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# Phosphatase Regulation of CFTR

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## CHAPTER 2

# Phosphatase Regulation of CFTR

John W. Hanrahan, Tang Zhu and L. Daniel Howell

### Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is tightly regulated by the opposing actions of protein kinases and phosphatases. Its phosphorylation and activation by protein kinases A (PKA) and C (PKC) have been studied in some detail but phosphatase regulation of the channel has received less attention. Several phosphatases may control CFTR in various cell types, however in epithelia most deactivation is mediated by a membrane-bound phosphatase with functional properties resembling those of PP2C, the prototypic member of the PPM gene family of serine/threonine phosphatases. The PP2C-like phosphatase requires Mg<sup>2+</sup>, is insensitive to the inhibitors okadaic acid and calyculin A, does not require Ca<sup>2+</sup> or calmodulin, and is inhibited non-specifically by phenylimidazothiazoles. It is closely associated with CFTR and can be co-immunoprecipitated or co-purified from cell lysates by affinity chromatography with, or without, pretreatment with chemical crosslinking reagents. Current efforts are directed towards identifying the phosphatase at the molecular level; i.e., determining if it is a novel isoform or alternatively spliced variant of a known PP2C isoform, or a new membrane-targeted phosphatase in the PPM family. Identifying and characterizing the phosphatase will open many new avenues of investigation into basic aspects of CFTR regulation, and may have clinical significance since the phosphatase is a potential target for pharmacotherapies to treat cystic fibrosis and secretory diarrhea.

### Introduction

CFTR is a non-rectifying, low-conductance chloride channel in epithelia and other cells which is activated in different preparations by cAMP-mobilizing agonists such as prostaglandin E<sub>1</sub>, epinephrine, vasoactive intestinal peptide, adenosine and secretin (see ref. 1). Gating of the CFTR pore is nucleotide dependent and tightly regulated by phosphorylation. In addition to its role in mediating plasma membrane chloride conductance, CFTR also influences the activity of other channels and transporters through mechanisms that are poorly understood and, in view of the large number and diversity of proteins affected, probably indirect. Some of these regulatory effects (e.g., down-regulation of epithelial sodium channels)<sup>2</sup> require phosphorylation of CFTR whereas others (e.g., upregulation of glutathione release)<sup>3</sup> apparently do not. Although phosphatases may control both chloride conductance and the regulatory effects of CFTR, this chapter focuses exclusively on their role in regulating CFTR channel activity.

### CFTR Phosphorylation

Activation of apical membrane chloride conductance by cAMP, the rate-limiting step for transepithelial chloride secretion, was established by the early work of M. Field, R. Frizzell, S.

Klyce and others (see ref. 4). It is now generally accepted that the channel mediating this conductance is CFTR, which is phosphorylated and strongly activated by protein kinase A (PKA).<sup>5-7</sup> Hyperstimulating PKA with forskolin leads to the addition of about 5 moles of PO<sub>4</sub> per mole of CFTR *in vivo*,<sup>8</sup> although this is a lower limit since it is likely that some ATP fails to acquire [<sup>32</sup>P]PO<sub>4</sub> at the  $\gamma$  position when cells are metabolically labeled. Higher stoichiometries are achieved *in vitro* through phosphorylation of additional low-affinity sites. This could be viewed as an artifact of the high kinase and low phosphatase activities present *in vitro*, but it could also be argued that phosphorylation of the low-affinity sites occurs *in vivo* but is not detected due to the technical limitations of *in vivo* experiments; i.e., the lower specific activity of [<sup>32</sup>P]ATP in cells and the labile nature of the phosphoryl groups at "weak" sites. Removing the nine strong dibasic consensus sequences on the R domain and one additional dibasic site proximal to the first nucleotide binding domain (NBD1) reduces *in vitro* phosphorylation by more than 90% and channel open probability by about half in excised membrane patches that are exposed to high PKA activity.<sup>9-11</sup> While the precise number and identity of the consensus sequences that are phosphorylated under physiological conditions remains uncertain, it is clear that PKA acts at multiple sites and that even the "weak" PKA sites are likely to have functional significance since they are almost perfectly conserved from cartilaginous fishes to humans.<sup>12</sup>

The mechanisms by which phosphorylation and dephosphorylation of the R domain regulate channel activity appear complex.<sup>13</sup> Phosphorylation induces only small changes in the secondary structure of a polypeptide comprising the R domain and distal region of NBD1,<sup>14</sup> and these are not observed when the distal part of NBD1 is not included in the construct.<sup>13</sup> Rather than regulating NBD function, phosphorylation may allow the efficient transduction of conformational changes induced by nucleotide interactions at the NBDs to the membrane domains (see ref. 1 for review). Adding dephosphorylated R domain inhibits wild-type CFTR channels incorporated into planar bilayers,<sup>15</sup> and pre-phosphorylating the exogenous domain with PKA prevents this inhibition.<sup>16</sup> Adding dephosphorylated R domain does not inhibit a mutant lacking the distal two thirds of the R domain when studied by patch clamping excised patches, however when pre-phosphorylated and added to excised patches, it does increase the spontaneous activity of this mutant.<sup>17</sup> While the latter implies a positive role of phosphorylated R domain in sustaining channel activity, the normal activity of "split" channels lacking the entire R domain when expressed in *Xenopus* oocytes argues strongly that the primary role of the R domain is as a negative regulator (when unphosphorylated) and that phosphorylated R domain is not essential for channel gating.<sup>18</sup> Mutating two potential PKA sites on the R domain (S737 and S768) enhances activation in oocytes, suggesting they may act like channel "brakes".<sup>19</sup> If so, this has interesting implications for phosphatases, which have a similar physiological role, since to remain phosphorylated (and inhibitory) in a particular cell type, those inhibitory sites would need to be either more resistant to the phosphatases in that cell, or more susceptible to basal PKA activity. Recent studies suggest a negatively charged, predicted helical region distal to the phosphorylation sites on the R domain (817-838) also plays a role in regulation by phosphorylation since deleting this region<sup>20</sup> causes the channel to become unresponsive to PKA. Removing negative charges in this region or disrupting its helical structure by mutagenesis abolishes inhibition by dephospho-R domain and stimulation by PKA, respectively.<sup>20</sup> Charge in the R domain has been considered a determinant of activation because a mutant containing aspartates at eight consensus dibasic PKA sites (to mimic phosphoserines) is spontaneously active.<sup>21</sup> However charge resulting from phosphoryl groups is probably not the only factor involved in regulating the R domain because mutants with aspartates<sup>21</sup> or glutamates<sup>22</sup> at PKA sites have low activity compared to phosphorylated wild-type channels, and altering the secondary structure of the R domain by mutagenesis without affecting charge is also sufficient to cause partial activation of the channel in bilayers.<sup>23</sup> Phosphorylation could

stimulate channel activity through several mechanisms; e.g., enhanced nucleotide binding, a more stable ATP-bound transition state at NBD1 or accelerated hydrolysis at NBD2,<sup>24</sup> or improved coupling of conformational changes to the channel gate. Consistent with the binding hypothesis, PKA phosphorylation causes a modest increase in the rate of hydrolysis and shifts its dependence to lower ATP concentrations.<sup>25</sup> The significance of such alterations in the apparent K<sub>M</sub> for hydrolysis need to be established, particularly when much of the gating does not depend on hydrolysis.<sup>22</sup> A shift in the [ATP] dependence of open probability is seen during patch clamp experiments when wild-type channels are phosphorylated by PKA,<sup>17</sup> or when low-phosphorylation mutants (e.g., 4SA, 10SA) are compared in excised patches with strongly phosphorylated wild-type channels.<sup>11</sup> However these functional effects would also be explained by a downstream mechanism in which dephosphorylation of the R domain hinders transduction of conformational changes from the NBDs to the transmembrane domains. Exposure to PKC (without PKA) causes a small increase in open probability that is not abolished by pre-treating excised patches with protein phosphatases,<sup>26</sup> however a more striking effect of PKC is to enhance the subsequent responsiveness of CFTR to PKA stimulation.<sup>6,27,28</sup> PKC increases the rate and magnitude of activation, and this is mediated by direct PKC phosphorylation of CFTR since it is abolished by mutating serines or threonines at all nine PKC consensus sequences between the Walker B consensus in NBD1 and TM7 of the second transmembrane domain to alanines.<sup>29</sup> These potential PKC sites, which are defined by the consensus R/K<sub>1-3</sub>, X<sub>2-0</sub>, S'/T', X<sub>2-0</sub>, R/K<sub>1-3</sub> include T582, T604, S641, T682, S686, S707, S790, T791, S809. Multiple PKC sites may be involved in modulating regulating activation by PKA, since PKC effects are reduced but not abolished by removing the only two (S686, S790) where phosphorylation has been directly demonstrated.<sup>8,28,30</sup> It seems unlikely that the mechanism by which PKC enhances PKA responses will be understood until the nature of PKA regulation is established.

### Deactivation of CFTR Channels by a Membrane-Bound Phosphatase

cAMP is a diffusible second messenger that regulates growth, metabolism and many other cellular functions not obviously related to transepithelial chloride secretion. CFTR must be efficiently dephosphorylated *in vivo* to maintain low resting chloride conductance and ensure that Cl<sup>-</sup> secretion is only increased by appropriate stimuli and is readily reversible. Tonic suppression of CFTR by a phosphatase would effectively raise its threshold for activation and minimize responses to irrelevant signals.

Evidence that CFTR is indeed regulated by a membrane-localized phosphatase came from the spontaneous "rundown" of channel activity that occurs after patches are excised from cAMP stimulated cells into bath solution lacking PKA.<sup>6</sup> Channels deactivate in about 10 seconds at 37°C when patches are isolated from Chinese hamster ovary cells (Fig. 1), or in 1–2 minutes when excised at room temperature.<sup>31</sup> The rundown is fully reversed by adding PKA catalytic subunit to the bath within the first few minutes after excision, and is more rapid than the reactivation induced by exposure to high PKA activity. Similar rundown is observed when patches are excised from an airway epithelial tumor cell line expressing endogenous CFTR<sup>32</sup> or an immortalized airway epithelial cell line transfected with CFTR cDNA,<sup>31</sup> therefore membrane-bound phosphatases probably regulate CFTR channels in cells that normally express them, not only heterologous expression systems. The extent of rundown and the relative importance of particular phosphatases may vary with cell type since it seems to be less dramatic in mouse fibroblasts,<sup>33</sup> insect cells,<sup>34</sup> or baby hamster kidney cells (BHK)<sup>35</sup> and has a large component that is sensitive to the membrane-impermeant PP2A inhibitor microcystin in guinea pig myocytes.<sup>36</sup> That said, CFTR-mediated currents in epithelial cells do have the same pharmacological properties as those described in patches excised from CHO and most other

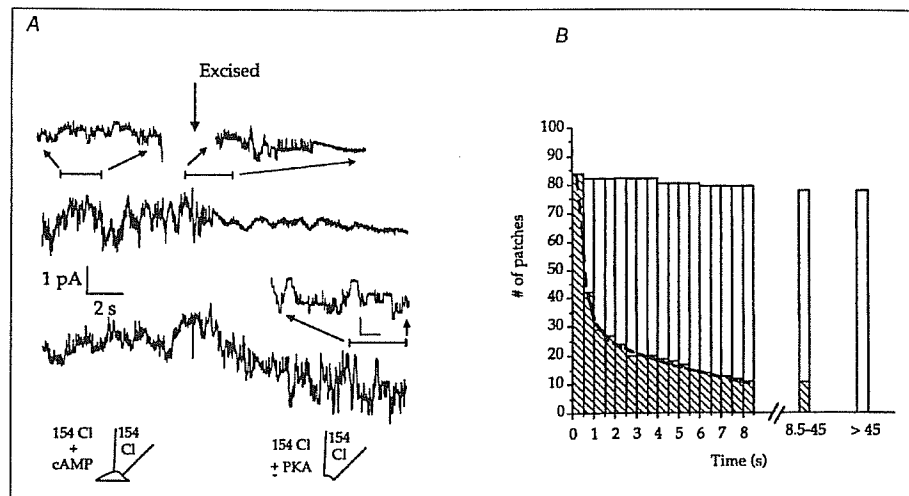


Figure 1. Rundown of CFTR channels mediated by a membrane-bound phosphatase. A, Effect of excising a membrane patch containing CFTR channels from a cAMP-stimulated CHO cell. The short traces above each recording show expanded views of the time intervals indicated by the horizontal lines. For the upper recording, the bath solution contained 0.5 mM ATP and 2 mM  $Mg^{2+}$  but no PKA, and the expanded segments show activity immediately before (left) and during excision (right). Note the rapid decline in channel activity after excision at 37°C. The lower recording shows an experiment using the same protocol, but with 180 nM PKA catalytic subunit in the bath. B, Number of patches with active channels as a function of time after excision in the absence (hatched bars) or presence (open bars) of 180 nM PKA, 0.5 mM ATP and 2 mM  $Mg^{2+}$  at 37°C. (from ref. 6)

cells<sup>6,31,35,37</sup> (see below). This implies that the predominant CFTR phosphatase in epithelia is the same membrane-bound enzyme that deactivates CFTR in excised patches from CHO and other cells, and provides the rationale for using rundown to assay its activity.<sup>31,35</sup> Most recent effort has focused on identifying it at the molecular level and cloning its cDNA.

### Characteristics of Protein Phosphatases

Mammalian Ser/Thr phosphatases were classified as type 1 (PP1) or type 2 (PP2A, PP2B, PP2C) based on their functional properties.<sup>38</sup> A more recent system based on gene relatedness places PP1, PP2A and PP2B in the "PPP family" along with several newly-cloned phosphatases (i.e., PP4, PP5, PP6, PP7; see below). PP2C and its orthologs such as ABI1 in *Arabidopsis* and PTC1 in *S. cerevisiae* are distinct and have been placed in the "PPM family" (for evolutionary analyses see<sup>39,40</sup>). The functional scheme (PP1, PP2A, etc) is used in this review because it is familiar and reflects the fact that the phosphatase regulating CFTR is still known by its functional properties rather than by its sequence.

The PP1 family of protein phosphatases dephosphorylates the  $\beta$ -subunit of phosphorylase kinase and is sensitive to the thermostable proteins Inhibitor 1 and Inhibitor 2 (see Table 1). PP1 holoenzymes are heterodimers of catalytic and accessory subunits. There are at least three catalytic subunit isoforms and about 50 known or putative accessory subunits. The PP1 $\delta$  catalytic subunit also has multiple splice variants. In most instances, a cleft in the PP1 catalytic subunit binds to a highly conserved sequence (RVxF) on an accessory subunit, which targets the holoenzyme to various cellular locations (see ref. 41).<sup>41</sup> PP1 has diverse functions that

Table 1. Summary of the functional characteristics of major protein phosphatase families<sup>a</sup>

Protein Phosphatases	Subunit Composition	Cofactors Needed	Inhibitors	Typical Substrates	CFTR (R Domain) Dephosphorylation <sup>b</sup>
PP1	Catalytic Accessory	-	Tautomycin Microcystin Calyculin A Okadaic acid Cantharidic acid Inhibitor 1,2	Phosphorylase kinase _ subunit	++
PP2A	A (structural) B (regulatory) C (catalytic)	-	Microcystin Okadaic acid Calyculin A Cantharidic acid	Phosphorylase kinase _ subunit	+++
PP2B	A (catalytic) B (regulatory)	$Ca^{2+}$ calmodulin	Deltamethrin Cyclosporin A FK506 Fenvalerate	Post-synaptic densities	+
PP2C	monomeric	$Mg^{2+}/Mn^{2+}$	-	AMP kinase MAP kinases Myosin light chains	+++

<sup>a</sup> PP1, PP2A, PP2B, and less well characterized PP4-PP7 (not shown) belong to the "PPP family" based on homology. PP2C is a member of the "PPM family".

<sup>b</sup> Number of crosses indicates relative efficiency of dephosphorylation using either phosphorylated full-length CFTR or GST-R domain fusion protein as the substrate.

include smooth muscle relaxation, pre-mRNA splicing, synaptic transmission, and the regulation of epithelial transport.

The PP2 family dephosphorylates the  $\alpha$ -subunit of phosphorylase kinase and is insensitive to the thermostable proteins Inhibitor 1 and Inhibitor 2. PP2 phosphatases have been further sub-divided into types 2A (PP2A), 2B (PP2B) and 2C (PP2C) according to their metal ion requirements and inhibitor sensitivities. Active PP2A does not require divalent cations or other cofactors for enzymatic activity and is a heterotrimer of "A" (structural), "B" (regulatory), and "C" (catalytic) subunits, all of which have multiple isoforms and splice variants. It is implicated in regulating metabolism, DNA replication, transcription, RNA splicing, translation, cell cycle progression and many other cellular functions. Of particular interest for CFTR research is its role in controlling signaling pathways, since it has been reported to upregulate four protein kinases and downregulate 29 others, including PKA and PKC.<sup>42</sup> This complicates the interpretation of any *in vivo* phosphorylation that might be induced by inhibitors such as calyculin A, even though they are relatively specific for PP2A when assayed *in vitro*. Phosphatases in the PP2B (calcineurin) sub-family have "A" (catalytic) and "B" (regulatory) subunits and require the binding of  $Ca^{2+}$  and calmodulin for enzymatic activity. PP2B regulates events at neuronal postsynaptic densities, microtubules of the cytoskeleton, and signal transduction in T cells, where it is a target for the immunosuppressive drugs cyclosporin and FK506. Several other protein phosphatases (PP3 – PP7) have been reported in mammalian cells, although the existence of PP3 has been questioned.<sup>43</sup> PP4-PP7 all belong to the PPP gene family

and have sequences resembling PP2A. PP7 is  $Mg^{2+}$  dependent and insensitive to okadaic acid, however its mass (75 kD) and restricted tissue distribution (retina) argue against a role in regulating CFTR (see below). For comprehensive accounts of serine/threonine phosphatases, the reader is directed to the many excellent general reviews of the early<sup>44-47</sup> and more recent literature.<sup>41-43,48-52</sup>

PP2C is the prototypic member of the PPM gene family and has no significant sequence homology with PP1, PP2A or PP2B<sup>53</sup> although its architecture and proposed catalytic mechanism are similar (see refs. 50, 54). First identified chromatographically as a glycogen synthase phosphatase,<sup>55</sup> it was later characterized as a divalent cation-dependent ( $Mg^{2+}=Mn^{2+}>Co^{2+}$ ) protein phosphatase of about 49 kDa.<sup>56</sup> An enzyme with properties resembling glycogen synthase phosphatase but which dephosphorylates phosphocasein<sup>57</sup> and myosin light chains<sup>58</sup> was characterized in cardiac and smooth muscle, respectively. cDNA cloning has identified seven PPMs in mammals (Fig. 2): a,<sup>53</sup> b,<sup>59</sup> pyruvate dehydrogenase phosphatase PDP,<sup>60</sup> g/FIN13,<sup>61,62</sup> d,<sup>63</sup> Ca-CaMK phosphatase<sup>64</sup> and Wip1.<sup>65</sup> The catalytic domains of all PPMs contain the signature sequence [LIVMFY]-[LIVMFYA]-[GSAC]-[LIVM]-[FYC]-D-G-H-[GAV]. The perfectly conserved aspartate sidechain and glycine carbonyl oxygen in PP2Cb (i.e., the underlined residues in the signature sequence) help coordinate metal ions in the active site. General features of the PPM family members are shown in a schematic alignment in Figure 2.

PP2Ca was purified to homogeneity from rat liver and turkey gizzard but is expressed in many other tissues including skeletal muscle, kidney, and cerebellum. There are several variants including the original  $\alpha$ -1,<sup>53</sup> and the much shorter  $\alpha$ -2<sup>66</sup> and  $\alpha$ -3 forms (A. Cherniack, S. Nicoloso, J. Buxton, A. Bose, M. Emoto, S. Waters, and M. Czech; NCBI accession # AF259672.1, 2000), which were cloned from different species. PP2Cb has at least six variants differing at their C termini, probably through alternative splicing;  $\alpha$ -1,<sup>59</sup>  $\beta$ -2,<sup>67</sup>  $\beta$ -3 and  $\beta$ -4,<sup>68</sup>  $\beta$ -5,<sup>69</sup> and  $\beta$ -6.<sup>70</sup> Fig. 3 shows an alignment of the C terminal amino acids of the  $\alpha$  and  $\beta$  variants.

PP2C $\beta$ 1 was identified in rat liver but is ubiquitously expressed. PP2C $\beta$ 2 is most abundant in mouse heart and brain whereas the PP2C $\beta$ 3 and PP2C $\beta$ 4 forms have been reported only in testes.<sup>68</sup> PP2C $\beta$ 5 is a "predicted" variant since it was originally identified by RT-PCR using an upstream primer that anneals about half way through the coding region, although Northern blots analyzed with a C-terminal cDNA probe suggest a full-length message is expressed in mouse testes and intestine.<sup>69</sup> Finally, PP2C $\beta$ 6 is the most divergent variant of the beta isoform, being only 74% similar to the others even without its long C-terminal tail. It is abundant in human skeletal muscle, heart and liver, but also detected in brain, placenta, kidney and pancreas.<sup>70</sup> Other members of the PPM family are not discussed in detail because they lack the epitope recognized by our antibody against the CFTR-associated phosphatase (see below) and because their cellular locations make it unlikely they regulate CFTR (e.g., PDP is mitochondrial; FIN13/PP2C $\beta$  and Wip1 are nuclear).

The catalytic domain of PP2Cs consists of the N-terminal ~290 amino acids, which are well conserved among isoforms. When the structure of PP2C $\alpha$ -1 was solved by X-ray crystallography it was found to have a novel fold consisting of a central beta-sandwich and two manganese ions surrounded by alpha-helices.<sup>54</sup>  $Mn^{2+}$ -bound water was postulated to act as a nucleophile during catalysis,<sup>50,54,71</sup> which would account for the strict dependence of enzymatic activity on  $Mg^{2+}$ ,  $Mn^{2+}$ , or  $Fe^{2+}$ .<sup>72,73</sup> Functional studies of mouse PP2C $\beta$ -1 mutants confirmed that metal ions bind at acidic amino acids in the fold (i.e., Glu37, Asp38, Asp60 and Asp239).<sup>74</sup> Other substrates of mammalian PP2Cs include AMP kinase,<sup>75</sup> moesin,<sup>76</sup> and kinases in stress-activated signaling pathways. For example the MAP kinase kinase kinase (MKKK) TAK1, which is activated by environmental stress and inflammatory cytokines, is down-regulated by PP2C $\beta$ -2<sup>52,77</sup> whereas the downstream kinases MKK and p38 MAPK are down-regulated by PP2C $\beta$ -1.<sup>66</sup> There may be many isoforms and splice variants of PP2C, each having a different set of phosphoprotein substrates.

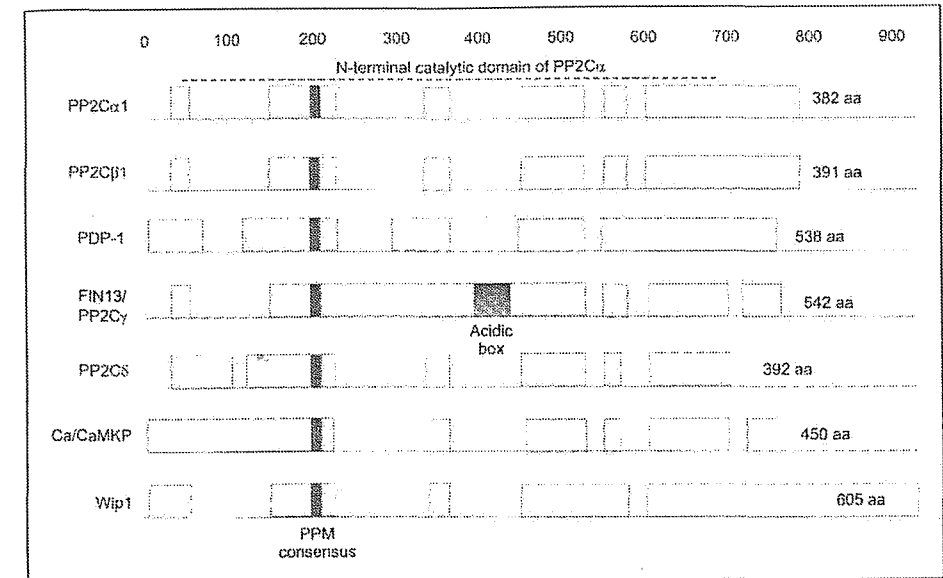


Figure 2. Similarity among representative members of the seven known PPM phosphatases. The boxes indicate regions of each polypeptide aligned with corresponding regions of other PPM phosphatases. The spaces between the boxes are gaps introduced by the alignment and therefore have no significance. Also shown are the relative locations of the PPM consensus ([LIVMFY]-[LIVMFYA]-[GSAC]-[LIVM]-[FYC]-D-G-H-[GAV]) and the acidic box of the Fin13/PP2C $\gamma$  are indicated. The total number of amino acids (aa) in each polypeptide is shown at right.

### Like Real Estate, What Counts Is Location, Location and Location

Serine/threonine phosphatases have broad and overlapping specificities when assayed *in vitro* (see ref. 44). Their specificity *in vivo* is often due to targeting to particular substrates by specific regulatory subunits or accessory proteins. For example in neurons, PP1 is targeted to AMPA-type glutamate receptors by a protein called spinophilin.<sup>78</sup> PP1 constitutively down-regulates AMPA currents and this is dependent on targeting. Introducing a phospho-peptide that disrupts the binding of PP1 also abolishes its ability to deactivate the AMPA current. Similar targeting of PP1 to glycogen particles is mediated by the accessory subunit GL, which has the consensus motif RVXF. This sequence is found on other PP1 targeting subunits and has recently been demonstrated near the amino terminus of NKCC1, the sodium-potassium-chloride co-transporter.<sup>79</sup> Altering the consensus abolishes PP1 co-immunoprecipitation with the cotransporter, slows dephosphorylation, and increases NKCC1-mediated ion transport. Like PP1, PP2A and PP2B are targeted by specific regulatory (i.e., B) subunits that influence its enzymatic activity *in vivo*.<sup>80</sup> Different isoforms of the regulatory subunit may have specific addresses; for example, PP2A is targeted to the microtubule associated protein Tau by B $\alpha$  and B $\beta$ , but not by the B' regulatory subunit.<sup>81</sup> Other substrates of PP2A include voltage-gated sodium channels, soluble CaMKII, and neurofilaments. Only a few PP2B targeting subunits have been identified to date. One is AKAP79 (A-kinase Anchoring Protein of 79 kD), which targets PP2B to postsynaptic densities and provides a scaffold for the binding of PKA and PKC.<sup>82</sup> Dynamin, plasma membrane Ca channels and IP3 receptors are also known substrates for PP2B.



Figure 3. Alignment of the carboxyl-terminal regions of PP2C  $\alpha$  and  $\beta$  isoforms, starting at amino acid position 276–281. Note the strong conservation of N-terminal amino acids <290, which are in the catalytic domain. The coils above the alignments indicate regions that are predicted to be  $\alpha$ -helical. PP2C $\alpha$ -2 and  $\alpha$ -3 are truncated and have distinct C-termini. PP2C $\beta$ s also differ in this region, however note that  $\beta$ -5 is a chimera of  $\beta$ -1 (AGDLEDSLVAL) and  $\beta$ -3 (FYQPSIAYS DN VFLL).

PP2C is unique in that dedicated targeting subunits or accessory proteins have not yet been identified in mammalian cells, although some structural and substrate selectivity is conferred by its C-terminal 75–90 amino acids, particularly the most distal region, which is distinct among isozymes and splice variants. Indirect evidence for this notion comes from the findings that 90 amino acids can be deleted from the C terminus of the  $\alpha$ -1 isoform without affecting its in vitro enzymatic activity,<sup>83</sup> and the ability of PP2C $\beta$ -1 to discriminate between phosphohistone and phosphocasein is lost when the C-terminal 12 amino acids are deleted.<sup>74</sup> The nucleus has been suggested as the “default” location of PP2C $\alpha$  within the cell, however this remains speculative (see discussion in ref. 84). PP2C $\beta$ 1 is detected in both cytosol and nucleus when transiently overexpressed in BHK or COS cells.<sup>84</sup> Cytosolic PP2C $\beta$ 1 washes out of the cell when the plasma membrane is permeabilized with digitonin, therefore it is probably a freely diffusible rather than membrane-bound enzyme. The cellular locations of variants PP2C $\beta$ 2–PP2C $\beta$ 6 have not been studied in detail. PP2C generally prefers substrates with phosphothreonine over those with phosphoserines. For example replacing phosphothreonine with phosphoserine in the synthetic phosphopeptide substrate RRATpVA reduces its rate of dephosphorylation by rabbit skeletal muscle PP2C $\beta$  approximately 20-fold.<sup>85</sup> Rabbit PP2C $\beta$  removes nearly all the phosphate from the threonine in RRSpTpVA before there is any detectable dephosphorylation of the phosphoserine. A preference for [<sup>32</sup>P]phospho-Thr over [<sup>32</sup>P]phospho-Ser is also observed when PKA-phosphorylated casein is used as the substrate. By contrast, mouse PP2C $\alpha$  and PP2C $\beta$  have only modest (i.e., 2–4 fold) preference for phosphothreonine when hydroxyl-methylglutaryl-coenzyme A is the substrate, and human PP2C $\alpha$  apparently dephosphorylates phosphoserine and phosphothreonine with equal efficiency.<sup>86</sup> PP2C activity is reduced when proline or negatively charged amino acids follow the phosphoryl group and increased by nearby phosphoryl groups. Neighboring sequence may influence which phosphatase acts at a particular site on CFTR as in the MAPK signaling pathway, where all three PP2C substrates have two phosphorylation sites separated by a single amino acid. In CFTR, this preference might cause closely spaced sites to be dephosphorylated by a PP2C-like phosphatase and widely separated sites by another phosphatase such as PP2A.<sup>86</sup> It is likely that sites on CFTR vary in their susceptibility to phosphatases. Indirect evidence for this comes from the observation that PKA-dependent channel activity runs down about seven times faster in excised membrane patches than does modulation by PKC (1.5 vs 10 min at room temperature). The pharmacological properties and metal ion dependence of the rundown of PKC modulation has not been studied systematically, but since only PP2C-like phosphatase has been found associated with CFTR (see below), it is probably mediated by the same membrane-bound phosphatase as the rundown in PKA-dependent activity.<sup>27</sup>

It should be mentioned that phosphatase targeting to the plasma membrane could be achieved even without specific proteins or targeting subunits. For example, the PRL family of protein-tyrosine phosphatases contains a consensus C-terminal “CAAX” sequence for prenylation (where C is cysteine, A is an aliphatic amino acid, and X any amino acid), which inserts into the inner leaflet of the plasma membrane as a lipid anchor. This system is complex in that at least two lipid moieties can be used as anchors: When “X” is a Met, Ser, or Gln residue the phosphatase becomes a farnesyltransferase substrate, but when it is a leucine the lipidation is by geranylgeranyl-transferase I.<sup>87</sup> Whether lipid anchors mediate targeting of serine/threonine phosphatases to the plasma membrane has not been investigated.

## Comparing the Deactivation of CFTR by Endogenous and Exogenous Protein Phosphatases

To help identify endogenous phosphatases that regulate CFTR, spontaneous deactivation of CFTR channels in excised patches has been compared with that induced by adding exogenous phosphatases. Fortunately channels in patches from baby hamster kidney (BHK) cells often do not exhibit rundown, making it possible to assay the effects of exogenous phosphatases.<sup>35</sup> Adding PP2A catalytic subunit from smooth muscle, PP2C $\alpha$  from turkey gizzard, or bovine intestinal alkaline phosphatase reduces channel activity by more than 90%, but at different rates depending on the phosphatase used. PP2C is most efficient, causing deactivation comparable to the rundown that is mediated by endogenous phosphatase in CHO and other cells. Deactivation by PP2A is somewhat slower, and that by alkaline phosphatase occurs after a delay of several minutes. By contrast, PP2B from bovine brain is much less effective in deactivating CFTR channels, and recombinant human PP1 has little effect, yielding the sequence PP2C > PP2A > PP2B >> PP1 for deactivation of channels by addition of exogenous phosphatases to excised patches. Exposure to PP2A or PP2C causes deactivation of macroscopic CFTR currents and dephosphorylates CFTR protein.<sup>33,37,88</sup> In addition to their similar time courses, spontaneous rundown and deactivation by exogenous PP2C are both magnesium dependent, and have similar effects on single channel kinetics.<sup>35</sup> Thus, as  $P_o$  declines during spontaneous rundown or exposure to exogenous PP2C, there is no obvious reduction in the mean duration of bursts of channel openings, and the fall in  $P_o$  is due to lengthening of the intervals between open bursts. By contrast, addition of exogenous PP2A causes slower deactivation, and this decline in  $P_o$  is accompanied by a decrease in burst duration. Interestingly, residual channel activity is observed even after prolonged exposure to purified PP2C or PP2A whereas spontaneous rundown is usually complete. This might reflect the involvement of multiple endogenous phosphatases during rundown that cannot be mimicked by individual enzymes. Alternatively, dephosphorylation by exogenous, soluble PP2C $\alpha$  may be inherently less effective than the membrane-bound form that is associated with CFTR. The properties of the membrane-bound phosphatase in excised patches, which are based mainly on single channel results obtained using patches from CHO from BHK cells, are entirely consistent with those of CFTR-mediated current across T84 epithelial cell monolayers, which is insensitive to high concentrations of the PP2A/PP1 inhibitor calyculin A (see below).

## Pharmacology Suggests the Membrane-Associated CFTR Phosphatase Is Related to PP2C

PP1 and PP2A are both sensitive to calyculin A at nanomolar concentrations. All the PP's except PP2C and PP7 are inhibited by okadaic acid, albeit at widely varying concentrations.<sup>89,90</sup> PP4 and PP6 resemble PP2A and are sensitive to the same inhibitors (reviewed by ref. 43). PP2B is distinguished by its sensitivity to deltamethrin, cyclosporin, and FK506.<sup>47,91</sup> Specific inhibitors of PP2C are not presently available.

Channel rundown is inhibited slightly or not all by okadaic acid<sup>6,31</sup> but is slowed four-fold by the phenylimidazothiazoles bromotetramisole and levamisole,<sup>31,92</sup> well known inhibitors of alkaline phosphatase isozymes from liver, bone and kidney (but not from intestine). Similar results are obtained whether patches from Chinese hamster ovary or human airway cells are used. These drugs stimulate mutant G551D channels that are processed and trafficked to the plasma membrane, although they do not respond to forskolin stimulation (Fig. 4). However, higher phenylimidazothiazole concentrations are needed to inhibit rundown or stimulate channel activity on intact cells ( $\geq 100 \mu\text{M}$ ) than to inhibit of alkaline phosphatases ( $\leq 11 \text{ mM}$ ; ref. 93, and PP1, PP2A, PP2B and PP2C are all sensitive to bromotetramisole at concentrations that stimulate CFTR.<sup>88,92</sup> These results establish that, at least in principle, phosphatase inhibitors

may benefit CF patients who have mutations that allow normal processing of CFTR, although more potent and selective inhibitors are clearly needed.

Studying excised patches could give a false impression regarding the relative importance of different phosphatases, since any soluble enzymes would be lost when deactivation is studied under these conditions. Perhaps the most compelling evidence that CFTR is down-regulated predominantly by the PP2C-like phosphatase in intact cells rather than by PP2A or other cytosolic phosphatases comes from the rapid decline in chloride current across T84 monolayers that occurs following washout of cpt-cAMP or forskolin. Deactivation of transepithelial current is unaffected by okadaic acid<sup>37</sup> or calyculin A,<sup>35</sup> even at toxic concentrations that would inhibit any contributions by PP1 or PP2A (Fig. 5).

Okadaic acid ( $10^{-8} \text{ M}$ ) does slow the deactivation of CFTR currents in permeabilized sweat ducts (when studied under low-Mg<sup>2+</sup> conditions that would inhibit any contribution by PP2C).<sup>94</sup> Partial inhibition of whole cell CFTR current deactivation in cardiac cells by microcystin or okadaic acid suggest PP2A can play a significant role in those cells.<sup>36</sup> However some results with transfected cells are difficult to reconcile. For example exogenous PP1 and PP2B have little effect on macroscopic CFTR current when added to patches excised from fibroblasts,<sup>33</sup> yet PP2B has been proposed as the endogenous phosphatase regulating heterologous CFTR channels in those cells based on their stimulation by cyclosporin A and deltamethrin.<sup>95</sup>

Dephosphorylation of individual sites has not yet been studied systematically by phosphopeptide mapping or mass spectrometry, although this will be essential to fully understand deactivation of CFTR, particularly when stimulatory and inhibitory sites weaken the correlation between total phosphorylation and channel activity.<sup>19</sup> However such studies would be most informative if carried out with the membrane-bound phosphatase. Moreover, if the phosphatase operates within a regulatory complex, dephosphorylation of critical sites on CFTR may depend not only on having the right phosphatase but also its orientation relative to that of the R domain. If dephosphorylation is not recapitulated in vitro using soluble enzymes, it may be necessary to study intact complexes.

## Evidence That CFTR and Its Phosphatase Are Part of a Regulatory Complex

Comparing deactivation by the endogenous phosphatase with that caused by exogenously added, purified enzymes could potentially be misleading if dephosphorylation efficiency depended on association of both proteins and their relative positions within a regulatory complex. The approach used in this laboratory has been to establish that CFTR and a phosphatase are indeed associated, and then identify the associated phosphatase using biochemical methods. Early speculation that the phosphatase might be physically associated with CFTR came from the striking deactivation of CFTR channels in excised patches.<sup>6,31,96</sup> That possibility was eventually tested directly using co-immunoprecipitation and crosslinking approaches.<sup>97</sup> Polyclonal antibodies were raised against four hydrophilic regions of PP2C $\alpha$ , and the one with highest affinity against PP2C $\alpha$  was purified and used for immunoprecipitations and immunoblotting. It recognizes several of the PP2C preparations that have been tested including PP2C $\alpha$  purified from turkey gizzard smooth muscle (from Dr. M. Pato, Univ. Saskatchewan, Canada), bacterially expressed human PP2C $\alpha$  (from Dr. P. T. W. Cohen, Univ. Dundee, UK),  $\alpha$  and  $\beta$  isoforms of mouse PP2C (from Dr. S. Tamura, Tohoku University, Sendai, Japan). When used for immunoblotting BHK and Calu-3 cells immunoprecipitated with the monoclonal anti-CFTR antibody M3A7,<sup>98</sup> it recognizes a polypeptide of about 44 kD, consistent with known  $\alpha$  and  $\beta$  isoforms of PP2C. PP1, PP2A and PP2B are not co-precipitated with CFTR under these conditions although they are readily detected in Western blots of cell lysates (Fig. 6).



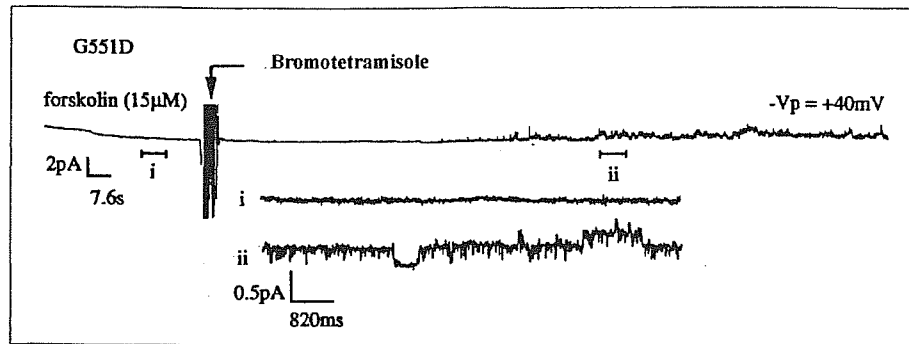


Figure 4. Effect of forskolin and the phenylimidazothiazole (-)-p-bromotetramisole on G551D mutant CFTR channels expressed on CHO cells. Channels were not responsive to 15  $\mu\text{M}$  forskolin (trace "i"), but were stimulated by addition of 1 mM bromotetramisole (trace "ii"). (from ref. 31)

In the converse experiment, anti-PP2C antibody co-precipitates CFTR protein from BHK membrane extracts, although the signal is weaker. Exposing BHK cell lysates to dithiobis[sulfosuccinimidyl propionate] (DTSSP), a bifunctional reagent with a 12 Å spacer arm, crosslinks CFTR molecules with a C-terminal histidine tag (CFTR<sub>His10</sub>) and PP2C into high molecular weight complexes that can be isolated by chelate chromatography. When the crosslinks are cleaved with DTT and proteins are separated on SDS-PAGE, only PP2C is the only phosphatase found to be co-purified with CFTR<sub>His10</sub>; the other major protein phosphatases types are not detected by Western blotting with anti-PP1, -PP2A or -PP2B antibodies. Under appropriate conditions, specific co-purification of CFTR and a PP2C-like phosphatase is observed without exposing the lysates to crosslinker suggesting they may exist in a stable complex, and similar results are obtained when the polyhistidine tag is at the amino- or carboxyl-terminus of CFTR. The membrane-bound phosphatase is still referred to as "PP2C-like" because defined only by its functional and immunological properties. It may be a splice variant of the PP2C $\alpha$  or  $\beta$  isoforms, a previously unidentified isozyme of PP2C, or perhaps a novel phosphatase that shares some characteristics with PP2C and happens to be recognized by the antibody. A proteomics approach based on co-purification of the phosphatase with CFTR without crosslinking mass spectrometry seems the most direct method for identification.<sup>99</sup> The main challenge has been the low expression of CFTR in mammalian cells and the low yield of phosphatase (< 0.2 moles PP2C / mole CFTR) in pull-downs.

The phosphatase may associate with CFTR directly like PP1 with the NKCC1 cotransporter as discussed above, or indirectly through an anchoring or scaffolding protein analogous to AKAPs (A-kinase anchoring proteins), which localize PKA near its substrates.<sup>100</sup> Both the PKA catalytic and type II (RII) regulatory subunits co-immunoprecipitate with CFTR, and endogenous kinase activity in immunoprecipitates is abolished by Ht31,<sup>101</sup> a peptide that mimics the amino terminus of RII and disrupts its interaction with AKAPs (see ref. 102 for review). Ezrin has been proposed as an AKAP for CFTR since it is expressed at the apical membrane of Calu-3 and T84 cells, can be co-immunoprecipitated with CFTR and binds to RII in overlay experiments.<sup>101</sup> It may also provide a link between PKA and E3KARP-CFTR.<sup>103</sup> Evidence that the association of PKA and CFTR has functional significance comes from the activation of CFTR channels in excised patches by cpt-cAMP alone, which indicates PKA holoenzyme is anchored near CFTR.<sup>104</sup> PKC $\epsilon$  also appears to be tethered near CFTR by the "regulator of C kinase" RACK1.<sup>105,106</sup> PP2C-like phosphatase probably does not associate with CFTR through one of the PDZ domain-containing proteins that have been found to interact with CFTR (i.e., EBP-50,<sup>107-110</sup> E3KARP,<sup>103</sup> CAP70<sup>111</sup> or CAL<sup>112</sup>). Mutating the C-terminal "TRL"

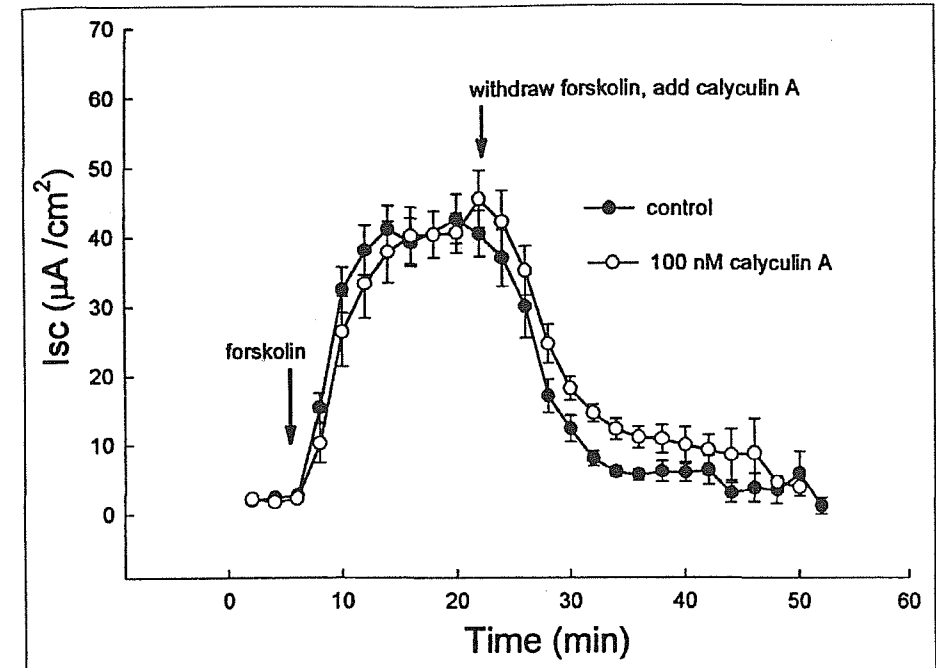


Figure 5. Effect of calyculin A on deactivation of the short-circuit current (Isc) across T84 monolayers after washing out forskolin (10  $\mu\text{M}$ ). Note that 100 nM calyculin A, which is eventually toxic, does not inhibit deactivation of the transepithelial chloride current after removal of forskolin. Similar results were obtained when 20 nM calyculin A was present continuously starting at time "0". (from ref. 35)

motif on CFTR to alanines, which disrupts PDZ domain binding, does not adversely affect the ability of CFTR<sub>His10</sub> to pull down phosphatase from BHK cell lysates.<sup>113</sup> Once the physical basis of interactions between the phosphatase and CFTR is identified, it should be possible to introduce mutations into CFTR or the phosphatase that disrupt the interaction and reveal its physiological role. Establishing whether phosphatase effects are exerted directly rather than via other proteins in the regulatory complex may ultimately require reconstitution of the CFTR-phosphatase complex from purified components.

### Is the Phosphatase Regulated?

Phosphatase activity could potentially be regulated through post-translational modifications of the phosphatase or through altered association with CFTR. This possibility remains speculative, however there are many precedents for regulation of serine/threonine phosphatases. The PP1 that dephosphorylates the cyclin-dependent kinase TPPR is itself inhibited by phosphorylation.<sup>114</sup> PP2A is inhibited when phosphorylated on threonine and tyrosine residues,<sup>115</sup> and is upregulated by methylation of its C-terminus.<sup>116</sup> Moreover, methylation of PP2A is stimulated by cAMP, suggesting an interesting feedback loop between the phosphatase and PKA.<sup>117</sup>

PP2C is phosphorylated in an isoform-specific manner in mammalian cells<sup>118</sup> and in yeast.<sup>119</sup> PP2C $\alpha$  becomes specifically phosphorylated on serines 375 and 377 when expressed heterologously in COS cells, sites that are phosphorylated by casein kinase II in vitro. Phosphorylation of PP2C $\alpha$  is also enhanced two-fold by okadaic acid in vivo whereas the PP2B inhibitor cyclosporin has no effect, suggesting that PP2C is phosphorylated by casein kinase II



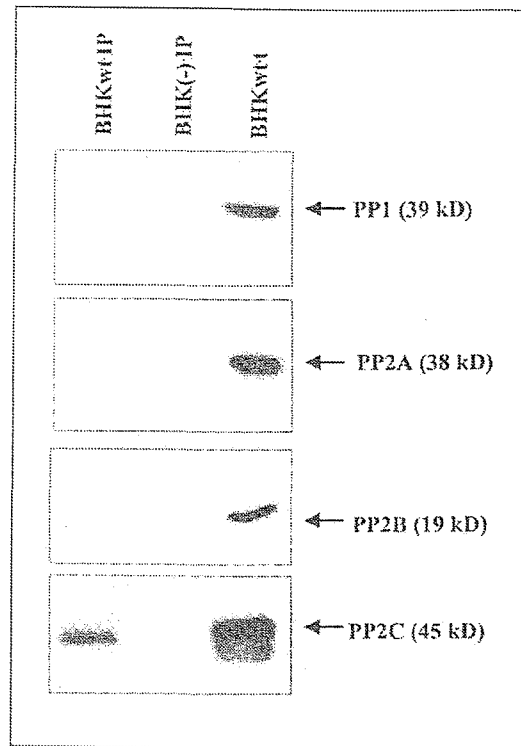


Figure 6. Specific co-precipitation of a PP2C-like phosphatase with CFTR. BHK cells were lysed, immunoprecipitated, and resolved on 10% SDS-PAGE. Bands corresponding to all four protein phosphatase types were detected at their expected molecular masses when BHK crude cell lysates expressing wild-type CFTR (BHKwt\_t) were immunoblotted using specific antibodies. None of the phosphatases were detectable when control cells lacking CFTR were precipitated using the anti-CFTR antibody M3A7. When cells expressing wild-type CFTR were immunoprecipitated with anti-CFTR antibody, PP2C was detected by immunoblotting but not the other phosphatases. (from ref. 97)

and dephosphorylated by PP1 and/or PP2A. The effect of such phosphorylation on the enzymatic activity of PP2C has not been studied in mammalian cells, however in yeast casein kinase II does regulate the PP2C orthologs Ptc2 and Ptc3 by phosphorylating serine residues in the conserved sequence (Ser-X-Ser-X-X-Glu/Asp) near their carboxyl termini.<sup>119</sup> Phosphorylation inhibits Ptc2 activity by 25% and increases the phosphatase activity of Ptc3 by 55%. Preliminary experiments with pulled down CFTR phosphatase have not revealed any effect of casein kinase II pretreatment on dephosphorylation.

A novel regulatory mechanism involving fatty acids and  $Mg^{2+}$  was described for PP2C $\alpha$  in bovine retina.<sup>120</sup> A hallmark of PP2C is its requirement for relatively high divalent cation concentration, somewhat higher than the free concentration of  $Mg^{2+}$  typically available inside cells 0.7 – 0.9 mM.<sup>121</sup> This  $Mg^{2+}$ -dependence is consistent with the inhibition of CFTR rundown when free  $[Mg^{2+}]$  is lowered from 2 mM to 0.5 mM.<sup>35</sup> The metal dependence of retinal PP2C is shifted to lower  $Mg^{2+}$  concentrations by specific mono- and polyunsaturated fatty acids. Thus arachidonic acid (500  $\mu$ M) causes a 10-fold stimulation of phosphatase activity when the  $Mg^{2+}$  concentration is 0.7 mM, and smaller stimulations are also observed for PP2C $\alpha$ . Interestingly, arachidonic acid reduces CFTR currents when added to the cytoplasmic side of patches, and this inhibition is not voltage dependent or influenced by extracellular  $Cl^-$  concentration suggesting it does not involve blockage of the pore.<sup>122</sup> CFTR inhibition by fatty acids follows the sequence linoleic  $\geq$  arachidonic  $\geq$  oleic > elaidic  $\geq$  palmitic  $\geq$  myristic, which is similar to the rank order of potency for inhibiting retinal PP2C $\alpha$ . Studies of the effects of unsaturated fatty acids on CFTR dephosphorylation are in progress. Interestingly, cis-unsaturated fatty acids also inhibit PKA,<sup>123</sup> hence they could downregulate CFTR channel activity through multiple mechanisms.

## Future Prospects on Phosphatase Regulation of CFTR

Precise identification of the membrane-bound phosphatase and the cloning of its cDNA are major goals and will be essential for understanding the physiological regulation of CFTR channel activity. Two types of information are presently available that can be used as "handles" for such an undertaking. The first is functional (i.e., its  $Mg^{2+}$  dependence, pharmacology, etc), properties that have been deduced mainly from studies of CFTR rundown in excised patches, and have focused our attention on PP2C and its relatives in the PPM family. The second is the physical association of the phosphatase with CFTR, which has now been demonstrated by two methods and should facilitate purification and sequencing of the main protein regulating CFTR, although other phosphatases such as PP2A may play a role in some tissues. The low expression of CFTR will make the proteomic strategy challenging, however identifying the phosphatase and cloning its cDNA will be worth the effort. Detailed biochemical studies of CFTR phosphorylation/dephosphorylation will become possible with recombinant enzyme. The amino acid sequence of the phosphatase may also suggest sites of interaction with CFTR or targeting molecules, or consensus sites for post-translational modifications that regulate its phosphatase activity. The phosphatase has been proposed as a potential target for pharmacotherapies to treat cystic fibrosis<sup>31,124</sup> and could be useful for treating those with CFTR mutations such as G551D that do not cause CFTR mislocalization, or as an adjunct to other therapies that only partially correct the chloride conductance defect. Conversely, activators of the phosphatase should inhibit CFTR-mediated chloride secretion in the gut and might find use in the treatment of secretory diarrhea, a major cause of infant mortality in the third world. Regardless, identifying and characterizing the phosphatase will break new ground and open many new avenues of research into the regulation of CFTR.

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## CHAPTER 3

# Control of Membrane Transport by the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

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### Abstract

In epithelial tissues of cystic fibrosis patients, the secretory Cl<sup>-</sup> transport is impaired, while the absorptive Na<sup>+</sup> transport is enhanced. The disease causing cystic fibrosis transmembrane conductance regulator (CFTR) is a protein expressed primarily in luminal membranes of secretory and absorptive epithelial cells, along with other proteins in charge of epithelial transport. After initial characterization of CFTR as a cAMP and protein kinase A regulated Cl<sup>-</sup> channel, numerous studies reported influences of CFTR on other independent membrane transport proteins, such as epithelial Na<sup>+</sup> channels, K<sup>+</sup> and Cl<sup>-</sup> channels, as well as electroneutral transporters such as the Na<sup>+</sup>/H<sup>+</sup> exchanger or the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> antiporter. Currently best examined is the regulation of the epithelial Na<sup>+</sup> channel ENaC by CFTR. The mechanisms for the inhibition of ENaC by CFTR are only slowly emerging. CFTR may control the membrane transport by other proteins not just by a single mechanism. CFTR's first nucleotide binding domain plays a central role, the Cl<sup>-</sup> transport by CFTR affects other transport proteins and CFTR is linked to some of these transport proteins via PDZ binding domain proteins, which may be co-localized in small functional membrane micro-domains. Moreover, a direct binding of CFTR to other transport proteins cannot be excluded. Thus, the puzzle around the functional relationship of CFTR, ENaC and other transport proteins, has not yet been solved and the results may not even be the same in each cell type.

### Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) plays a vital role for epithelial Cl<sup>-</sup> transport in both absorptive and secretory epithelial cells.<sup>140,221</sup> CFTR is the only relevant luminal Cl<sup>-</sup> channel in the intestinal epithelium and probably also in sweat duct epithelial cells, and it is the important secretory channel in the airways.<sup>140,199</sup> Mutations in the CFTR gene lead to a defect in Cl<sup>-</sup> secretion in these epithelial tissues. It has been proposed to be the cause for the clinical symptoms observed in cystic fibrosis.<sup>140,199</sup> However, some of the transport defects observed in either in vivo measurements in cystic fibrosis patients, in isolated tissues from CF patients or in transgenic mice, carrying CFTR mutations, could not easily be reconciled with the concept of a defective Cl<sup>-</sup> conductance as the only reason for the transport defects observed in CF.<sup>23,88,140</sup>