can directly associate with the CFTR[1-25] peptide which also binds filamins. Calmodulin (1 μ g) was incubated with the indicated peptides for 30 minutes at 37°C. Calmodulin was then resolved using native acrylamide gel electrophoresis and visualized using coomassie blue. Peptide binding is observed as an increase in the apparent molecular weight of calmodulin. We observe an increase in the apparent molecular weight of calmodulin at all concentrations tested for a peptide corresponding to the calmodulin binding domain of the calmodulin-dependent protein kinase (CaM kinase), which is known to interact with calmodulin with a nanomolar affinity (Figure 5.1A). Furthermore, we observe concentration dependent binding of the CFTR[1-25] peptide, but not another N-terminal peptide, CFTR[42-66]. Calmodulin interacts with CFTR in a calcium-dependent manner with a micromolar affinity ($K_d = 1.6 \mu\text{M}$) (Figure 5.1B). The CFTR-calmodulin interaction has not been validated *in vivo*, but offers an interesting mechanism by which the CFTR-filamin interaction may be regulated.

While we have not yet explored a scaffolding role for filamins, it is likely that filamins also recruit additional regulatory factors into a complex with CFTR. This is a reasonable hypothesis because filamins are known to compartmentalize receptors and ion channels with signal transduction elements. For example, the interaction between FLN-A and β -integrins is critical for the formation of focal adhesions following extracellular matrix engagement. FLN-A provides integrins with a direct link to the actin and recruits cytoskeletal regulatory proteins such as Rho, Rac, CDC42, RalA, Trio, and PAK to the

Figure 5.1. Calmodulin can also interact with the CFTR N-terminus

A. Calmodulin (1 μg) was incubated with CFTR[1-25], CFTR[42-66] calmodulin binding domain peptides at the indicated molar excess. Calmodulin was separated on a non-denaturing acrylamide gels and visualized by coomassie blue staining. The CFTR[1-25] peptide clearly interacts with calmodulin as, at increasing concentrations of peptide, calmodulin is observed to increase in molecular weight. Calmodulin does not bind to CFTR[42-66] at any concentration tested. Calmodulin is completely bound to the CaM kinase peptide at all concentrations. This is not surprising given the high affinity for this interaction (K_d ranges from 2 and 0.02 nM). B. The interaction between CFTR[1-25] and calmodulin was analyzed by SPR. Calmodulin interacts with CFTR in a concentration dependent manner with an a $K_d = 1.6 \mu\text{M}$. No binding was observed for the CFTR[42-66] peptide. Furthermore, no binding was observed in the absence of calcium. As such, all assays were done in the presence of 500 nM calcium.

A



B

complex (Stossel et al., 2001). Recently, Tu et al. identified additional components of the FLN-A and β -integrin complex (Tu et al., 2003). The mitogen-inducible gene-2 (Mig-2) recruits the LIM domain-containing protein migfilin to FLN-A which is required for ECM-dependent cell spreading. Thus, filamins function as scaffolding proteins to link extracellular and intracellular signaling events by coupling receptor activation to cytosolic signal transduction and cytoskeletal rearrangements.

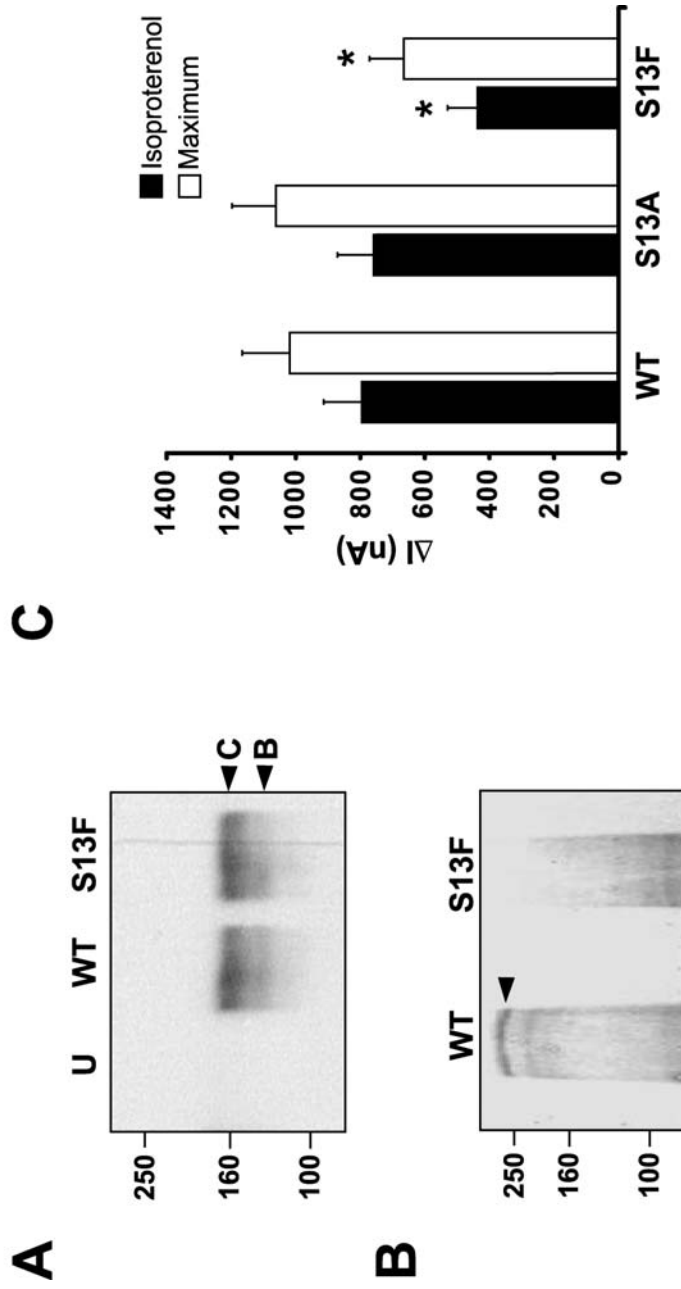
In future studies we will explore the role of filamins in the regulation of CFTR channel activity in polarized epithelial cells. In preliminary experiments, we studied CFTR channel activity in *Xenopus* oocytes, a heterologous system where S13F CFTR is efficiently processed (Figure 5.2A). We first confirmed that filamins are expressed in oocytes and were able to bind human CFTR. Using the CFTR[1-25] and CFTR[1-25/S13F] peptides in pull-down assays from oocyte cell extracts, we find a high molecular weight band, consistent with the size of human FLNs, purified with CFTR[1-25] but not CFTR[1-25/S13F] (Figure 5.2B). This band was excised, digested with trypsin, and analyzed by nano-electrospray MS/MS. Using the tandem MS spectra, we derived the amino acid sequences from five of the peptides analyzed. When searched against human protein databases, the *Xenopus* peptide sequences matched hFLN-A (~80% identical). Therefore, we proceeded with functional studies to compare basal and stimulated CFTR currents in injected oocytes. Interestingly, S13F CFTR functions differed from WT CFTR in two ways. First, S13F CFTR maximal currents stimulated by forskolin/IBMX were decreased by ~45% relative to wild-type CFTR (Figure 5.2C). This is consistent with our data presented in chapter 4 which demonstrate that filamins regulate the surface expression of CFTR. We also examined CFTR channel activity stimulated by a relevant receptor signaling pathway by co-injecting the β_2 -adrenergic

receptor in oocytes. The β_2 -adrenergic receptor potently stimulates CFTR via adenylyl cyclase activation which increases cAMP and results in PKA phosphorylation of CFTR (Uezono et al., 1993). We find that the fractional stimulation of S13F CFTR through activation of co-injected β_2 -adrenergic receptor was 40% compared to 78% for wild-type and S13A CFTR (Figure 1C). Despite potential difference between the number of wild-type and S13F CFTR channels on the cell surface, these data indicate that S13F CFTR is less responsive to activation via a receptor mediated pathway.

It is intriguing to speculate that filamins may compartmentalize CFTR with upstream activators. Our lab has previously shown that the cellular machinery capable of generating cAMP, including the adenosine receptor and membrane bound adenylyl cyclases, are compartmentalized with CFTR in the apical membrane of airway epithelia. Given that filamins directly associate with numerous G-protein coupled receptors including the D2-dopamine receptor (Li et al., 2000), calcitonin receptor (Seck et al., 2003), calcium-sensing receptor (Pi et al., 2002), and mu opioid receptor (Onoprishvili et al., 2003), it will be important to ask whether receptors relevant to CFTR, such as β_2 -adrenergic or adenosine receptors also bind to filamins.

Filamins may also regulate CFTR by spatially confining the channel in the plasma membrane. By SPT, we observe that CFTR is mobile on the cell surface, but undergoes periods of transient confinement. The importance of these transient confinements is not well understood. An emerging hypothesis is that receptors and signaling molecules are compartmentalized in membrane micro-domains, which are regulated by the lipid composition of the plasma membrane and the underlying cytoskeleton (Kusumi and Sako, 1996; Sheets et al., 1997). Perhaps the most well characterized membrane micro-domains

Figure 5.2 . CFTR requires filamin binding for efficient receptor mediated activation
(A) Representative oocytes were lysed and analyzed for CFTR expression by western blot. Both WT and S13F CFTR are expressed at similar levels and there is no apparent difference in the ratio of maturely versus immaturely glycosylated forms. (B) The CFTR[1-25] peptide, but not the S13F mutant, interacts with a *Xenopus* protein identified by mass spectrometry to be >80% homologous to human filamin (C) CFTR chloride conductance was measured by two-electrode voltage clamp in *Xenopus* oocytes expressing either wild-type or S13F CFTR and the β 2AR. CFTR activation was measured following β 2AR stimulation (Isoproterenol, open bars) followed by treatment with IBMX/forskolin. White bars represent maximal CFTR activity (Isoproterenol response + IBMX/forskolin).



are lipid rafts. However, the exact nature of this compartment remains elusive, suggesting that rafts are highly dynamic. Current hypotheses suggest that lipid rafts are compartments enriched in cholesterol, GPI-anchored proteins, signaling molecules such as Src kinases, and caveolin (Anderson and Jacobson, 2002). Like other GPI-anchored proteins, we find that CFTR is compartmentalized into TCZ. Although this is some evidence for the partitioning of CFTR into lipid rafts, we find that cholesterol depletion or Src kinase inhibition, maneuvers that inhibit the transient confinement of raft proteins, have no effect on CFTR confinement (data not shown). Furthermore, we do find that disrupting filamin binding to CFTR, using the S13F mutant, significantly disrupts the transient confinement of CFTR. Thus, we hypothesize that the confinement of CFTR is coordinated by interactions with the cytoskeleton. An interesting hypothesis is that the transient confinement of CFTR represents the compartmentalization of the channel with regulatory proteins. It will be interesting to examine whether the confinement of CFTR in TCZs correlates with changes in channel activity.

In addition to the studying how filamins individually regulate CFTR, we are also interested in how they cooperate with other CFTR-interacting proteins. In chapter 4, we proposed a model for the apical polarity of CFTR which depends upon a dual N- and C-terminal protein interaction. We also predict that filamins and PDZ proteins may coordinately regulate the membrane mobility of CFTR and its organization with signaling molecules. We are continuing to study CFTR by SPT and are examining how the CFTR confinement dually regulated by N- and C-terminal cytoskeletal attachments. Interestingly, more than half of the ion channels and receptors identified as filamin binding proteins also

Table 3. Filamins and PDZ proteins may coordinately regulate their binding partners

A table of all known filamin binding proteins which additionally interact, or are predicted to interact, with PDZ proteins. References: (Bomberger et al., 2005; Enz, 2002; Enz and Croci, 2003; Gupta et al., 2004; Jeanneteau et al., 2004; Leonoudakis et al., 2004; Leonoudakis et al., 2001; Li et al., 2000; Liu et al., 1997; Nehring et al., 2000; Sampson et al., 2003; Seck et al., 2003; Short et al., 1998; Sun et al., 2000b; Wang et al., 2000; Xu et al., 1999; Zhang et al., 1998).

FLN Interactors	COOH	PDZ Binding Proteins
CFTR	DTRL	NHERFS-1, -2, -3, CAL, SNX-27
Kir2.1	ESEI	PSD-95, SAP-97, CASK, Veli, Mint-1
Kv2.4	VSAL	Not Determined
Presenilin I	QLYI	Omni Protease, PSAP
Furin	QSAL	Not Determined
Metabotropic Glutamate Receptor 7	NLVI	PICK-1
Calcitonin Receptor	ESSA	NHERF-1
Dopamine (D2) Receptor	ILHC	GIPC, Shank

contain PDZ binding motifs (Table 3). Thus, filamins and PDZ proteins may coordinately regulate the activities and membrane trafficking of their binding partners.

In summary, we have presented evidence that CFTR is regulated by direct interactions with PP2A and filamins. In addition to furthering our understanding of normal CFTR physiology, the PP2A and filamin interactions may provide useful insights for the design of future therapeutic approaches aimed at treating CF. For example, there is a growing interest in developing pharmacological treatments, such as chemical chaperones, which stabilize folding mutants like $\Delta F508$ CFTR, allowing them to exit the ER. In cell culture models, rescued $\Delta F508$ CFTR reaches the cell surface and has chloride channel activity (Denning et al., 1992). However, several studies have demonstrated that rescued $\Delta F508$ CFTR does not function to the capacity of the wild-type channel due to altered gating and decreased plasma membrane stability (Gentzsch et al., 2004; Swiatecka-Urban et al., 2005a; Swiatecka-Urban et al., 2005b). These defects highlight our need to understand the processes which regulate CFTR activity, trafficking, and surface expression. Given that PP2A inhibition increases CFTR channel activity and ASL height in primary airway epithelia, this enzyme may provide a good drug target to augment the activity of mutant channels (Thelin et al., 2005). Furthermore, targeting signaling pathways that favor the CFTR-filamin interaction may promote retention of the channel at the cell surface. Thus, in future experiments it will be important to study these interactions in the context of mutant CFTR proteins in polarized epithelia.

References

- Alaiya, A.A., M. Oppermann, J. Langridge, U. Roblick, L. Egevad, S. Brindstedt, M. Hellstrom, S. Linder, T. Bergman, H. Jornvall, and G. Auer. 2001. Identification of proteins in human prostate tumor material by two-dimensional gel electrophoresis and mass spectrometry. *Cell Mol Life Sci.* 58:307-11.
- Ameen, N.A., C. Marino, and P.J. Salas. 2003. cAMP-dependent exocytosis and vesicle traffic regulate CFTR and fluid transport in rat jejunum in vivo. *Am J Physiol Cell Physiol.* 284:C429-38.
- Anderson, M.P., H.A. Berger, D.P. Rich, R.J. Gregory, A.E. Smith, and M.J. Welsh. 1991a. Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell.* 67:775-84.
- Anderson, M.P., D.P. Rich, R.J. Gregory, A.E. Smith, and M.J. Welsh. 1991b. Generation of cAMP-activated chloride currents by expression of CFTR. *Science.* 251:679-82.
- Anderson, R.G., and K. Jacobson. 2002. A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science.* 296:1821-5.
- Anilkumar, G., S.A. Rajasekaran, S. Wang, O. Hankinson, N.H. Bander, and A.K. Rajasekaran. 2003. Prostate-specific membrane antigen association with filamin A modulates its internalization and NAALADase activity. *Cancer Res.* 63:2645-8.
- Annesley, T.M. 2003. Ion suppression in mass spectrometry. *Clin Chem.* 49:1041-4.
- Arthur, J.F., E.E. Gardiner, M. Matzaris, S.G. Taylor, L. Wijeyewickrema, Y. Ozaki, M.L. Kahn, R.K. Andrews, and M.C. Berndt. 2005. Glycoprotein VI is associated with GPIb-IX-V on the membrane of resting and activated platelets. *Thromb Haemost.* 93:716-23.
- Azam, M., S.S. Andrabi, K.E. Sahr, L. Kamath, A. Kuliopulos, and A.H. Chishti. 2001. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol.* 21:2213-20.
- Baldursson, O., L.S. Ostedgaard, T. Rokhlina, J.F. Cotten, and M.J. Welsh. 2001. Cystic fibrosis transmembrane conductance regulator Cl⁻ channels with R domain deletions and translocations show phosphorylation-dependent and -independent activity. *J Biol Chem.* 276:1904-10.
- Bannykh, S.I., G.I. Bannykh, K.N. Fish, B.D. Moyer, J.R. Riordan, and W.E. Balch. 2000. Traffic pattern of cystic fibrosis transmembrane regulator through the early exocytic pathway. *Traffic.* 1:852-70.

- Barnes, A.P., G. Livera, P. Huang, C. Sun, W.K. O'Neal, M. Conti, M.J. Stutts, and S.L. Milgram. 2005. Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium. *J Biol Chem.* 280:7997-8003.
- Baukrowitz, T., T.C. Hwang, A.C. Nairn, and D.C. Gadsby. 1994. Coupling of CFTR Cl⁻ channel gating to an ATP hydrolysis cycle. *Neuron.* 12:473-82.
- Bear, C.E., F. Duguay, A.L. Naismith, N. Kartner, J.W. Hanrahan, and J.R. Riordan. 1991. Cl⁻ channel activity in *Xenopus* oocytes expressing the cystic fibrosis gene. *J Biol Chem.* 266:19142-5.
- Becker, W., H. Kentrup, S. Klumpp, J.E. Schultz, and H.G. Joost. 1994. Molecular cloning of a protein serine/threonine phosphatase containing a putative regulatory tetratricopeptide repeat domain. *J Biol Chem.* 269:22586-92.
- Becq, F., T.J. Jensen, X.B. Chang, A. Savoia, J.M. Rommens, L.C. Tsui, M. Buchwald, J.R. Riordan, and J.W. Hanrahan. 1994. Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc Natl Acad Sci U S A.* 91:9160-4.
- Benharouga, M., M. Haardt, N. Kartner, and G.L. Lukacs. 2001. COOH-terminal truncations promote proteasome-dependent degradation of mature cystic fibrosis transmembrane conductance regulator from post-Golgi compartments. *J Cell Biol.* 153:957-70.
- Benharouga, M., M. Sharma, J. So, M. Haardt, L. Drzymala, M. Popov, B. Schwapach, S. Grinstein, K. Du, and G.L. Lukacs. 2003. The role of the C terminus and Na⁺/H⁺ exchanger regulatory factor in the functional expression of cystic fibrosis transmembrane conductance regulator in nonpolarized cells and epithelia. *J Biol Chem.* 278:22079-89.
- Berger, H.A., M.P. Anderson, R.J. Gregory, S. Thompson, P.W. Howard, R.A. Maurer, R. Mulligan, A.E. Smith, and M.J. Welsh. 1991. Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J Clin Invest.* 88:1422-31.
- Berger, H.A., S.M. Travis, and M.J. Welsh. 1993. Regulation of the cystic fibrosis transmembrane conductance regulator Cl⁻ channel by specific protein kinases and protein phosphatases. *J Biol Chem.* 268:2037-47.
- Bilan, F., V. Thoreau, M. Nacfer, R. Derand, C. Norez, A. Cantereau, M. Garcia, F. Becq, and A. Kitzis. 2004. Syntaxin 8 impairs trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibits its channel activity. *J Cell Sci.* 117:1923-35.
- Bomberger, J.M., W.S. Spielman, C.S. Hall, E.J. Weinman, and N. Parameswaran. 2005. Receptor activity-modifying protein (RAMP) isoform-specific regulation of adrenomedullin receptor trafficking by NHERF-1. *J Biol Chem.* 280:23926-35.

- Borchers, C., C.E. Parker, L.J. Deterding, and K.B. Tomer. 1999. Preliminary comparison of precursor scans and liquid chromatography-tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer. *J Chromatogr A*. 854:119-30.
- Bradbury, N.A., J.A. Clark, S.C. Watkins, C.C. Widnell, H.S.t. Smith, and R.J. Bridges. 1999. Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. *Am J Physiol*. 276:L659-68.
- Bradbury, N.A., J.A. Cohn, C.J. Venglarik, and R.J. Bridges. 1994. Biochemical and biophysical identification of cystic fibrosis transmembrane conductance regulator chloride channels as components of endocytic clathrin-coated vesicles. *J Biol Chem*. 269:8296-302.
- Brewis, N.D., A.J. Street, A.R. Prescott, and P.T. Cohen. 1993. PPX, a novel protein serine/threonine phosphatase localized to centrosomes. *Embo J*. 12:987-96.
- Brown, C.R., L.Q. Hong-Brown, J. Biwersi, A.S. Verkman, and W.J. Welch. 1996. Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. *Cell Stress Chaperones*. 1:117-25.
- Chang, X.B., L. Cui, Y.X. Hou, T.J. Jensen, A.A. Aleksandrov, A. Mengos, and J.R. Riordan. 1999. Removal of multiple arginine-framed trafficking signals overcomes misprocessing of delta F508 CFTR present in most patients with cystic fibrosis. *Mol Cell*. 4:137-42.
- Chang, X.B., J.A. Tabcharani, Y.X. Hou, T.J. Jensen, N. Kartner, N. Alon, J.W. Hanrahan, and J.R. Riordan. 1993. Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J Biol Chem*. 268:11304-11.
- Chappe, V., D.A. Hinkson, T. Zhu, X.B. Chang, J.R. Riordan, and J.W. Hanrahan. 2003. Phosphorylation of protein kinase C sites in NBD1 and the R domain control CFTR channel activation by PKA. *J Physiol*. 548:39-52.
- Cheng, J., B.D. Moyer, M. Milewski, J. Loffing, M. Ikeda, J.E. Mickle, G.R. Cutting, M. Li, B.A. Stanton, and W.B. Guggino. 2002. A Golgi-associated PDZ domain protein modulates cystic fibrosis transmembrane regulator plasma membrane expression. *J Biol Chem*. 277:3520-9.
- Cheng, J., H. Wang, and W.B. Guggino. 2005. Regulation of cystic fibrosis transmembrane regulator trafficking and protein expression by a Rho family small GTPase TC10. *J Biol Chem*. 280:3731-9.

- Cheng, S.H., R.J. Gregory, J. Marshall, S. Paul, D.W. Souza, G.A. White, C.R. O'Riordan, and A.E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*. 63:827-34.
- Cheng, S.H., D.P. Rich, J. Marshall, R.J. Gregory, M.J. Welsh, and A.E. Smith. 1991. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*. 66:1027-36.
- Chilvers, M.A., and C. O'Callaghan. 2000. Local mucociliary defence mechanisms. *Paediatr Respir Rev*. 1:27-34.
- Christodoulides, N., S. Feng, J.C. Resendiz, M.C. Berndt, and M.H. Kroll. 2001. Glycoprotein Ib/IX/V binding to the membrane skeleton maintains shear-induced platelet aggregation. *Thromb Res*. 102:133-42.
- Clarke, L.L., L.R. Gawenis, T.C. Hwang, N.M. Walker, D.B. Gruis, and E.M. Price. 2004. A domain mimic increases DeltaF508 CFTR trafficking and restores cAMP-stimulated anion secretion in cystic fibrosis epithelia. *Am J Physiol Cell Physiol*. 287:C192-9.
- Clarke, L.L., B.R. Grubb, S.E. Gabriel, O. Smithies, B.H. Koller, and R.C. Boucher. 1992. Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science*. 257:1125-8.
- Cormet-Boyaka, E., A. Di, S.Y. Chang, A.P. Naren, A. Tousson, D.J. Nelson, and K.L. Kirk. 2002. CFTR chloride channels are regulated by a SNAP-23/syntaxin 1A complex. *Proc Natl Acad Sci U S A*. 99:12477-82.
- Cormet-Boyaka, E., M. Jablonsky, A.P. Naren, P.L. Jackson, D.D. Muccio, and K.L. Kirk. 2004. Rescuing cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by transcomplementation. *Proc Natl Acad Sci U S A*. 101:8221-6.
- Cranmer, S.L., I. Pikovski, P. Mangin, P.E. Thompson, T. Domagala, M. Frazzetto, H.H. Salem, and S.P. Jackson. 2005. Identification of a unique filamin A binding region within the cytoplasmic domain of glycoprotein Ibalph. *Biochem J*. 387:849-58.
- Crump, C.M., C.H. Hung, L. Thomas, L. Wan, and G. Thomas. 2003. Role of PACS-1 in trafficking of human cytomegalovirus glycoprotein B and virus production. *J Virol*. 77:11105-13.
- Dahan, D., A. Evagelidis, J.W. Hanrahan, D.A. Hinkson, Y. Jia, J. Luo, and T. Zhu. 2001. Regulation of the CFTR channel by phosphorylation. *Pflugers Arch*. 443 Suppl 1:S92-6.
- Delaney, S.J., E.W. Alton, S.N. Smith, D.P. Lunn, R. Farley, P.K. Lovelock, S.A. Thomson, D.A. Hume, D. Lamb, D.J. Porteous, J.R. Dorin, and B.J. Wainwright. 1996. Cystic

- fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *Embo J.* 15:955-63.
- Denning, G.M., M.P. Anderson, J.F. Amara, J. Marshall, A.E. Smith, and M.J. Welsh. 1992. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature.* 358:761-4.
- Dozier, C., M. Bonyadi, L. Baricault, L. Tonasso, and J.M. Darbon. 2004. Regulation of Chk2 phosphorylation by interaction with protein phosphatase 2A via its B' regulatory subunit. *Biol Cell.* 96:509-17.
- Dubois, C., B. Steiner, N. Kieffer, and S.C. Reigner. 2003. Thrombin binding to GPIIb/IIIa induces platelet aggregation and fibrin clot retraction supported by resting alphaIIb beta3 interaction with polymerized fibrin. *Thromb Haemost.* 89:853-65.
- Dyson, J.M., A.D. Munday, A.M. Kong, R.D. Huysmans, M. Matzaris, M.J. Layton, H.H. Nandurkar, M.C. Berndt, and C.A. Mitchell. 2003. SHIP-2 forms a tetrameric complex with filamin, actin, and GPIb-IX-V: localization of SHIP-2 to the activated platelet actin cytoskeleton. *Blood.* 102:940-8.
- Egan, M.E., J. Glockner-Pagel, C. Ambrose, P.A. Cahill, L. Pappoe, N. Balamuth, E. Cho, S. Canny, C.A. Wagner, J. Geibel, and M.J. Caplan. 2002. Calcium-pump inhibitors induce functional surface expression of Delta F508-CFTR protein in cystic fibrosis epithelial cells. *Nat Med.* 8:485-92.
- Egan, M.E., M. Pearson, S.A. Weiner, V. Rajendran, D. Rubin, J. Glockner-Pagel, S. Canny, K. Du, G.L. Lukacs, and M.J. Caplan. 2004. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science.* 304:600-2.
- Eidelman, O., C. Guay-Broder, P.J. van Galen, K.A. Jacobson, C. Fox, R.J. Turner, Z.I. Cabantchik, and H.B. Pollard. 1992. A1 adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells. *Proc Natl Acad Sci U S A.* 89:5562-6.
- Englund, G.D., R.J. Bodnar, Z. Li, Z.M. Ruggeri, and X. Du. 2001. Regulation of von Willebrand factor binding to the platelet glycoprotein Ib-IX by a membrane skeleton-dependent inside-out signal. *J Biol Chem.* 276:16952-9.
- Enz, R. 2002. The actin-binding protein Filamin-A interacts with the metabotropic glutamate receptor type 7. *FEBS Lett.* 514:184-8.
- Enz, R., and C. Croci. 2003. Different binding motifs in metabotropic glutamate receptor type 7b for filamin A, protein phosphatase 1C, protein interacting with protein kinase C (PICK) 1 and syntenin allow the formation of multimeric protein complexes. *Biochem J.* 372:183-91.

- Eskandari, S., E.M. Wright, M. Kreman, D.M. Starace, and G.A. Zampighi. 1998. Structural analysis of cloned plasma membrane proteins by freeze-fracture electron microscopy. *Proc Natl Acad Sci U S A*. 95:11235-40.
- Farinha, C.M., P. Nogueira, F. Mendes, D. Penque, and M.D. Amaral. 2002. The human DnaJ homologue (Hdj)-1/heat-shock protein (Hsp) 40 co-chaperone is required for the in vivo stabilization of the cystic fibrosis transmembrane conductance regulator by Hsp70. *Biochem J*. 366:797-806.
- Feng, S., X. Lu, and M.H. Kroll. 2005. Filamin A binding stabilizes nascent glycoprotein Ibalpha trafficking and thereby enhances its surface expression. *J Biol Chem*. 280:6709-15.
- Feng, S., J.C. Resendiz, X. Lu, and M.H. Kroll. 2003. Filamin A binding to the cytoplasmic tail of glycoprotein Ibalpha regulates von Willebrand factor-induced platelet activation. *Blood*. 102:2122-9.
- Fischer, H., B. Illek, and T.E. Machen. 1998. Regulation of CFTR by protein phosphatase 2B and protein kinase C. *Pflugers Arch*. 436:175-81.
- Fischer, H., and T.E. Machen. 1994. CFTR displays voltage dependence and two gating modes during stimulation. *J Gen Physiol*. 104:541-66.
- Fischer, H., and T.E. Machen. 1996. The tyrosine kinase p60c-src regulates the fast gate of the cystic fibrosis transmembrane conductance regulator chloride channel. *Biophys J*. 71:3073-82.
- Fouassier, L., M.T. Nichols, E. Gidey, R.R. McWilliams, H. Robin, C. Finnigan, K.E. Howell, C. Housset, and R.B. Doctor. 2005. Protein kinase C regulates the phosphorylation and oligomerization of ERM binding phosphoprotein 50. *Exp Cell Res*. 306:264-73.
- Fu, J., H.L. Ji, A.P. Naren, and K.L. Kirk. 2001. A cluster of negative charges at the amino terminal tail of CFTR regulates ATP-dependent channel gating. *J Physiol*. 536:459-70.
- Gadsby, D.C., and A.C. Nairn. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol Rev*. 79:S77-S107.
- Ganeshan, R., A. Di, D.J. Nelson, M.W. Quick, and K.L. Kirk. 2003. The interaction between syntaxin 1A and cystic fibrosis transmembrane conductance regulator Cl⁻ channels is mechanistically distinct from syntaxin 1A-SNARE interactions. *J Biol Chem*. 278:2876-85.

- Gentzsch, M., X.B. Chang, L. Cui, Y. Wu, V.V. Ozols, A. Choudhury, R.E. Pagano, and J.R. Riordan. 2004. Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol Biol Cell*. 15:2684-96.
- Gimeno-Alcaniz, J.V., and P. Sanz. 2003. Glucose and type 2A protein phosphatase regulate the interaction between catalytic and regulatory subunits of AMP-activated protein kinase. *J Mol Biol*. 333:201-9.
- Glogauer, M., P. Arora, D. Chou, P.A. Janmey, G.P. Downey, and C.A. McCulloch. 1998. The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J Biol Chem*. 273:1689-98.
- Gorlin, J.B., R. Yamin, S. Egan, M. Stewart, T.P. Stossel, D.J. Kwiatkowski, and J.H. Hartwig. 1990. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J Cell Biol*. 111:1089-105.
- Gravante, B., A. Barbuti, R. Milanesi, I. Zappi, C. Viscomi, and D. DiFrancesco. 2004. Interaction of the pacemaker channel HCN1 with filamin A. *J Biol Chem*. 279:43847-53.
- Gregory, R.J., S.H. Cheng, D.P. Rich, J. Marshall, S. Paul, K. Hehir, L. Ostedgaard, K.W. Klinger, M.J. Welsh, and A.E. Smith. 1990. Expression and characterization of the cystic fibrosis transmembrane conductance regulator. *Nature*. 347:382-6.
- Grubb, B.R. 1995. Ion transport across the jejunum in normal and cystic fibrosis mice. *Am J Physiol*. 268:G505-13.
- Grubb, B.R. 2002. Bioelectric measurement of CFTR function in mice. *Methods Mol Med*. 70:525-35.
- Grubb, B.R., S.E. Gabriel, A. Mengos, M. Gentzsch, S.H. Randell, A.M. Van Heeckeren, M.R. Knowles, M.L. Drumm, J.R. Riordan, and R.C. Boucher. 2005. SERCA Pump Inhibitors Do Not Correct Biosynthetic Arrest of Δ F508CFTR in Cystic Fibrosis. *Am J Respir Cell Mol Biol*.
- Gu, M., X. Xi, G.D. Englund, M.C. Berndt, and X. Du. 1999. Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin α (IIb) β (3) using a reconstituted mammalian cell expression model. *J Cell Biol*. 147:1085-96.
- Guggino, W.B. 2001. Cystic fibrosis salt/fluid controversy: in the thick of it. *Nat Med*. 7:888-9.
- Gupta, S., R. Singh, P. Datta, Z. Zhang, C. Orr, Z. Lu, G. Dubois, A.S. Zervos, M.H. Meisler, S.M. Srinivasula, T. Fernandes-Alnemri, and E.S. Alnemri. 2004. The C-terminal tail of presenilin regulates Omi/HtrA2 protease activity. *J Biol Chem*. 279:45844-54.

- Haardt, M., M. Benharouga, D. Lechardeur, N. Kartner, and G.L. Lukacs. 1999. C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. *J Biol Chem.* 274:21873-7.
- Haggie, P.M., B.A. Stanton, and A.S. Verkman. 2002. Diffusional mobility of the cystic fibrosis transmembrane conductance regulator mutant, delta F508-CFTR, in the endoplasmic reticulum measured by photobleaching of GFP-CFTR chimeras. *J Biol Chem.* 277:16419-25.
- Haggie, P.M., B.A. Stanton, and A.S. Verkman. 2004. Increased diffusional mobility of CFTR at the plasma membrane after deletion of its C-terminal PDZ binding motif. *J Biol Chem.* 279:5494-500.
- Hallows, K.R., V. Raghuram, B.E. Kemp, L.A. Witters, and J.K. Foskett. 2000. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest.* 105:1711-21.
- He, H.J., S. Kole, Y.K. Kwon, M.T. Crow, and M. Bernier. 2003. Interaction of filamin A with the insulin receptor alters insulin-dependent activation of the mitogen-activated protein kinase pathway. *J Biol Chem.* 278:27096-104.
- Howell, L.D., R. Borchardt, J. Kole, A.M. Kaz, C. Randak, and J.A. Cohn. 2004. Protein kinase A regulates ATP hydrolysis and dimerization by a CFTR (cystic fibrosis transmembrane conductance regulator) domain. *Biochem J.* 378:151-9.
- Hu, W., M. Howard, and G.L. Lukacs. 2001. Multiple endocytic signals in the C-terminal tail of the cystic fibrosis transmembrane conductance regulator. *Biochem J.* 354:561-72.
- Huang, P., E.R. Lazarowski, R. Tarran, S.L. Milgram, R.C. Boucher, and M.J. Stutts. 2001. Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells. *Proc Natl Acad Sci U S A.* 98:14120-5.
- Huang, P., K. Trotter, R.C. Boucher, S.L. Milgram, and M.J. Stutts. 2000. PKA holoenzyme is functionally coupled to CFTR by AKAPs. *Am J Physiol Cell Physiol.* 278:C417-22.
- Hwang, T.C., M. Horie, and D.C. Gadsby. 1993. Functionally distinct phospho-forms underlie incremental activation of protein kinase-regulated Cl⁻ conductance in mammalian heart. *J Gen Physiol.* 101:629-50.
- Janssens, V., and J. Goris. 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J.* 353:417-39.

- Jeanneteau, F., J. Diaz, P. Sokoloff, and N. Griffon. 2004. Interactions of GIPC with dopamine D2, D3 but not D4 receptors define a novel mode of regulation of G protein-coupled receptors. *Mol Biol Cell*. 15:696-705.
- Jia, Y., C.J. Mathews, and J.W. Hanrahan. 1997. Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *J Biol Chem*. 272:4978-84.
- Kanaji, T., S. Russell, and J. Ware. 2002. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. *Blood*. 100:2102-7.
- Knowles, M.R., and R.C. Boucher. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest*. 109:571-7.
- Kottgen, M., T. Benzing, T. Simmen, R. Tauber, B. Buchholz, S. Feliciangeli, T.B. Huber, B. Schermer, A. Kramer-Zucker, K. Hopker, K.C. Simmen, C.C. Tschucke, R. Sandford, E. Kim, G. Thomas, and G. Walz. 2005. Trafficking of TRPP2 by PACS proteins represents a novel mechanism of ion channel regulation. *Embo J*. 24:705-16.
- Kovacsovics, T.J., and J.H. Hartwig. 1996. Thrombin-induced GPIb-IX centralization on the platelet surface requires actin assembly and myosin II activation. *Blood*. 87:618-29.
- Kreda, S.M., M. Mall, A. Mengos, L. Rochelle, J. Yankaskas, J.R. Riordan, and R.C. Boucher. 2005. Characterization of wild-type and deltaF508 cystic fibrosis transmembrane regulator in human respiratory epithelia. *Mol Biol Cell*. 16:2154-67.
- Kubesch, P., T. Dork, U. Wulbrand, N. Kalin, T. Neumann, B. Wulf, H. Geerlings, H. Weissbrodt, H. von der Hardt, and B. Tummeler. 1993. Genetic determinants of airways' colonisation with *Pseudomonas aeruginosa* in cystic fibrosis. *Lancet*. 341:189-93.
- Kusumi, A., and Y. Sako. 1996. Cell surface organization by the membrane skeleton. *Curr Opin Cell Biol*. 8:566-74.
- Kwak, K.B., S.S. Chung, O.M. Kim, M.S. Kang, D.B. Ha, and C.H. Chung. 1993. Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim Biophys Acta*. 1175:243-9.
- Lambert, M., D. Choquet, and R.M. Mege. 2002. Dynamics of ligand-induced, Rac1-dependent anchoring of cadherins to the actin cytoskeleton. *J Cell Biol*. 157:469-79.
- Lee, G.M., A. Ishihara, and K.A. Jacobson. 1991. Direct observation of brownian motion of lipids in a membrane. *Proc Natl Acad Sci U S A*. 88:6274-8.

- Leonoudakis, D., L.R. Conti, C.M. Radeke, L.M. McGuire, and C.A. Vandenberg. 2004. A multiprotein trafficking complex composed of SAP97, CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. *J Biol Chem.* 279:19051-63.
- Leonoudakis, D., W. Mailliard, K. Wingerd, D. Clegg, and C. Vandenberg. 2001. Inward rectifier potassium channel Kir2.2 is associated with synapse-associated protein SAP97. *J Cell Sci.* 114:987-98.
- Li, M., J.C. Bermak, Z.W. Wang, and Q.Y. Zhou. 2000. Modulation of dopamine D(2) receptor signaling by actin-binding protein (ABP-280). *Mol Pharmacol.* 57:446-52.
- Li, M., H. Guo, and Z. Damuni. 1995a. Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry.* 34:1988-96.
- Li, M., A. Makkinje, and Z. Damuni. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem.* 271:11059-62.
- Li, Z., B. Taylor-Blake, A.R. Light, and M.F. Goy. 1995b. Guanylin, an endogenous ligand for C-type guanylate cyclase, is produced by goblet cells in the rat intestine. *Gastroenterology.* 109:1863-75.
- Liedtke, C.M., C.H. Yun, N. Kyle, and D. Wang. 2002. Protein kinase C epsilon-dependent regulation of cystic fibrosis transmembrane regulator involves binding to a receptor for activated C kinase (RACK1) and RACK1 binding to Na⁺/H⁺ exchange regulatory factor. *J Biol Chem.* 277:22925-33.
- Liu, G., L. Thomas, R.A. Warren, C.A. Enns, C.C. Cunningham, J.H. Hartwig, and G. Thomas. 1997. Cytoskeletal protein ABP-280 directs the intracellular trafficking of furin and modulates proprotein processing in the endocytic pathway. *J Cell Biol.* 139:1719-33.
- Loiselle, D.R., W.R. Thelin, C.E. Parker, N.N. Dicheva, B.A. Kesner, V. Mocanu, F. Wang, S.L. Milgram, M.R. Warren, and C.H. Borchers. 2005. Improved protein identification through the use of unstained gels. *J Proteome Res.* 4:992-7.
- Loo, D.T., S.B. Kanner, and A. Aruffo. 1998a. Filamin binds to the cytoplasmic domain of the beta1-integrin. Identification of amino acids responsible for this interaction. *J Biol Chem.* 273:23304-12.
- Loo, M.A., T.J. Jensen, L. Cui, Y. Hou, X.B. Chang, and J.R. Riordan. 1998b. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *Embo J.* 17:6879-87.

- Lukacs, G.L., G. Segal, N. Kartner, S. Grinstein, and F. Zhang. 1997. Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation. *Biochem J.* 328 (Pt 2):353-61.
- Luo, J., M.D. Pato, J.R. Riordan, and J.W. Hanrahan. 1998. Differential regulation of single CFTR channels by PP2C, PP2A, and other phosphatases. *Am J Physiol.* 274:C1397-410.
- Ma, J., J.E. Tasch, T. Tao, J. Zhao, J. Xie, M.L. Drumm, and P.B. Davis. 1996. Phosphorylation-dependent block of cystic fibrosis transmembrane conductance regulator chloride channel by exogenous R domain protein. *J Biol Chem.* 271:7351-6.
- Ma, T., J.R. Thiagarajah, H. Yang, N.D. Sonawane, C. Folli, L.J. Galiotta, and A.S. Verkman. 2002. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest.* 110:1651-8.
- Mall, M., B.R. Grubb, J.R. Harkema, W.K. O'Neal, and R.C. Boucher. 2004. Increased airway epithelial Na⁺ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med.* 10:487-93.
- Matsui, H., B.R. Grubb, R. Tarran, S.H. Randell, J.T. Gatzky, C.W. Davis, and R.C. Boucher. 1998a. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell.* 95:1005-15.
- Matsui, H., S.H. Randell, S.W. Peretti, C.W. Davis, and R.C. Boucher. 1998b. Coordinated clearance of periciliary liquid and mucus from airway surfaces. *J Clin Invest.* 102:1125-31.
- McCright, B., A.M. Rivers, S. Audlin, and D.M. Virshup. 1996. The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem.* 271:22081-9.
- Meacham, G.C., Z. Lu, S. King, E. Sorscher, A. Tousson, and D.M. Cyr. 1999. The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *Embo J.* 18:1492-505.
- Meyer, S.C., S. Zuerbig, C.C. Cunningham, J.H. Hartwig, T. Bissell, K. Gardner, and J.E. Fox. 1997. Identification of the region in actin-binding protein that binds to the cytoplasmic domain of glycoprotein IB α . *J Biol Chem.* 272:2914-9.
- Milewski, M.I., J.E. Mickle, J.K. Forrest, D.M. Stafford, B.D. Moyer, J. Cheng, W.B. Guggino, B.A. Stanton, and G.R. Cutting. 2001. A PDZ-binding motif is essential but

- not sufficient to localize the C terminus of CFTR to the apical membrane. *J Cell Sci.* 114:719-26.
- Mitsuhashi, S., H. Shima, N. Tanuma, S. Sasa, K. Onoe, M. Ubukata, and K. Kikuchi. 2005. Protein phosphatase type 2A, PP2A, is involved in degradation of gp130. *Mol Cell Biochem.* 269:183-7.
- Mohler, P.J., S.M. Kreda, R.C. Boucher, M. Sudol, M.J. Stutts, and S.L. Milgram. 1999. Yes-associated protein 65 localizes p62(c-Yes) to the apical compartment of airway epithelia by association with EBP50. *J Cell Biol.* 147:879-90.
- Molloy, S.S., L. Thomas, C. Kamibayashi, M.C. Mumby, and G. Thomas. 1998. Regulation of endosome sorting by a specific PP2A isoform. *J Cell Biol.* 142:1399-411.
- Moyer, B.D., J. Denton, K.H. Karlson, D. Reynolds, S. Wang, J.E. Mickle, M. Milewski, G.R. Cutting, W.B. Guggino, M. Li, and B.A. Stanton. 1999. A PDZ-interacting domain in CFTR is an apical membrane polarization signal. *J Clin Invest.* 104:1353-61.
- Moyer, B.D., M. Duhaime, C. Shaw, J. Denton, D. Reynolds, K.H. Karlson, J. Pfeiffer, S. Wang, J.E. Mickle, M. Milewski, G.R. Cutting, W.B. Guggino, M. Li, and B.A. Stanton. 2000. The PDZ-interacting domain of cystic fibrosis transmembrane conductance regulator is required for functional expression in the apical plasma membrane. *J Biol Chem.* 275:27069-74.
- Nakamura, F., J.H. Hartwig, T.P. Stossel, and P.T. Szymanski. 2005a. Ca²⁺ and calmodulin regulate the binding of filamin A to actin filaments. *J Biol Chem.* 280:32426-33.
- Nakamura, F., R. Pudas, O. Heikkinen, P. Permi, I. Kilpelainen, A.D. Munday, J.H. Hartwig, T.P. Stossel, and J. Ylanne. 2005b. The structure of the GPIb-filamin A complex. *Blood.*
- Naren, A.P., B. Cobb, C. Li, K. Roy, D. Nelson, G.D. Heda, J. Liao, K.L. Kirk, E.J. Sorscher, J. Hanrahan, and J.P. Clancy. 2003. A macromolecular complex of beta 2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci U S A.* 100:342-6.
- Naren, A.P., A. Di, E. Cormet-Boyaka, P.N. Boyaka, J.R. McGhee, W. Zhou, K. Akagawa, T. Fujiwara, U. Thome, J.F. Engelhardt, D.J. Nelson, and K.L. Kirk. 2000. Syntaxin 1A is expressed in airway epithelial cells, where it modulates CFTR Cl⁻ currents. *J Clin Invest.* 105:377-86.
- Naren, A.P., D.J. Nelson, W. Xie, B. Jovov, J. Pevsner, M.K. Bennett, D.J. Benos, M.W. Quick, and K.L. Kirk. 1997. Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature.* 390:302-5.

- Naren, A.P., M.W. Quick, J.F. Collawn, D.J. Nelson, and K.L. Kirk. 1998. Syntaxin 1A inhibits CFTR chloride channels by means of domain-specific protein-protein interactions. *Proc Natl Acad Sci U S A.* 95:10972-7.
- Nehring, R.B., E. Wischmeyer, F. Doring, R.W. Veh, M. Sheng, and A. Karschin. 2000. Neuronal inwardly rectifying K(+) channels differentially couple to PDZ proteins of the PSD-95/SAP90 family. *J Neurosci.* 20:156-62.
- Nelson, W.J., R. Wilson, D. Wollner, R. Mays, H. McNeill, and K. Siemers. 1992. Regulation of epithelial cell polarity: a view from the cell surface. *Cold Spring Harb Symp Quant Biol.* 57:621-30.
- Oceandy, D., B. McMorran, R. Schreiber, B.J. Wainwright, and K. Kunzelmann. 2003. GFP-tagged CFTR transgene is functional in the G551D cystic fibrosis mouse colon. *J Membr Biol.* 192:159-67.
- Oceandy, D., B.J. McMorran, S.N. Smith, R. Schreiber, K. Kunzelmann, E.W. Alton, D.A. Hume, and B.J. Wainwright. 2002. Gene complementation of airway epithelium in the cystic fibrosis mouse is necessary and sufficient to correct the pathogen clearance and inflammatory abnormalities. *Hum Mol Genet.* 11:1059-67.
- Ohta, Y., T.P. Stossel, and J.H. Hartwig. 1991. Ligand-sensitive binding of actin-binding protein to immunoglobulin G Fc receptor I (Fc gamma RI). *Cell.* 67:275-82.
- Onji, T., M. Takagi, and N. Shibata. 1987. Calpain abolishes the effect of filamin on the actomyosin system in platelets. *Biochim Biophys Acta.* 912:283-6.
- Onoprishvili, I., M.L. Andria, H.K. Kramer, N. Ancevska-Taneva, J.M. Hiller, and E.J. Simon. 2003. Interaction between the mu opioid receptor and filamin A is involved in receptor regulation and trafficking. *Mol Pharmacol.* 64:1092-100.
- Oppermann, M., N. Cols, T. Nyman, J. Helin, J. Saarinen, I. Byman, N. Toran, A.A. Alaiya, T. Bergman, N. Kalkkinen, R. Gonzalez-Duarte, and H. Jornvall. 2000. Identification of foetal brain proteins by two-dimensional gel electrophoresis and mass spectrometry comparison of samples from individuals with or without chromosome 21 trisomy. *Eur J Biochem.* 267:4713-9.
- Ostedgaard, L.S., O. Baldursson, D.W. Vermeer, M.J. Welsh, and A.D. Robertson. 2000. A functional R domain from cystic fibrosis transmembrane conductance regulator is predominantly unstructured in solution. *Proc Natl Acad Sci U S A.* 97:5657-62.
- Ostedgaard, L.S., C. Randak, T. Rokhlina, P. Karp, D. Vermeer, K.J. Ashbourne Excoffon, and M.J. Welsh. 2003. Effects of C-terminal deletions on cystic fibrosis transmembrane conductance regulator function in cystic fibrosis airway epithelia. *Proc Natl Acad Sci U S A.* 100:1937-42.

- Parker, C.E., M.R. Warren, D.R. Loiselle, N.N. Dicheva, C.O. Scarlett, and C.H. Borchers. 2005. Identification of components of protein complexes. *Methods Mol Biol.* 301:117-51.
- Pasyk, E.A., and J.K. Foskett. 1995. Mutant (delta F508) cystic fibrosis transmembrane conductance regulator Cl⁻ channel is functional when retained in endoplasmic reticulum of mammalian cells. *J Biol Chem.* 270:12347-50.
- Peter, K., K. Varga, Z. Bebok, C.M. McNicholas-Bevensee, L. Schwiebert, E.J. Sorscher, E.M. Schwiebert, and J.F. Collawn. 2002. Ablation of internalization signals in the carboxyl-terminal tail of the cystic fibrosis transmembrane conductance regulator enhances cell surface expression. *J Biol Chem.* 277:49952-7.
- Peters, K.W., J. Qi, S.C. Watkins, and R.A. Frizzell. 1999. Syntaxin 1A inhibits regulated CFTR trafficking in xenopus oocytes. *Am J Physiol.* 277:C174-80.
- Petrecca, K., D.M. Miller, and A. Shrier. 2000. Localization and enhanced current density of the Kv4.2 potassium channel by interaction with the actin-binding protein filamin. *J Neurosci.* 20:8736-44.
- Pfaff, M., S. Liu, D.J. Erle, and M.H. Ginsberg. 1998. Integrin beta cytoplasmic domains differentially bind to cytoskeletal proteins. *J Biol Chem.* 273:6104-9.
- Pi, M., R.F. Spurney, Q. Tu, T. Hinson, and L.D. Quarles. 2002. Calcium-sensing receptor activation of rho involves filamin and rho-guanine nucleotide exchange factor. *Endocrinology.* 143:3830-8.
- Picciano, J.A., N. Ameen, B.D. Grant, and N.A. Bradbury. 2003. Rme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator. *Am J Physiol Cell Physiol.* 285:C1009-18.
- Pind, S., J.R. Riordan, and D.B. Williams. 1994. Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem.* 269:12784-8.
- Poulsen, J.H., H. Fischer, B. Illek, and T.E. Machen. 1994. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A.* 91:5340-4.
- Prince, L.S., K. Peter, S.R. Hatton, L. Zaliauskiene, L.F. Cotlin, J.P. Clancy, R.B. Marchase, and J.F. Collawn. 1999. Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator requires a tyrosine-based signal. *J Biol Chem.* 274:3602-9.
- Prince, L.S., R.B. Workman, Jr., and R.B. Marchase. 1994. Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc Natl Acad Sci U S A.* 91:5192-6.

- Puchelle, E., O. Bajolet, and M. Abely. 2002. Airway mucus in cystic fibrosis. *Paediatr Respir Rev.* 3:115-9.
- Quinton, P.M. 1999. Physiological basis of cystic fibrosis: a historical perspective. *Physiol Rev.* 79:S3-S22.
- Raghuram, V., H. Hormuth, and J.K. Foskett. 2003. A kinase-regulated mechanism controls CFTR channel gating by disrupting bivalent PDZ domain interactions. *Proc Natl Acad Sci U S A.* 100:9620-5.
- Raghuram, V., D.D. Mak, and J.K. Foskett. 2001. Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction. *Proc Natl Acad Sci U S A.* 98:1300-5.
- Ramjeesingh, M., J.F. Kidd, L.J. Huan, Y. Wang, and C.E. Bear. 2003. Dimeric cystic fibrosis transmembrane conductance regulator exists in the plasma membrane. *Biochem J.* 374:793-7.
- Reczek, D., M. Berryman, and A. Bretscher. 1997. Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. *J Cell Biol.* 139:169-79.
- Reddy, M.M., and P.M. Quinton. 1996. Deactivation of CFTR-Cl conductance by endogenous phosphatases in the native sweat duct. *Am J Physiol.* 270:C474-80.
- Riordan, J.R., J.M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.L. Chou, and et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 245:1066-73.
- Sako, Y., A. Nagafuchi, S. Tsukita, M. Takeichi, and A. Kusumi. 1998. Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. *J Cell Biol.* 140:1227-40.
- Sampson, L.J., M.L. Leyland, and C. Dart. 2003. Direct interaction between the actin-binding protein filamin-A and the inwardly rectifying potassium channel, Kir2.1. *J Biol Chem.* 278:41988-97.
- Sarkadi, B., D. Bauzon, W.R. Huckle, H.S. Earp, A. Berry, H. Suchindran, E.M. Price, J.C. Olson, R.C. Boucher, and G.A. Scarborough. 1992. Biochemical characterization of the cystic fibrosis transmembrane conductance regulator in normal and cystic fibrosis epithelial cells. *J Biol Chem.* 267:2087-95.

- Scott, G.K., F. Gu, C.M. Crump, L. Thomas, L. Wan, Y. Xiang, and G. Thomas. 2003. The phosphorylation state of an autoregulatory domain controls PACS-1-directed protein traffic. *Embo J.* 22:6234-44.
- Scott, R.O., W.R. Thelin, and S.L. Milgram. 2002. A novel PDZ protein regulates the activity of guanylyl cyclase C, the heat-stable enterotoxin receptor. *J Biol Chem.* 277:22934-41.
- Seck, T., R. Baron, and W.C. Horne. 2003. Binding of filamin to the C-terminal tail of the calcitonin receptor controls recycling. *J Biol Chem.* 278:10408-16.
- Seibert, F.S., Y. Jia, C.J. Mathews, J.W. Hanrahan, J.R. Riordan, T.W. Loo, and D.M. Clarke. 1997. Disease-associated mutations in cytoplasmic loops 1 and 2 of cystic fibrosis transmembrane conductance regulator impede processing or opening of the channel. *Biochemistry.* 36:11966-74.
- Seibert, F.S., P. Linsdell, T.W. Loo, J.W. Hanrahan, D.M. Clarke, and J.R. Riordan. 1996. Disease-associated mutations in the fourth cytoplasmic loop of cystic fibrosis transmembrane conductance regulator compromise biosynthetic processing and chloride channel activity. *J Biol Chem.* 271:15139-45.
- Seibert, F.S., J.A. Tabcharani, X.B. Chang, A.M. Dulhanty, C. Mathews, J.W. Hanrahan, and J.R. Riordan. 1995. cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J Biol Chem.* 270:2158-62.
- Sharma, C.P., R.M. Ezzell, and M.A. Arnaout. 1995. Direct interaction of filamin (ABP-280) with the beta 2-integrin subunit CD18. *J Immunol.* 154:3461-70.
- Sheets, E.D., G.M. Lee, R. Simson, and K. Jacobson. 1997. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. *Biochemistry.* 36:12449-58.
- Short, D.B., K.W. Trotter, D. Reczek, S.M. Kreda, A. Bretscher, R.C. Boucher, M.J. Stutts, and S.L. Milgram. 1998. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J Biol Chem.* 273:19797-801.
- Smith, F.D., G.S. Oxford, and S.L. Milgram. 1999. Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem.* 274:19894-900.
- Smith, J.J., S.M. Travis, E.P. Greenberg, and M.J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell.* 85:229-36.

- Song, Y., N.D. Sonawane, D. Salinas, L. Qian, N. Pedemonte, L.J. Galiotta, and A.S. Verkman. 2004. Evidence against the rescue of defective DeltaF508-CFTR cellular processing by curcumin in cell culture and mouse models. *J Biol Chem.* 279:40629-33.
- Stossel, T.P., J. Condeelis, L. Cooley, J.H. Hartwig, A. Noegel, M. Schleicher, and S.S. Shapiro. 2001. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol.* 2:138-45.
- Stutts, M.J., B.C. Rossier, and R.C. Boucher. 1997. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. *J Biol Chem.* 272:14037-40.
- Sun, F., M.J. Hug, N.A. Bradbury, and R.A. Frizzell. 2000a. Protein kinase A associates with cystic fibrosis transmembrane conductance regulator via an interaction with ezrin. *J Biol Chem.* 275:14360-6.
- Sun, F., M.J. Hug, C.M. Lewarchik, C.H. Yun, N.A. Bradbury, and R.A. Frizzell. 2000b. E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells. *J Biol Chem.* 275:29539-46.
- Swiatecka-Urban, A., C. Boyd, B. Coutermarsh, K.H. Karlson, R. Barnaby, L. Aschenbrenner, G.M. Langford, T. Hasson, and B.A. Stanton. 2004. Myosin VI regulates endocytosis of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem.* 279:38025-31.
- Swiatecka-Urban, A., A. Brown, S. Moreau-Marquis, J. Renuka, B. Coutermarsh, R. Barnaby, K.H. Karlson, T.R. Flotte, M. Fukuda, G.M. Langford, and B.A. Stanton. 2005a. The short apical membrane half-life of rescued {Delta}F508-cystic fibrosis transmembrane conductance regulator (CFTR) results from accelerated endocytosis of {Delta}F508-CFTR in polarized human airway epithelial cells. *J Biol Chem.* 280:36762-72.
- Swiatecka-Urban, A., M. Duhaim, B. Coutermarsh, K.H. Karlson, J. Collawn, M. Milewski, G.R. Cutting, W.B. Guggino, G. Langford, and B.A. Stanton. 2002. PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem.* 277:40099-105.
- Swiatecka-Urban, A., S. Moreau-Marquis, D.P. Maceachran, J.P. Connolly, C.R. Stanton, J.R. Su, R. Barnaby, A. O'Toole G, and B.A. Stanton. 2005b. Pseudomonas aeruginosa Inhibits Endocytic Recycling of the Cystic Fibrosis Transmembrane Conductance Regulator in Polarized Human Airway Epithelial Cells. *Am J Physiol Cell Physiol.*

- Tabcharani, J.A., X.B. Chang, J.R. Riordan, and J.W. Hanrahan. 1991. Phosphorylation-regulated Cl⁻ channel in CHO cells stably expressing the cystic fibrosis gene. *Nature*. 352:628-31.
- Takafuta, T., G. Wu, G.F. Murphy, and S.S. Shapiro. 1998. Human beta-filamin is a new protein that interacts with the cytoplasmic tail of glycoprotein Ibalpha. *J Biol Chem*. 273:17531-8.
- Tarran, R., B. Button, M. Picher, A.M. Paradiso, C.M. Ribeiro, E.R. Lazarowski, L. Zhang, P.L. Collins, R.J. Pickles, J.J. Fredburg, and R.C. Boucher. 2005. Normal and cystic fibrosis airway surface liquid homeostasis: The effects of phasic shear stress and viral infections. *J Biol Chem*.
- Tarran, R., B.R. Grubb, D. Parsons, M. Picher, A.J. Hirsh, C.W. Davis, and R.C. Boucher. 2001. The CF salt controversy: in vivo observations and therapeutic approaches. *Mol Cell*. 8:149-58.
- Tarran, R., M.E. Loewen, A.M. Paradiso, J.C. Olsen, M.A. Gray, B.E. Argent, R.C. Boucher, and S.E. Gabriel. 2002. Regulation of murine airway surface liquid volume by CFTR and Ca²⁺-activated Cl⁻ conductances. *J Gen Physiol*. 120:407-18.
- Tehrani, M.A., M.C. Mumby, and C. Kamibayashi. 1996. Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. *J Biol Chem*. 271:5164-70.
- Thelin, W.R., M. Kesimer, R. Tarran, S.M. Kreda, B.R. Grubb, J.K. Sheehan, M.J. Stutts, and S.L. Milgram. 2005. The Cystic Fibrosis Transmembrane Conductance Regulator Is Regulated by a Direct Interaction with the Protein Phosphatase 2A. *J Biol Chem*. 280:41512-20.
- Thornton, D.J., J.K. Sheehan, H. Lindgren, and I. Carlstedt. 1991. Mucus glycoproteins from cystic fibrotic sputum. Macromolecular properties and structural 'architecture'. *Biochem J*. 276 (Pt 3):667-75.
- Tien, X.Y., T.A. Brasitus, M.A. Kaetzel, J.R. Dedman, and D.J. Nelson. 1994. Activation of the cystic fibrosis transmembrane conductance regulator by cGMP in the human colonic cancer cell line, Caco-2. *J Biol Chem*. 269:51-4.
- Travis, S.M., H.A. Berger, and M.J. Welsh. 1997. Protein phosphatase 2C dephosphorylates and inactivates cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A*. 94:11055-60.
- Tu, Y., S. Wu, X. Shi, K. Chen, and C. Wu. 2003. Migfilin and Mig-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. *Cell*. 113:37-47.

- Uezono, Y., J. Bradley, C. Min, N.A. McCarty, M. Quick, J.R. Riordan, C. Chavkin, K. Zinn, H.A. Lester, and N. Davidson. 1993. Receptors that couple to 2 classes of G proteins increase cAMP and activate CFTR expressed in *Xenopus* oocytes. *Receptors Channels*. 1:233-41.
- Varga, K., A. Jurkuvenaite, J. Wakefield, J.S. Hong, J.S. Guimbellot, C.J. Venglarik, A. Niraj, M. Mazur, E.J. Sorscher, J.F. Collawn, and Z. Bebok. 2004. Efficient intracellular processing of the endogenous cystic fibrosis transmembrane conductance regulator in epithelial cell lines. *J Biol Chem*. 279:22578-84.
- Vastiau, A., L. Cao, M. Jaspers, G. Owsianik, V. Janssens, H. Cuppens, J. Goris, B. Nilius, and J.J. Cassiman. 2005. Interaction of the protein phosphatase 2A with the regulatory domain of the cystic fibrosis transmembrane conductance regulator channel. *FEBS Lett*. 579:3392-6.
- Verkman, A.S. 2001. Lung disease in cystic fibrosis: is airway surface liquid composition abnormal? *Am J Physiol Lung Cell Mol Physiol*. 281:L306-8.
- Wang, K., and S.J. Singer. 1977. Interaction of filamin with f-actin in solution. *Proc Natl Acad Sci U S A*. 74:2021-5.
- Wang, S., R.W. Raab, P.J. Schatz, W.B. Guggino, and M. Li. 1998. Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). *FEBS Lett*. 427:103-8.
- Wang, S., H. Yue, R.B. Derin, W.B. Guggino, and M. Li. 2000. Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. *Cell*. 103:169-79.
- Wang, X., J. Matteson, Y. An, B. Moyer, J.S. Yoo, S. Bannykh, I.A. Wilson, J.R. Riordan, and W.E. Balch. 2004. COPII-dependent export of cystic fibrosis transmembrane conductance regulator from the ER uses a di-acidic exit code. *J Cell Biol*. 167:65-74.
- Ward, C.L., and R.R. Kopito. 1994. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J Biol Chem*. 269:25710-8.
- Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell*. 83:121-7.
- Weihing, R.R. 1988. Actin-binding and dimerization domains of HeLa cell filamin. *Biochemistry*. 27:1865-9.
- Weixel, K.M., and N.A. Bradbury. 2000. The carboxyl terminus of the cystic fibrosis transmembrane conductance regulator binds to AP-2 clathrin adaptors. *J Biol Chem*. 275:3655-60.

- Welsh, M.J., and A.E. Smith. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*. 73:1251-4.
- Widdicombe, J.G. 1989. Airway mucus. *Eur Respir J*. 2:107-15.
- Williamson, D., I. Pikovski, S.L. Cranmer, P. Mangin, N. Mistry, T. Domagala, S. Chehab, F. Lanza, H.H. Salem, and S.P. Jackson. 2002. Interaction between platelet glycoprotein Ibalpha and filamin-1 is essential for glycoprotein Ib/IX receptor anchorage at high shear. *J Biol Chem*. 277:2151-9.
- Wine, J.J. 1999. The genesis of cystic fibrosis lung disease. *J Clin Invest*. 103:309-12.
- Wollner, D.A., and W.J. Nelson. 1992. Establishing and maintaining epithelial cell polarity. Roles of protein sorting, delivery and retention. *J Cell Sci*. 102 (Pt 2):185-90.
- Wood, H.G., and R.E. Barden. 1977. Biotin enzymes. *Annu Rev Biochem*. 46:385-413.
- Xiang, Y., S.S. Molloy, L. Thomas, and G. Thomas. 2000. The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct trans-Golgi network/endosomal compartments. *Mol Biol Cell*. 11:1257-73.
- Xu, W., Z. Xie, D.W. Chung, and E.W. Davie. 1998. A novel human actin-binding protein homologue that binds to platelet glycoprotein Ibalpha. *Blood*. 92:1268-76.
- Xu, X., Y. Shi, X. Wu, P. Gambetti, D. Sui, and M.Z. Cui. 1999. Identification of a novel PSD-95/Dlg/ZO-1 (PDZ)-like protein interacting with the C terminus of presenilin-1. *J Biol Chem*. 274:32543-6.
- Yang, I.C., T.H. Cheng, F. Wang, E.M. Price, and T.C. Hwang. 1997. Modulation of CFTR chloride channels by calyculin A and genistein. *Am J Physiol*. 272:C142-55.
- Yang, Y., S. Janich, J.A. Cohn, and J.M. Wilson. 1993. The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. *Proc Natl Acad Sci U S A*. 90:9480-4.
- Yoo, J.S., B.D. Moyer, S. Bannykh, H.M. Yoo, J.R. Riordan, and W.E. Balch. 2002. Non-conventional trafficking of the cystic fibrosis transmembrane conductance regulator through the early secretory pathway. *J Biol Chem*. 277:11401-9.
- Zhang, H., K.W. Peters, F. Sun, C.R. Marino, J. Lang, R.D. Burgoyne, and R.A. Frizzell. 2002. Cysteine string protein interacts with and modulates the maturation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem*. 277:28948-58.
- Zhang, W., S.W. Han, D.W. McKeel, A. Goate, and J.Y. Wu. 1998. Interaction of presenilins with the filamin family of actin-binding proteins. *J Neurosci*. 18:914-22.

- Zhang, W., I. Mannan, S. Schulz, S.J. Parkinson, A.E. Alekseev, L.A. Gomez, A. Terzic, and S.A. Waldman. 1999. Interruption of transmembrane signaling as a novel antisecretory strategy to treat enterotoxigenic diarrhea. *Faseb J.* 13:913-22.
- Zhu, T., D. Dahan, A. Evagelidis, S. Zheng, J. Luo, and J.W. Hanrahan. 1999. Association of cystic fibrosis transmembrane conductance regulator and protein phosphatase 2C. *J Biol Chem.* 274:29102-7.
- Zielenski, J., and L.C. Tsui. 1995. Cystic fibrosis: genotypic and phenotypic variations. *Annu Rev Genet.* 29:777-807.