A Study of Protein Phosphatase 2C in Cystic Fibrosis

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Jan. 10, 2007

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Masters of Science

Your file Votre référence
ISBN: 978-0-494-32839-2
Our file Notre référence
ISBN: 978-0-494-32839-2

ACKNOWLEDGMENTS

I would like to thank Dr. John Hanrahan for his supervision and guidance. I would also like to express my gratitude to Dr. Hanrahan for bringing me to the North American Cystic Fibrosis Conference in Baltimore. I would like to give a special thanks to Yishan Luo, Jie Liao and Alex Evagelidis for their collaboration on this project, Robert Renaud graciously provided a French translation of the abstract for this thesis. Furthermore, I have to thank Dr. Michel Tremblay and Isabelle Aubry, who provided me kindly help in phosphatase assay. Finally, I would like to thank all the members of the Hanrahan Lab, for their help and cooperation during my time in the lab.

ABSTRACT

Activity of the cystic fibrosis transmembrane conductance regulator (CFTR) is tightly regulated by cAMP-dependent phosphorylation, but the protein phosphatases (PP) that dephosphorylate and inactivate CFTR remain poorly understood. Differential regulation of single CFTR channels by protein phosphatase 2C (PP2C), protein phosphatase 2A (PP2A) and other phosphatases has been reported. The results suggest multiple protein phosphatases are required for complete CFTR deactivation, and that PP2C may be the primary phosphatase regulating CFTR in human airway and colonic epithelia, two major sites of disease.

The goal of this project was to identify PP2C isoforms that regulate CFTR. Six isoforms of PP2C reported previously in mouse were recently recloned in this laboratory with epitope tags, and their interaction with CFTR was assessed by co-immunoprecipitation. Preliminary results indicated that MB2.1, one of the PP2Cβ isoforms, and MA3, one of the PP2Cα isoforms, interact with CFTR. To overexpress these phosphatases, I developed two inducible systems in which MB2.1 or MA3 gene expression is controlled by a promoter that is activated upon treatment of cells with ponasterone A, an ecdysone analogue. Although

specific PP2C inhibitors are not presently available, mutagenesis and enzyme assays have identified residues that are essential for its enzymatic activity. I made PP2Cα and PP2Cβ mutants which are predicted to act as dominant negative inhibitors, and have stably transfected them using the inducible gene expression system. Furthermore, I showed that MB2.1 and MA3 are functionally expressed in the inducible expression system and that site-directed mutagenesis inactivated their phosphatase activity as assayed using phosphatase *p*-nitrophenyl phosphate (*p*NPP) as a substrate. Baby Hamster Kidney (BHK) cells stably expressing CFTR + inducible MB2.1, MA3 or their dominant negative mutants, are presently being used in iodide efflux assays to study the downregulation of CFTR by MB2.1 and MA3. Based on results, we exclude PP2C β regulating CFTR. However, if PP2C α is the phosphatase which dephosphorylates CFTR can not be tested because of iodide loading problem.

ABRÉGÉ

L'activité du canal chlorure CFTR (Cystic Fibrosis Transmembrane conductance Regulator) est finement régulée par les phosphorylations dépendantes de la voie de l'AMPc, mais les protéines phosphatases (PP) qui déphosphorylent et inactivent le CFTR restent mal comprises. Diverses régulations du canal CFTR par la protéine phosphatase 2C (PP2C), la protéine phosphatase 2A (PP2A) ainsi que par d'autres phosphatases ont été rapportées. Des résultats suggèrent que le recrutement de plusieurs protéines phosphatases est exigé pour une déphosphorylation complète du CFTR et que la PP2C pourrait être la principale phosphatase régulant le CFTR au niveau des voies aériennes humaine et de l'épithelium du colon, deux sites majeurs de la maladie.

Le but de ce projet est d'identifier les isoformes de PP2C qui régulent la protéine CFTR. Six isoformes de PP2C étudiées précédemment chez la souris ont été re-clonées récemment au laboratoire et leur interaction avec le CFTR a été évaluée par co-immunoprécipitation. Nos résultats préliminaires indiquent que le MB2.1, une des isoformes de PP2Cβ, ainsi que MA3, une des isoformes de PP2Cα, interagissent l'un et l'autre avec le CFTR. Pour sur-exprimer ces

phosphatases, j'ai développé deux systèmes inductibles dans lesquels l'expression des gènes MB2.1 ou MA3 est contrôlée par un promoteur qui est activé par traitement des cellules avec la ponasterone A, un analogue de l'ecdysone. Aucun inhibiteur spécifique de la PP2C n'est actuellement disponible, bien que des expériences de mutagenèse et des analyses enzymatiques aient identifié des résidus essentiels pour son activité enzymatique. J'ai construit les mutants de PP2Cα et PP2Cβ présentés comme agissant en tant qu'inhibiteur dominant négatif et j'ai réalisé une transfection stable de ces mutants en utilisant un système inductible d'expression de gènes. En outre, j'ai montré que les isoformes MB2.1 et MA3 sont fonctionnellement exprimées en système inductible d'expression et que la mutagenèse dirigée sur un site spécifique inactive leur activité de phosphatase comme analysé en utilisant la phosphatase *p*-nitrophenyl phosphate (*p*NPP) comme substrat. Les cellules BHK (Baby Hamster Kidney) exprimant de manière stable le CFTR + le MB2.1 inductible, MA3 inductible ou leurs mutants dominants négatifs, sont présentement utilisées en efflux d'iodure pour étudier la perte de régulation du CFTR par MB2.1 et MA3. Ces expériences devraient fournir de nouvelles perspectives dans la régulation du CFTR et pourraient valider une cible thérapeutique pour le traitement de la fibrose kystique.

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Chapter 1 Introduction

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive lethal disorder that affects approximately 1/2500 births among Caucasian populations (Collins et al., 1990). Approximately 1/20 Caucasians are carriers of the defective gene. The mutation leads to dense mucous secretions of epithelial cells in the intestine, respiratory system, pancreas, gall bladder and sweat glands. Poor airway clearance combined with excessive mucus production and chronic infections cause obstructive lung disease and lead to respiratory failure that is the major cause of mortality in CF. Another serious consequence ensues in the digestion system, where defective anion secretion leads to a failure of the pancreas to produce bicarbonate-rich fluid, which causes plugging of the pancreatic ducts with digestive enzymes and mucus and severe malabsorption. Most CF patients fail to produce pancreatic enzymes and are classified as pancreatic insufficient (PI). In sweat glands, failure to reabsorb NaCl results in greatly elevated salt concentration in the sweat and this is widely used as a diagnostic test for the disease. In the reproductive system, CF disrupts the normal secretion of ion and water by the epididymal epithelium that provides an

optimal fluid environment for sperm maturation and transport, causing infertility in most adult male CF patients. Finally, meconium ileus (MI), an obstruction of the distal intestine caused by accumulation of thickened meconium, occurs in 10–20% of CF newborns.

CF follows simple autosomal recessive transmission, and homozygotes express this syndrome fully. Heterozygotes have no recognizable clinical symptoms. The clinical course of the disease varies widely (Bienvenu, 1997): some patients die very young because of meconium ileus or respiratory complications, while others have a mild form of the disease and live into their forties.

CF is due to the mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is located on chromosome 7q31.2 and is approximately 190 kb of genomic DNA (Ellsworth et al., 2000; Riordan et al., 1989). More than 1,400 mutations have been described in CFTR (Cystic Fibrosis Genetic Analysis Consortium. Cystic Fibrosis Mutation Data Base). The types of mutations have been arbitrarily grouped into six classes by Vankeerberghen (Vankeerberghen et al., 2002): class one, full-length CFTR is not produced; class two, defective processing; class three, defective regulation; class four, defective conductance; class five, partly defective production or

processing; and class six, defective regulation of other channels. However, the relationship between different genotypes and clinical phenotypes is still unclear.

1.2 Structure and function of CFTR

CFTR is a member of the largest transporter gene family -- the ATP-binding cassette (ABC) superfamily. ABC proteins transport a wide variety of substrates across the lipid bilayer including sugars, amino acids, metal ions, peptides and proteins, and a large number of hydrophobic compounds and metabolites. This transport is an essential function in all living cells, and not surprisingly, mutations in these genes cause or contribute to several human genetic disorders including cystic fibrosis, neurological disease and the drug resistance of cancers (Higgins, 2001).

After identification of the primary amino acid sequence of CFTR, Riordan et al. proposed a structure that was based on comparing CFTR with other members of ABC transporter family (Riordan et al., 1989). In this model, CFTR is composed of two motifs, each containing a membrane spanning domain (MSD) consisting of six transmembrane helices and connected to two intracellular nucleotide binding domains (NBD1 and NBD2). However, unlike other ABC transporters, CFTR also has a large cytosolic regulatory (R) domain that contains multiple

phosphorylation sites and many charged amino acids. This R domain links the two MSD-NBD halves of CFTR. In general the pore region of the channel is made up of the interaction between the two membrane spanning domains, and the opening of this channel is stimulated by phosphorylation of its R domain and ATP binding and/or hydrolysis at the NBDs. In the proposed transmembrane topology, which is supported by experimental evidence, 77% of the protein is in the cytoplasm including N-terminal and C-terminal tails, NBDs, R. domain and intracellular loops, 19 % in membrane-spanning segments, and 4% in extracellular loops, which (except for the M1-M2 and M7-M8 loops) are very short. M7-M8 extracellular loop contains two N-linked glycosylation sites (Denning et al., 1992; Chang et al., 1994; Chen and Zhang, 1996).

The N-terminal tail of CFTR consists of residues 1 to 80 present on the cytoplasmic side of the plasma membrane (Riordan *et al.*, 1989). Structurally, little is known about the N-terminus of CFTR, except that residues 46 to 63 form an α-helix (Naren et al., 1999). Sequences within the N-terminal tail can directly bind to syntaxin 1A, a member of the syntaxin family of membrane fusion regulators that is expressed highly in neurons and to a lesser extent in intestinal epithelial cells. CFTR-mediated chloride currents are inhibited by this direct protein-protein

interaction (Naren et al., 1998). Residues 46 to 63 in the N-terminal tail, which are predicted to form an α helix on the basis of secondary structure features and circular dichroism spectral analysis, can modulate channel activity by interacting with the R domain (Naren et al., 1999). Mutations in the N-terminal tail compromised biogenesis and enhanced endocytosis compared with wild-type CFTR (Jurkuvenaite et al., 2006).

The MSDs are made up of twelve transmembrane helices which are assumed to be α-helix or β-sheet conformation. A focus of research on MSDs has been on the identification of residues that line the pore and that influence conduction. Mutation of specific residues in M1, M5, M6, and M12 altered conductance, permeation, and open-channel block, which suggests that these transmembrane segments may line the CFTR pore (Akabas et al., 1994; Anderson et al., 1991; Cheung and Akabas, 1996; Mansoura et al., 1998; McDonough et al., 1994; Sheppard et al., 1993; Tabcharani et al., 1993). In addition, when reconstituted into planar lipid bilayers, peptides with sequences representing M2 and M6 formed a CFTR-like pore. This result suggests that M2 may also contribute to the pore (Oblatt-Montal et al., 1994).

Two NBDs of CFTR contain a number of highly conserved sequences that were predicted to bind and hydrolyze intracellular MgATP.

These are Walker A (GXXGXGKT/S) and B (R/KX_7hhhhD) motifs (X = any amino acid, h = hydrophobic residue), as well as a short signature sequence (LSGGQ) (Sheppard and Welsh, 1999). The Walker A lysine interacts with either the α or γ-phosphate of ATP and is essential for ATP hydrolysis, the Walker B aspartate coordinate Mg^{2+} in MgATP and is required for ATP binding. The LSGGQ motif plays a key role in GTP hydrolysis (Sheppard and Welsh, 1999). The mechanism by which ATP controls channel opening and closing through the NBDs is only partially understood. The fact that the overall amino acid homology between the two NBDs of CFTR is only 29% initially suggested that the two NBDs may have distinct functions. Based on the further structural and functional studies, a model was suggested (Gadsby and Nairn, 1999; Sheppard and Welsh, 1999; Gadsby and Nairn, 1999). In this model, ATP hydrolysis at NBD1 initiates a burst of channel activity, whereas ATP hydrolysis at NBD2 terminates it. However, more recent findings suggest that ATP hydrolysis is not strictly required for opening or closing the CFTR channel. For example, mutating the conserved Walker A lysine in NBD1 reduced ATPase activity by roughly 50% but had no effect on channel activity (Ramjeesingh et al., 1999). It is now generally accepted that the NBDs form a dimer when nucleotides bind (Gadsby and Nairn, 1999), and that

MgATP binds stably at NBD1 and turns over rapidly at NBD2 (Aleksandrov et al., 2000) although recent data indicate that NBD2 functions predominantly as an adenylate kinase rather than an ATPase (Gross et al., 2006).

When the CFTR cDNA was cloned, the R domain was defined as the region (aa 590-830) encoded by exon 13 (Riordan et al., 1989). More recent studies suggest that the N-terminal R domain boundary begins between residues 622 and 634, and that the C-terminal boundary ends in the region of residue 835 (Chan et al., 2000). The R domain has little well-ordered secondary structure, with only 5% helical content, and the rest random coil (Ostedgaard et al., 2000). Phosphorylation and dephosphorylation of the R-domain is the main mechanism by which CFTR chloride channel activity is controlled, and will be discussed in more detail later in this chapter.

The C-terminus of CFTR (C-tail) begins after NBD2 at approximately residue 1424 and extends to residue 1480. The C-tail is not essential for channel function but does contain motifs that are important for the trafficking and localization of the molecule within the cell. One of the best characterized is the PDZ-domain binding motif, which is composed of the last three residues (TRL). CFTR interacts with several

different PDZ-domain-containing proteins and these interactions may play roles in apical localization (Milewski et al., 2001), trafficking through the Golgi apparatus (Cheng et al., 2002) and channel regulation (Raghuram et al., 2001; Wang et al., 2000), association with other proteins in a regulatory complex in proximity to protein kinase A (Huang et al., 2001). Interaction with PDZ domain proteins greatly restricts the lateral diffusion of CFTR in the plasma membrane (Bates et al., 2006).

1.3 CFTR biogenesis and degradation

Biogenesis of CFTR begins with targeting of the nascent chain-ribosome complexes to the ER followed by translocation and integration of transmembrane domains into the lipid bilayer (Kopito, 1999). Rescent research suggested that on the ER, TMD1 is firstly translated and integrated, which is followed by the translation and folding of NBD1. Folded NBD1 docks with the integrated TMD, and NBD and TMD are both stabilized by this interaction allowing the subsequent translation, folding and assembly of TMD2 and NBD2 (Sheppard et al., 1996; Gregory et al., 1991; Pollet et al., 2000; Thibodeau et al., 2005; Du et al., 2005). After correct folding and addition of N-linked "core" glycosylation (i.e. oligosaccharides containing mannose) in the ER, CFTR is exported in COPII (coatomer protein)-coated vesicles that later fuse to ER/Golgi

intermediate compartment (ERGIC) (Aridor et al., 1995; Schekman and Orci, 1996). Within the ERGIC, CFTR is incorporated into anterograde COPI vesicles for transport to the Golgi in which CFTR glycosylation is further processed to "complex" glycosylated oligosaccharides by Golgi-associated mannosidases and glycosyl transferases, leading to the addition of polyactosamines such as sialic acid and galactose (Orci et al., 1997; O'Riordan et al., 2000). Correctly folded CFTR is then transported to the cell membrane or recycled back to the ER in COPI vesicles (Orci et al., 1997).

Normally folded CFTR is exported from the ER in a COPII-dependent fashion (Yoo et al., 2002). Several lines of evidence suggest that misfolded CFTR polypeptide chains are rapidly degraded by the ubiquitin-proteasome pathway (Ward et al., 1995) Proteasomal degradation of misfolded proteins requires polyubiquitination via the ubiquitin conjugating enzymes (E2s) and ubiquitin protein ligases (E3s). The latter class of enzymes is thought to provide the specificity of the degradation process. It has been shown that in mammalian cells, CHIP (carboxyl terminus of the Hsc70-interacting protein) interacts with Hsc70 and functions as an E3 enzyme and triggers the partial degradation of CFTR (Meacham et al., 2001). The CHIP ubiquitin ligase is tightly

embedded in the cellular co-chaperone network that modulates Hsc70activity. CHIP was found to cooperate with the Hsc70 co-chaperone BAG-1 during proteasomal sorting of the glucocorticoid hormone receptor (Demand et al., 2001). Recent studies have found that the cochaperone HspBP1 attenuates the ubiquitin ligase activity of CHIP in the presence Hsc70. As a consequence, HspBP1 interferes with CHIP-induced degradation of the immature forms of CFTR and stimulates CFTR maturation (Alberti et al., 2004). Based on these results a CHIP modulation mechanism was suggested (Alberti et al., 2004) in which Hsp70 cochaperones BAG-1 and HspBP1 interact with CHIP and have opposing actions: BAG-1 stimulating CHIP-induced degradation and HspBP1 inhibiting the ubiquitin ligase activity of CHIP.

1.4 CFTR channel activity is tightly regulated by phosphorylation

1.4.1 Protein Serine/Threonine phosphatases

In eukaryotic cells, two major families of protein phosphatases are protein serine/threonine and protein tyrosine phosphatases. The tyrosine phosphatases, a family of intracellular and receptor-like enzymes, are important in signaling pathways that involve tyrosine kinase-linked oncogenes and growth factor receptors. In general, protein serine/threonine phosphatases are specific for dephosphorylation of

serine and threonine residues although low level of tyrosine phosphatase activity has also been reported ((Goris et al., 1988; Chen et al., 1989).

Mammalian serine/threonine phosphatases were classified as type 1 (PP1) or type 2 (PP2A, PP2B, PP2C) based on biochemical differences in the activity and regulation of individual enzymes (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983). The criteria for this classification system is the relative activity toward the α- and β-subunits of phosphorylase kinase and sensitivity to the thermostable protein Inhibitor 1 and Inhibitor 2. PP1 phosphatases are defined as those that preferentially dephosphorylate β-subunit of phosphorylase kinase and are sensitive to both Inhibitor 1 and Inhibitor two. PP2 phosphatases preferentially dephosphorylate the α-subunit of phosphorylase kinase and are insensitive to either inhibitor. PP2 enzymes are further divided based on their structural and regulatory properties: type 2A phosphatase (PP2A) is a class of oligomeric enzymes with no obvious requirements for ions or cofactors; type 2B phosphatase (PP2B), also known as calcineurin, requires Ca^{2+}/calmodulin; type 2C phosphatase (PP2C) is dependent on relatively high (mM) concentrations of Mg^{2+}. In a more recent system, protein phosphatases are defined into three structurally-distinct gene families; PPP, PPM and PTPs. The PPP and PPM families

dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acid. Accordingly, PP1, PP2A and PP2B along with several newly-cloned phosphatases (i.e., PP4, PP5, PP6, and PP7) belong to the PPP family, and PP2C with its orthologs such as ABI1 in Arabidopsis and PTC1 in S. cerevisae are placed in the PPM family (Barford et al., 1998).

PP1 consists of multimeric structures which are composed of a catalytic subunit (PPc) complexed to regulatory subunits that are different in distinct PP1 holoenzymes. Cloning revealed that the catalytic subunits of PP1 can be differentiated into α (330 amino acids), δ (327 amino acids), and γ (actually two splicing variants: γ_1 323 amino acids and γ_2 337 amino acids) forms which are derived from three different genes (Sasaki et al., 1990). In general, the PP1 catalytic subunit specific binds to a highly conserved sequence (RVxF) on a regulatory subunit, which associates with different targets in various cellular locations (Cohen, 2002). Several regulatory subunits (also called regulatory proteins) are known, which include I_1, I_2, DARPP-32, G_L, R_{GL}, and so on. The regulatory subunit can change (that is, inhibit or augment) the activity, substrate selectivity, or subcellular localization of the catalytic subunit. PP1 has diverse functions

including glycogen metabolism, muscle contraction, cell cycle progression, neuronal activities, and the splicing of RNA.

PP2A consists of "A" (structural) and "C" (catalytic) subunits, which are constitutively expressed (Mayer-Jaekel et al., 1992) and a "B" (regulatory) subunit, which is expressed differentially (Mayer et al., 1991), interacts with AC complexes, and activates PP2A activity by conferring distinct enzymatic properties to the complexes. The cellular localization and substrate specificity of PP2A is determined by the unique combination of A and B subunit. PP2A catalytic subunits exist in two isoforms called Cα (309 amino acids) and Cβ (309 amino acids, different from PP2Aα in 8 amino acids). The expression of Cα is higher than Cβ at both the protein and mRNA level (Kitagawa et al., 1988; Kitagawa et al., 1988b). The A structural subunit is encoded by one of two genes α and β, which are 86% identical. The B regulatory subunit is distinguished into the highly divergent B, B′, B″, and B‴ families. All three subunits have multiple isoforms and splice variants and thus constitute at least 75 distinct holoenzymes. The specific roles of these holoenzmes remain unclear, although PP2A regulates diverse processes that include metabolism, cell signaling, the cell cycle, and the control of telomerase. PP1 and PP2A are specifically and potently inhibited by okadaic Acid, microcystin, calyculinA

and canthardic acid, toxins that are widely used to identify phosphatases mediating dephosphorylation by PP1 and PP2A (MacKintosh, 1994).

PP2B, also called calcineurin, consists of an "A" (catalytic) subunit, which has an N-terminal catalytic domain and a C-terminal regulatory region containing binding sites for "B" (regulatory) subunits and calmodulin. The extreme C-terminus of the A subunit also has an autoinhibitory sequence. In the presence of physiological concentration of Ca^{2+}, camodulin forms a complex with "B" subunits and induces a conformational change of the "B" subunit which results in stimulation of the A subunit phosphatase activity. There are three mammalian genes for the A subunit termed Aα, Aβ and Aγ. Differential splicing of Aα generates two transcripts (α1 and α2). The Aβ gene is alternatively spliced to three transcripts β1, β2 and β3 (Ueki et al., 1992). Two different B subunits genes are known called Bα (two splicing variants Bα1 and Bα2) and Bβ. (Ueki et al., 1992; Chang et al., 1994). PP2B is involved in Ca^{2+}-dependent signalling pathways and regulates events at neuronal postsynaptic densities, microtubules of cytoskeleton, and signal transduction in T cells. Deltamethrin, cyclosporin A, FK506 and fenvalerate are commonly used PP2B inhibitors.

PP2C is a monomeric, divalent cation-dependent (Mg^{2+}=Mn^{2+}>Co^{2+}) protein phosphatase of ~ 49 kDa (Binstock and Li, 1979). The crystal structure of mammalian PP2Cα revealed two domains: an N-terminal catalytic domain (residues 1-290) with six α-helices and eleven β-strands, and a 90-residue C-terminal domain of three α-helices. The catalytic domain is composed of a central β-sandwich that binds two manganese ions and is surrounded by α-helices (Das et al., 1996). The catalytic domain of PP2C contains the signature sequence of the PPM gene family ([LIVMFY]-[LIVMFYA]-[GSAC]-[LIVM]-[FYC]-D-G-H-[GAV]), which helps coordinate metal ions in the active site. The N-terminal catalytic domain is well conserved among isoforms, whereas the C-terminal domain is highly variant among isoforms and is thought to be the targeting part of the enzyme. Two isoforms were first identified in rabbit skeletal muscle and rabbit liver, and amino acid sequencing of demonstrated that they were the products of distinct genes termed PP2Cα and PP2Cβ (McGowan et al., 1987; McGowan and Cohen, 1987). There are several splicing variants of PP2Cα including the original α1, and the much shorter α2 and α3 variants, which are broadly expressed in liver, skeletal muscle, kidney and cerebellum (Tamura et al., 1989; Takekawa et al., 1998). PP2Cβ has at least six splicing variants differing at their C

termini called β1, β2, β3, β4, β5 and β6. PP2Cβ1 and PP2Cβ2 were first cloned from mouse melanocyte cDNA. Although the mRNA of PP2Cβ 1 is expressed ubiquitously in various tissues, that of PP2Cβ2 is expressed exclusively in brain and heart (Terasawa et al., 1993). Later PP2Cβ3, PP2Cβ4 and PP2Cβ5 were also cloned from mouse melanocyte cDNA. Finally, PP2Cβ6 was cloned from a human liver cDNA library which codes for a protein homologous to other mammalian PP2Cβs at the N-terminus, but which has an extended C-terminus that is unique amongst the PP2Cs. Northern blot showed that PP2Cβ6 is widely expressed and most abundant in heart and skeletal muscle (Marley et al., 1998). Although studies on the functions of PP2C have suggested that PP2C is involved in the regulation of AMP-activated protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, and stress-activated kinase pathways, the details of PP2Cs activity are still unclear. Variable tissue expression of the different isoforms suggests a difference in their physiological roles. For example, PP2Cβ2 can down-regulate the MAP kinase kinase kinase (MKKK) TAK1, whereas the downstream kinases MKK and p38 MAPK are down-regulated by PP2Cβ1 (Tamura et al., 2002; Takekawa et al., 1998; Hanada et al., 2001). A major problem in determining the functions of PP2C has been the lack of specific PP2C inhibitors.

1.4.2 Phosphorylation of CFTR

Opening and closing of the CFTR channel is controlled by ATP hydrolysis (Gadsby and Nairn, 1999). However, CFTR channels will not open even in the presence of millimolar MgATP unless the R-domain is phosphorylated (Anderson et al., 1991; Nagel et al., 1992; Tabcharani et al., 1991). It is well accepted that PKA is the main kinase regulating CFTR, although PKC enhances PKA dependent activation and other kinases may also be involved.

1.4.2.1 PKA

The most striking feature of the R domain is the presence of multiple consensus PKA phosphorylation sites that are highly conserved from fish to humans (Dahan et al., 2001). *In vitro,* PKA phosphorylates Ser-660, Ser-700, Ser-712, Ser-737, Ser-753, Ser-768, Ser-795, and Ser-813 (Cheng et al., 1991; Picciotto et al., 1992). In contrast, when CFTR is activated by cAMP in cells, phosphorylation is limited to Ser-660, Ser-700, Ser-737, Ser-795, and Ser-813, which results in PKA dependent stimulation (Cheng et al., 1991; Picciotto et al., 1992; Cohn et al., 1992). Although CFTR channel regulation involves phosphorylation of multiple serines, mutation of the major sites reduces but does not abolish phosphorylation-stimulated activity, and no single serine is required for

stimulation. CFTR still can be activated by PKA after mutegenesis of all the strong PKA phosphorylation sites (Chang et al., 1993; Seibert et al., 1995). The reason for the numerous PKA phosphorylation sites is unknown. More recent research has further proven that the phosphorylation sites in the R domain have both stimulatory and inhibitory effects (Vais et al., 2004).

The mechanism by which PKA phosphorylation regulates CFTR channel activity is still unclear. R domain is thought to inhibit CFTR when it is unphosphorylated. In support of this, overexpression of R domain inhibited CFTR activity in 9/HTEo cells (Perez et al., 1996); addition of unphosphorylated R-domain to the cytoplasmic side of the channel in planar bilayers inhibited channel activity; and expression of ΔR split CFTR channels induced constitutive channel activity that could be abolished by coexpression of R domain (Chappe et al., 2005), although the role of the R domain remains controversial (Csanady et al., 2000; King and Sorscher, 2000; Baldursson et al., 2001; Winter and Welsh, 1997; Ma et al., 1997; Ostedgaard et al., 2000b). R domain still works when attached at C terminus. PKA phosphorylation of R domain is believed to relieve the effect of this inhibition. The R-domain undergoes significant secondary structure changes when it is phosphorylated by PKA (Dulhanty and

Riordan, 1994). Moreover ATR-FTIR spectroscopy suggests that R domain phosphorylation can induce a large conformational change of CFTR that could correspond either to a displacement of the R domain or long range conformational changes e.g. those transmitted from the phosphorylation sites to the nucleotide binding domains and the transmembrane segments (Grimard et al., 2004). Recent studies indicate that phosphorylation of the R domain enhances interaction of the R domain with other regions of CFTR, perhaps in the N-terminal half (Chappe et al., 2005).

It has been proposed that R domain phosphorylation increases the affinity of CFTR NBDs for ATP. Experimentally, PKA increased the activity of wild-type channels more at low ATP concentrations than at high concentrations (Winter and Welsh, 1997), and there was an inverse correlation between the amount of PKA phosphorylation (i.e. the number of sites phosphorylated) and the ATP concentrations required for channel activation (Mathews et al., 1998). In the summary, the basic mechanism of phosphorylation regulation by PKA remains unsolved; however most data suggest that the R domain inhibits channel activity when unphosphorylated, and that PKA relieved this inhibition by a mechanism in

which the R domain interacts more tightly with other domains, perhaps enhancing ATP affinity.

1.4.2.2 PKC

The putative PKC phosphorylation sites on CFTR include T582, T604, S641, T682, S686, S707, S790, T791, and S809, which are between the Walker B consensus in NBD1 and TMD7 of the second transmembrane domain (Chappe et al., 2003). PKC alone induces a slight activation of CFTR, a small percentage (<20%) of that observed during PKA-dependent stimulation (Berger et al., 1993; Tabcharani et al., 1991). This small affect is consistent with the finding that PKC phosphorylation did not change R domain conformation (Dulhanty and Riordan, 1994). PKC phosphorylation is thought to increase CFTR activation by PKA (Jia et al., 1997). One possible mechanism for this is by direct phosphorylation of CFTR. Direct evidence for this comes from the effect of mutating serines and threonines at the nine PKC consensus sequences on the R domain. These mutations weaken CFTR activation by PKA in excised patches and slow stimulation of iodide efflux from intact cells (Dulhanty and Riordan, 1994; Chappe et al., 2003). An alternative mechanism is through PKC phosphorylation of ancillary proteins. The binding and cross-linking of two C-terminal tails of CFTR by two PSD-

95/Dlg/ZO-1(PDZ) domains of Na^+/H^+ exchanger regulatory factor (NHERF, also known as EBP50) enhances CFTR gating (Raghuram et al., 2001). PKC phosphorylates Ser-162 within the PDZ domain of NHERF which disrupts the interaction between CFTR and NHERF. Consequently, phosphorylated NHERF is unable to stimulate CFTR (Raghuram et al., 2003)

1.4.2.3 Other kinases

Although PKA and PKC are the major kinases regulating CFTR, other kinases can also stimulate channel activity. In excised patches, $p60^{c\text{-}src}$ phosphorylated and stimulated CFTR. Moreover, coexpression of CFTR and $p60^{c\text{-}src}$ induced constitutive channel activity (Fischer and Machen, 1996). Elevation of CFTR activity in lymphocytes during the G1 phase of the cell cycle suggests involvement of cell cycle-dependent kinases (Mohler et al., 1999).

1.4.3 Dephosphrylation of CFTR

CFTR is a non-rectifying, low-conductance chloride channel on the apical side of epithelia and other cells. To maintain low resting chloride conductance and ensure that Cl^- secretion is only increased by appropriate stimuli and is readily reversible, CFTR must be efficiently dephosphorylated in vivo.

Early studies of channel activity in cell-attached patches showed that CFTR channels were activated within 40s when forskolin and IBMX were added to increase intracellular levels of cAMP. When agonists were removed, current returned to basal values within 100 s, and currents could be reversibly activated by repeated addition of agonist. In cell-free patches, CFTR was activated by PKA catalytic subunit and this activation was reversible on repeated addition of agonist (Berger et al., 1991). Similar rundown and reversible activation were observed when patches were excised from an airway epithelial cell line expressing endogenous CFTR (Haws et al., 1994). The rundown of CFTR is probably not due to an irreversible loss of agents or enzymes critical for channel function, because activity was readily restored by phosphorylation. Thus CFTR must be dephophorylated by a phosphatase such that the balance between opposing kinase and phosphatase activity determines the level of channel activity.

1.4.3.1 Alkaline phosphatase

Alkaline phosphatase was an early candidate for the phosphatase that dephosphorylates CFTR in excised patches because when cell-free patches performed on Chinese hamster ovary (CHO) stably expressing CFTR, exogenous alkaline phosphatase decreased channel open

probability (P_o) by more than 90 % within 2 minutes in the continued presence of PKA (Tabcharani et al., 1991). Furthermore, (-)-*p*-bromotetramisole (Br-t) a well known inhibitor of liver, bone, and kidney (LBK) alkaline phosphatases, slowed the rundown of CFTR channels in excised membrane patches and reduced the dephosphorylation of CFTR protein in isolated membranes. In unstimulated cells, CFTR channels were activated by exposure to phosphatase inhibitors alone (Becq et al., 1994). However, it was subsequently found that rundown and dephosphorylation were both inhibited by alkaline inhibitor phenylimidazothiazoles such as (-)-*p*-Br-t (Becq et al., 1994; Becq et al., 1996), only at much higher concentrations than needed to inhibit alkaline phosphatases. Also, the stereoisomer (+)-*p*-Br-t, which is widely used as an inactive analog of (-)-*p*-Br-t and does not inhibit alkaline phosphatases, also slowed CFTR channel rundown (Luo et al., 2000). Furthermore, both enantiomers of Br-t inhibited PP2A and PP2C activity by >90% when radiolabeled phosphocasein was used as the substrate, and also inhibited dephosphorylation of radiolabeled GST-R domain fusion protein by PP2A and PP2C. Thus, it seems that the rundown of CFTR is not due to dephosphorylation by alkaline phosphatases which are specifically inhibited by (-)-*p*-Br-t.

1.4.3.2 Protein phosphatases

To help identify the protein phosphatases involved in regulating CFTR, the effects of exogenous phosphatases on single CFTR channels were tested. In patches that were excised from baby hamster kidney (BHK) cells overexpressing CFTR, channel activity was reduced by more than 90% after adding PP2A catalytic subunit from smooth muscle or PP2Cα.from turkey gizzard. PP2B from bovine brain caused weak deactivation whereas recombinant human PP1 had no detectable effect. Furthermore, PP2A and PP2C had distinct effects on channel gating: PP2A caused a dramatic shortening of burst duration similar to that reported in patches from cardiac cells during deactivation of CFTR by endogenous phophatases; P_O declined during exposure to exogenous PP2C without any change in mean burst duration. Interestingly, even after prolonged exposure to exogenous PP2A or PP2C, CFTR activity was still not abolished completely whereas spontaneous rundown was usually complete (Luo et al., 1998). These results indicated that multiple protein phosphatases may be required for complete deactivation of CFTR, and that PP2A and PP2C are two potent regulators of CFTR activity.

Early evidence of CFTR deactivation by PP2A came from studies with okadaic acid, a PP1 and PP2A inhibitor. Okadaic acid inhibited spontaneous deactivation of CFTR currents in permeabilized sweat ducts, and ~40% of the deactivation after forskolin washout was blockable by okadaic acid or microcystin in guinea pig cardiac myocytes (Hwang et al., 1993; Reddy and Quinton, 1996). More recently, a regulatory subunit of the PP2A *PR65* was identified as an interacting partner of the CFTR R domain (AA 672-855) in yeast two hybrid (Vastiau et al., 2005; Thelin et al., 2005). Physical interaction of *PR65* and R domain was also demonstrated by pull-down and co-immunoprecipitation experiments. In order to determine the functional role of PP2A-dependent dephophorylation of CFTR, a GFP vector expressing PR1-10, the domain in PR65 that binds to CFTR but can not associate with catalytic and structure subunits, was transfected into Caco-2 cells. Overexpression of PR1-10 significantly delayed deactivation of cAMP-activated Cl^- currents through CFTR channels in whole-cell current measurements (Vastiau et al., 2005; Thelin et al., 2005). In Cassiman's lab, using nanoliquid chromatography MS/MS, PP2A was found physically associating with the COOH terminus of CFTR. Furthermore, these PP2A subunits localized to the apical surface of airway epithelia consistent with the localization of

CFTR in these tissues, and PP2A co-precipitated with endogenous CFTR from Calu-3 cell membrane. Further evidence came from functional assays performed on airway epithelia expressing endogenous CFTR: inhibitors of PP2A slowed rundown of basal CFTR currents, albeit modestly, and increased channel activity in excised patches of airway epithelia. PP2A inhibition in well differentiated human bronchiaepithelial cells also caused a CFTR –dependent increase in the airway surface liquid (Thelin et al., 2005).

Specific inhibitors of PP2C are not presently available. Early speculation that PP2C deactivates CFTR came from the fact that channel rundown is inhibited slightly or not all by okadaic acid in excised patches (Becq et al., 1994; Tabcharani et al., 1991). Those results did not exclude regulation by PP2A on the cell, since it is a soluble enzyme and would be lost when deactivation is studied under these conditions. Perhaps the most compelling evidence against PP2A playing a major role came from the fact that okadaic acid and calyculin A had little effect on the run-down of CFTR after washout of cpt-cAMP in intact human airway epithelia and T84 human colonic epithelia. Furthermore, in polarized epithelial monolayers stably expressing CFTR, Cl^- current was activated by cpt-cAMP, inhibited by removing the cAMP agonists and the deactivation of

CFTR was not prevented or slowed by adding okadaic acid. However, coexpression of PP2C with CFTR results in lower total Cl^- current (Travis et al., 1997). Interaction of CFTR with PP2C was eventually tested directly using co-immunoprecipitation and crosslinking approaches (Zhu et al., 1999). CFTR was immunoprecipitated from BHK cells using the anti-CFTR monoclonal antibody M3A7, and the immunoprecipitated proteins were analyzed by Western blotting with antibodies specific for PP1, PP2A, PP2B and PP2C. PP2C was present in total cell lysates (BHKwt-t) and was pulled down by anti-CFTR antibody, and was conspicuous in the immunoprecipitate (BHKwt-IP). PP1, PP2A and PP2B were not detected in immunoprecipitated cells expressing CFTR (BHKwt-IP) or in control cells (BHK (-)-IP), but were easily observed in total cell lysates (BHKwt-t). In the converse experiment, CFTR was immunoprecipitated from solubilized BHK membranes by anti-PP2C antibody. To further test the interaction between CFTR and protein phosphatases they were cross-linked by the reagent DTSSP. Addition of DTSSP to lysed BHK cells expressing $CFTR_{His10}$ resulted in cross-linking of $CFTR_{His10}$ into high molecular weight complexes. To identify cross-linked proteins, CFTR and associated proteins were pulled down on Ni^{2+}-NTA-agarose from DTSSP-treated lysates and western blots were then probed with antibodies

specific for PP1, PP2A, PP2B and PP2C. The results showed that PP2C was the only phosphatase that was cross-linked with CFTR.

1.5 CFTR regulation by protein phosphatase 2C -- Outline and hypothesis

All previous studies suggested that PP2C is an important protein serine/threonine phosphatase which dephosphorylates CFTR but the molecular identity of the PP2C remains uncertain. Recently, six PP2C isoforms were recloned in this laboratory from mouse L cells as part of a "brute-force" approach to identifying the phosphatase. There were three reasons for using this cell line: 1) most reported PP2C isoform sequences are from mouse; 2) the levels of CFTR expression in transfected L cells are comparable to those in epithelial cells expressing endogenous CFTR and 3) deactivation of Cl^- current in L cells has properties similar to those in epithelia. The initial co-immunoprecipitation experiments suggested that MB2.1, one of the PP2Cβ isoforms, and MA3, one of the PP2Cα isoforms, may interact with CFTR.

Based on that unpublished work, I hypothesized that MA3 and/or MB2.1 might be the PP2C isoform that dephophorylates CFTR. To test that hypothesis these PP2C variants were coexpressed with CFTR and their functional effects on CFTR regulation were examined. PP2C overexpression is toxic to cells and it is not possible to generate cell lines

stably overexpressing PP2C, therefore I developed an inducible expression system for PP2C (BHK cell lines). In this system, MB2.1 or MA3 gene expression is controlled by a promoter that is activated upon treatment of cells with ponasterone A, an ecdysone analogue.

Previous mutagenesis and enzyme assays suggested that residues H62/D282 in MA3 and the equivalent amino acids in MB2.1 (H62/D284 are essential for the activity of PP2Cα1 activity (Jackson et al., 2003). To knock down PP2C activity specifically I mutagenized MA3 (H62Q and D282N) and MB2.1 (H62Q and H62Q, D286N), which are predicted to have a dominant negative effect, and stably expressed the mutants in the inducible gene expression systems.

Phosphatase assays (pNPP assay) proved that MA3 and MB2.1 were indeed functionally expressed in inducible systems and that site-directed mutagenesis inactivated both MA3 and MB2.1. Finally, co-localization of CFTR with the cloned phosphatases and the effect of MB2.1, MA3 and mutants on CFTR channel activity in the inducible systems were investigated in order to understand their functional roles.

Chapter 2 Materials and Methods

2.1 Materials

Chemicals were from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. The laboratory of J. Riordan (Mayo Foundation and SC Johnson Medical Research Center, Mayo Clinic, Scottsdale, AZ) generously provided the CFTR antibody 450 as well as the pNUT/WT CFTR plasmid. Mouse monoclonal antibody anti-hemagglutinin (HA) was purchased from Covance (Berkeley, CA) and rat monoclonal antibody anti-HA High Affinity was purchased from Roche Applied Science (Indianapolis, IN). Goat anti-mouse IgG secondary antibody labelled with horseradish peroxidase, Cy3-conjugated goat anti-rat second antibody and Cy5-conjugated goat anti-mouse secondary antibody were purchased from Jackson ImmunoResearch Lab. (West Grove, PA). The QuikChange® Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Fugene 6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). Dr. Annick Guyot provided a BHK/pVgRXR stable cell line stably expressing the ecdysone-activated receptor VgRXR. The plasmids pIND, Zeocin and ponasterone A were

purchased from Invitrogen (Burlington, ON, Canada). calyculin A was purchased from Alexis Biochemicals (San Diego, CA).

2.2 Molecular biology

Plasmid DNA was prepared from Top10 bacteria using mini or midi prep kits (Qiagen, Santa Clarita, CA). Transformations of plasmid DNA into competent Top10 cells (*E. coli*) used the heat-shock method and followed established protocols. Restriction enzyme digests and DNA ligations followed standard methods (Sambrook et al., 1989) or those described by the manufacturer (New England Biolabs). Agarose gel electrophoresis was performed using 0.8 % gels. DNA fragments were extracted from the agarose using the QIAEX II Extraction kit (Qiagen). The polymerase chain reaction (PCR) was carried out using a PTC-100 programmable thermal controller (MJ Research Inc., Las Vegas, NV. Primers were purchased from Gibco BRL (Burlington, Ontario, Canada). DNA sequencing was performed by the McGill University and Genome Quebec Innovation Centre.

2.3 Mutagenesis and construction

HA tagged PP2C isoforms were re-cloned from mouse L cells and inserted into pcDNA3 vector by Leticia Sanchez in this lab. There are no specific inhibitors of PP2C phosphatases that can be used to establish

their functional roles. As an alternative approach, we used site-directed mutagenesis to generate dominant negative mutant versions of MA3 and MB2.1. The crystal structure of human PP2Cα suggests that the target phosphate coordinates to the metal centers M1 and M2 through hydrogen-bonding interactions with water ligands, as well as electrostatic interaction with Arg-33. Asp-60 bridges the two metal ions, while Asp-239, Asp-282, and Gly-61 (backbone carbonyl) make direct contact with the metal centers. Although conserved residues His-40 and His-62 do not make contact with this coordination shell, they are within the active site pocket (Figure1) (Das et al., 1996). These residues have been mutated in the α1 isoform previously to investigate their role in catalysis and metal/substrate binding, and the kinetic properties of the mutants have been analyzed in detail (Jackson et al., 2003). D282N and H62Q mutants displayed low enzyme activity and normal substrate affinity when compared with wild-type PP2Cα. Based on those results, we generated two PP2Cα mutants (MA3-D282N and MA3-H62Q) as putative dominant negative mutants of MA3 in this project. Since catalytic domains of PP2Cα and PP2Cβ are well conserved, two mutants likely to function as dominant negative inhibitor of MB2.1 (MB2.1-D286N and MB2.1-D286N, H62Q) were also

generated based on sequence comparison of PP2Cα and PP2Cβ (Figure 2) (Wenk et al., 1992).

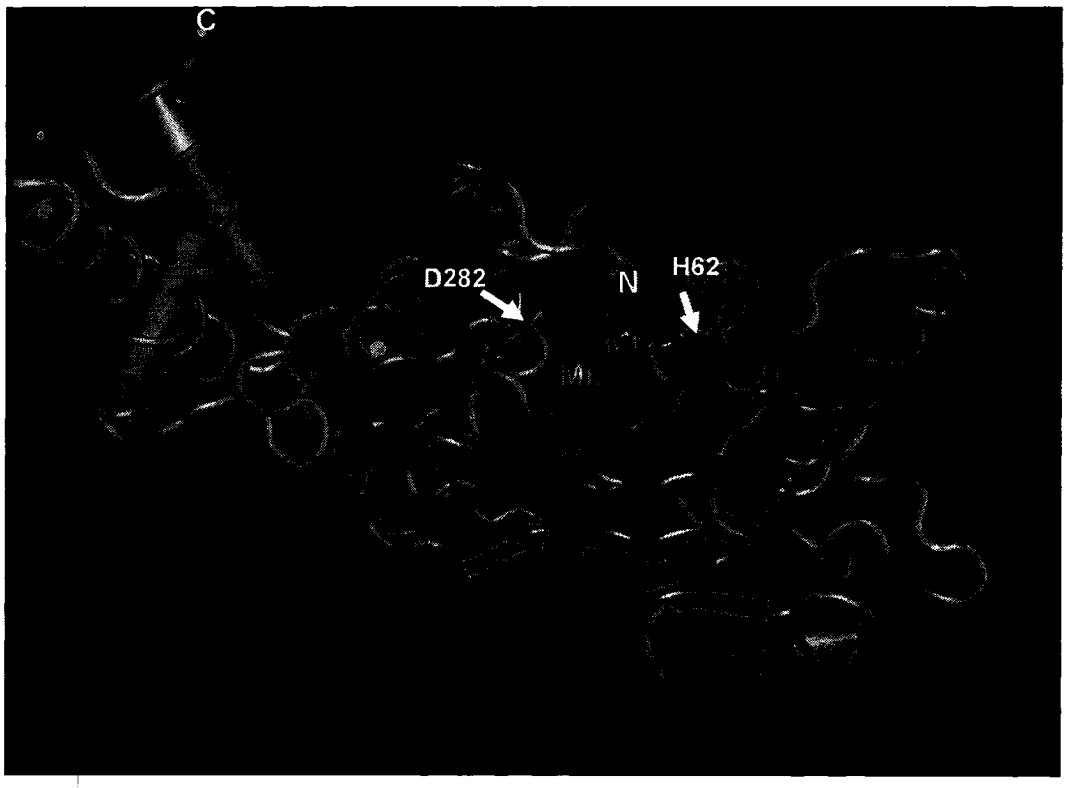
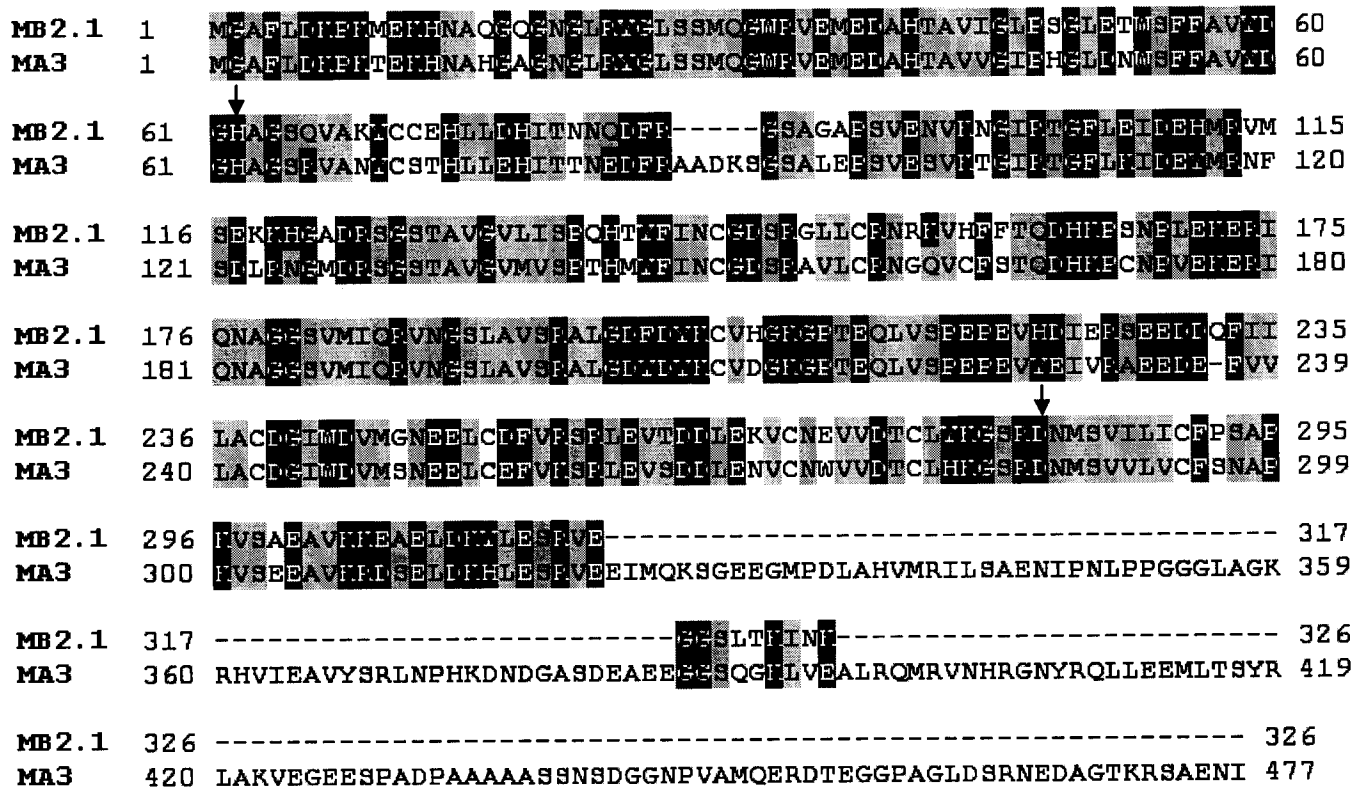


Figure 1 Published Crystal structure of human α 1 PP2C at 2.0 Å resolution

Mutated residues D282 and H62 (as white arrows), the two manganese ions (as gray spheres), and the phosphate ion are shown (Das et al., 1996).

Figure 2 Sequence comparisons between mouse PP2C1 (alpha) and mouse PP2C2 (beta)

The colour boxes mark regions of identity between the isoforms; the arrows show conservative amino acid exchanges. Polar positive amino acids (H, K, R) are blue, polar negative amino acids (D, E) are red, polar neutral amino acids (S,T N, Q) are green, non-polar aliphatic amino acids are grey, and non-polar aromiatic amino acids are magenta (F, W, Y), brown (P, G) and yellow (C).


MB2.1 1 MGAFLDRPTMEHHNAQSQGNGLPYGLSSMQGWPVEMEDAHTAVIGLPSGLETMSFFAVTI 60
MA3 1 MGAFLDRPTEHHNAHSAGNGLPYGLSSMQGWPVEMEDAHTAVVGIPHGLLNMSFFAVTI 60
MB2.1 61 GHAGSQVAKRCCEHLLDHITNNQLFP-----GSAGAPSVENVRNGIPTGFLEIDEHMRVM 115
MA3 61 GHAGSPVANTCSTHLLEHITTNEIFPAADKSGSALEPSVESVTTGIPTGFLHIDEHMRNF 120
MB2.1 116 SEKHHGADPSGSTAVGVLISPQHTAFINCGLSPGLLCPNRHVHFFTQDHMPSNPLEHERI 175
MA3 121 SLLPNGMDRSGSTAVGVMVSPTHMTFINCGLSPAVLCPNGQVCFSTQDHKPCNPVEHERI 180
MB2.1 176 QNAGGSVMIQRVNGSLAVSRALGDFDYKCVHGNGPTEQLVSPEPEVHDIEPSEEDQFII 235
MA3 181 QNAGGSVMIQRVNGSLAVSRALGDTDYKCVDGNGETEQLVSPEPEVTEIVPAEELE-FVV 239
MB2.1 236 LACDGIMLVMGNEELCDFVPSPLEVTDDLEKVCNEVVDTCLYNGSRDNMSVILICFPSAP 295
MA3 240 LACDGIMLVMSNEELCEFVTSPLEVSDDLENVCNWVVDTCLHRGSRDNMSVVLVCFSNAP 299
MB2.1 296 RVSAEAVHREAELDKYLESRVE---------- 317
MA3 300 RVSEEAVHRDSELDKHLESRVEEIMQKSGEEGMPDLAHVMRILSAENIPNLPPGGGLAGK 359
MB2.1 317 ----------GGSLTNING---------- 326
MA3 360 RHVIEAVYSRLNPHKDNDGASDEAEEGGSQGTLVEALRQMRVNHRGNYRQLLEEMLTSYR 419
MB2.1 326 ---------- 326
MA3 420 LAKVEGEESPADPAAAAASSNSDGGNPVAMQERDTEGGPAGLDSRNEDAGTKRSAENI 477

Individual point mutations H62Q and D282N were subsequently introduced into wild-type MA3 using the QuickChange (Stratagene) mutagenesis kit, which was also used to create the wild-type MB2.1 mutant D286N. The following forward (F) and reverse (R) primers: MA3-D282N (F, CATAAGGGAAGTCGAAATAACATGAGTGTTG;
R, CAACACTCATGTTATTTCGACTTCCCTTATG), MA3-H62Q
(F, GCTGTATATGATGGGCAAGCTGGTTCTCAGGTTGC;
R, GCAACCTGAGAACCAGCTTGCCCATCATATACAGC),
MB2.1-D286N (F, CATAAGGGAAGTCGAAATAACATGAGTGTTG;
R, CAACACTCATGTTATTTCGACTTCCCTTATG) were used for mutagenesis, with pcDNA3-MA3 and pcDNA3-MB2.1 serving as templates. The same approach was used to construct the MB2.1 double mutant MB2.1-D286N, H62Q with pcDNA3-MB2.1-D286N as template and the following forward (F) and reverse (R) primers: F, GCAATTTATGACGGTCAAGCTGGATCCCGAGTGGC,
R, GCCACTCGGGATCCAGCTTGACCGTCATAAACTGC. PCR protocols were those provided by Stratagene. A typical PCR protocol included one cycle of denaturation at 95°C (30 sec), and 14 cycles beginning with denaturation at 95°C (30 sec), annealing at 55°C (1 min), and elongation

at 68°C (7 min). After PCR, methylated, nonmutated parental DNA was digested by 2 µl Dpn I restriction enzyme at 37°C for 2 hours and transformed into Top10 competent cells. The final constructs were confirmed by sequencing.

Since there are two unique restriction sites precisely flanking the region that encode the wild-type and mutated phosphatases, plasmids were restricted using Kpn I and Xba I and the digestion products (5.4 kb pcDNA3 vector and 1.0~1.5 kb wild-type phosphatases or mutants) were separated by 0.8% agarose gel electrophoresis. The 1.0~1.5kb fragments (with Kpn I and Xba I sticky ends) were extracted from the gel and ligated into the ecdysone-inducible expression vector pIND. The final products (pIND/MA3, pIND/MB2.1, pIND/MA3-H62Q, pIND/MA3-D282N, pIND/MB2.1-D286N, and pIND/MB2.1-D286N, H62Q) were further confirmed by restriction digestion and sequencing.

2.4 Transfection and selection of wild-type PP2C and mutants in BHK cells.

BHK/pVgRXR cells were cultured in F12-DMEM medium supplemented with 5% F.B.S, 100 I.U penicillin, 100 µg/ml streptomycin containing 250 µg/ml Zeocin, in a humidified incubator at 37°C and 5% CO2. Medium and supplements were from Life Technologies (Burlington,

Ontario, Canada). After 24 h subculture, 70%~80% confluent cells were transfected with pNUT/wt CFTR plasmid using Fugen6 transfection reagent. The cells were selected by BHK/pVgRXR cell medium with selection reagent 500 μM methotrexate (MTX). Six surviving single colonies were selected 10-14 days after transfection and propagated in selective medium. The same method was used to transfect BHK/pVgRXR, and pNUT-wtCFTR stable cell lines with pIND/MA3, pIND/MB2.1, pIND/MA3-H62Q, pIND/MA3-D282N, pIND/MB2.1-D286N, and pIND/MB2.1-D286N, H62Q. Six stable cell lines were obtained after selection by 400 μg/ml G418. Constitutive expression of CFTR and regulated expression of wild-type phosphatases and mutants were verified by Western blotting.

2.5 Protein detection

Expression of wild-type phosphatases and mutants was induced by 10 μM ponasterone A for 24 hours. Non-induced cells were used as negative controls. Cells were washed in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO4, 1.4 mM KH_2PO4, pH 7.4), harvested by scraping with a rubber policeman, and lysed (15-30 min, on ice) in RIPA buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.08% Deoxycholic Sodium, 20 mM Tris-HCl and protease inhibitor cocktail from

Roche Applied Science, Indianapolis, IN at pH 8.0). Insoluble cellular debris was removed by centrifugation of lysates (15,000g, 15 min at 4°C). Concentration of total cellular protein content was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Harvested cells that were not lysed immediately were stored at –20°C for up to two months. Lysates were diluted 1:1 with 2X sample buffer (4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 125 mM Tris-HCl, 0.2% bromophenol blue, pH 6.8), separated in 10% or 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% non-fat skim milk in TTBS (1.21 g/L Tris, 8.8 g/L NaCl, 0.2% TWEEN20, pH8.0) for 1 h, and then incubated with the specified primary antibodies, mouse anti-CFTR (450, 1:5000) or mouse monoclonal antibody anti-HA tag (1:1000) in TBS (1.21 g/L Tris, 8.8 g/L NaCl, pH8.0). After extensive washes with TTBS, blots were incubated with the secondary antibody, a goat anti-mouse IgG labelled with horseradish peroxidase at a dilution of 1:1000. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) kit (Amersham Biosciences Corp. Piscataway, NJ) and exposed to X-ray film.

2.6 Immunoprecipitation and phosphatase assay

After induction by ponasterone A for 24 hours, cells were washed in PBS twice and harvested by scraping with a rubber policeman. To maintain phosphatase activity, cells were lysed (1 h, on ice) in EVF buffer (0.2% Triton X-100, 0.15 mol NaCl, 20 mM Tris-HCl, and protease inhibitor cocktail). Lysate was then centrifuged at 15,000g for 15 min at 4°C, supernatant was collected, and total protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit. Soluble proteins in the supernatant were immunoprecipitated immediately to avoid freezing and thawing of the samples. Protein G Sepharose 4 fast flow (Amersham Biosciences Corp. Piscataway, NJ) was washed five times in EVF buffer without protease inhibitor cocktail to remove sodium azide. Lysate (9 mg of protein) was then incubated with 180 µl of protein G Sepharose beads, 60µl of mouse monoclonal antibody anti-HA tag (1:200) in 9 ml EVF buffer on a rotator for 5-8 h at 4 °C. After that, unbound antibody and protein were washed away with PBS buffer. Because activity of protein phosphatase is sensitive to low pH elution buffer, immunocomplexes were used for further phosphatase assay.

To analyze the phosphatase activity of MA3, MB2.1 and mutants, pNPP was used as a substrate. *p*NPP assay were performed under four

conditions: 1) phosphatase buffer with high Mn^{2+}, 2) phosphatase buffer without Mn^{2+}, 3) in the presence of calyculin A, 4) control, i.e. empty beads incubation with phosphatase buffer containing high Mn^{2+}. After immunoprecipitation, 150 µl of protein G Sepharose beads and phosphatase complex were divided into three aliquots for the first three conditions. One aliquot was washed with phosphatase buffer (50 mM Tris, 0.01 mM EDTA, 1% β-mercaptoethanol, 1 mg/ml BSA, 10 mM MnCl2) twice, and incubated with 100 µl phosphatase buffer containing 10 mM pNPP, incubated at 30 °C for 1 h and terminated by the addition of 0.25 M NaOH. Absorbance was determined at 405 nm using a spectrophotometer. For the second condition, phosphatase buffer without Mn^{2+} was used to replace phosphatase buffer. 100 nM calyculin A was added to phosphatase buffer in the third condition.

2.7 Immunolocalization

Cells stably transfected with wild-type MA3 and MB2.1 (both in pIND) were plated at low density on glass coverslips, induced with ponasterone A for 24 h, and fixed with 2% paraformaldehyde. After permeabilization and blocking with 0.1% Triton X-100/2% BSA in PBS, they were incubated in fresh solution containing 0.1% Triton X-100, 0.2% BSA, the anti-CFTR antibody 450 diluted 1:1000, in PBS for 1 h at room

temperature, and then washed and incubated with a secondary antibody (Cy5-conjugated goat anti-mouse at 1:100 diluted in PBS/0.1% Triton X-100/0.2% BSA). In one set of experiments, cells were washed and incubated with the rat monoclonal anti-HA tag antibody diluted 1: 100 in PBS/0.1% Triton X-100/0.2% BSA and then with Cy3-conjugated goat anti-rat secondary antibody (1:200). After the final wash, slides were mounted, dried, and viewed using a Zeiss LSM 510/META confocal microscope. Cells expressing CFTR and Na^+/H^+ exchanger regulatory factor (NHERF) with HA tag served as positive controls.

2.8 Iodide efflux

Cells were cultured in 24-well plates and induced with ponasterone A for 24 h. In the iodide efflux experiment, cells were loaded with 500 µl iodide loading buffer (136 mM NaI, 3mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM HEPES, pH 7.4) for 1 h at room temperature. Extracellular NaI was washed away with eight washes of iodide efflux buffer (136 mM $NaNO_3$ replacing 136 mM NaI in the loading buffer). Samples were collected by completely replacing the efflux buffer (210 µl volume) with fresh solution at 1 min intervals. The first three samples were averaged to establish the baseline efflux rate, then cpt-cAMP was added and samples were collected every minute for 9 min in the continued

presence of cpt-cAMP (i.e. the efflux buffer used for subsequent replacements also contained cpt-cAMP at the same concentration). For controls, the same amount of H_2O without agonists was added to the efflux buffer. Iodide concentration was determined in each aliquot using an iodide-sensitive electrode (Orion Research Inc., Boston, MA) and converted to iodide content (i.e. the amount of iodide released during a 1 min interval in nanomoles). Each time point was then normalized to un-stimulated controls. Cumulative efflux curves were plotted to show the total amount of iodide (nmol) that had been collected up to each time point indicated, i.e. the cumulative iodide effluxes calculated by adding the amount collected during each sample period to the total already collected during the preceding sample periods. The mean iodide content of samples collected before cpt-cAMP addition was subtracted from those measured after addition to correct for background leakage, which accounts for the slight decrease at later time points. To investigate the effect of wild type phosphatases and mutants on CFTR activity, different conditions were compared at multiple cpt-cAMP concentrations. In some experiments, 100 μM calyculin A were added to the iodide buffer and efflux buffer to inhibit PP2A. At the end of each experiment, expression of wild type and mutant phosphatases was assessed by Western blotting.

2.9 Statistical methods

All data in phosphatase assay and iodide efflux are presented ae means ± SE. The data from phosphatase assay with only one variable were analyzed with a one-way ANOVA, and a two-way ANOVA was applied to data from iodide efflux with two variables. A Bonferroni multiple comparison test was applied after the ANOVA analysis. A value of $P < 0.05$ was considerded significant.

Chapter 3 Results

3.1 Generation of inducible BHK expression systems for wild-type and mutant phosphatases MA3 and MB2.1

PP2C is involved in several essential signal pathways in cells, regulating enzymes such as AMP-activated protein kinase, Ca^{2+}/Calmodulin-dependent protein kinase II, and various stress-activated kinase pathways. Overexpression of PP2C is therefore toxic to cells, making it impossible to generate stable cell lines that constitutively overexpress PP2C. To overcome this problem, an inducible system was used in this project to prepare BHK cell lines expressing MA3 and MB2.1. The inducible system utilizes a heterodimer of the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) that binds a hybrid ecdysone response element (E/GRE) in the presence of the synthetic ecdysone analog ponasterone A (No et al., 1996). HA-tagged MA3 and MB2.1 were subcloned into the pIND vector, which contain five modified E/GREs. Each modified E/GRE contains one half-site from the natural ecdysone response element and one half-site from the glucocorticoid response element. pIND-MA3 and pIND-MB2.1 were then transfected into a BHK cell line already stably expressing VFTR and pVgRXR vector, which

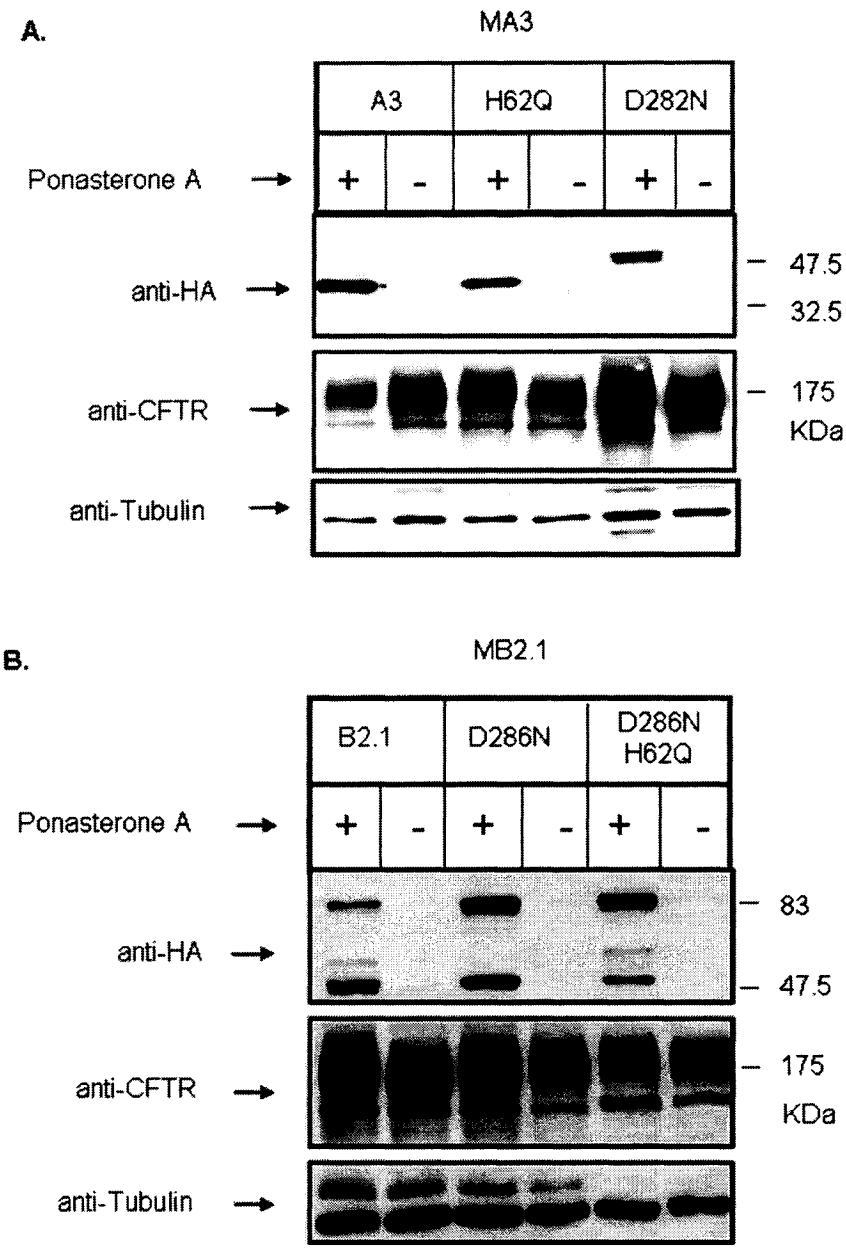
encodes VgEcR and RXR. VgEcR contains the glucocortoid response element binding domain and the VP 16 transactivation domain, while RXR recognizes ecdysone response element. In the presence of ponasterone A, RXR and VgEcR associate together and bind to the hybrid ecdysone response element (E/GRE) on pIND-MA3 or pIND-MB2.1. Transcription is activated from the minimal heat shock promoter via the VP 16 transactivation domain in the modified VgEcR. Expression of HA-tagged MA3 and MB2.1 polypeptides was assessed by probing Western blots with anti-HA antibody (Figure 3).

Expression of HA-tagged MA3 and MB2.1 was detectable after 24 hours induction with ponasterone A (Figure 3 A and B, first lane of upper panel). No signals were obtained when cells were not induced (second lane), confirming that expression of the tagged proteins depends on induction (Figure 3 A and B, second lane of upper panels). MA3 and MB2.1 were predicted to be ~45 kDa based on the number of amino acids. A band was obtained for MA3 was at 45 kDa as expected; however, MB2.1 had two bands, one at ~45 kDa and the other at ~83 kDa band. All cell lines stably expressed CFTR, and the level of CFTR expression was not affected systematically by the expression of MA3 or MB2.1 (Figure 3 A and B, first lane of middle panels).

Putative dominant negative MA3 and MB2.1 constructs were subcloned into the pIND vector and stably transfected into BHK cells to enable side-by-side comparison with overexpressed wild-type protein (Figure 3). Expression of the mutants was strongly induced by ponasterone A, and no expression was detected in cells that were not induced (Figure 3 A and B, last two lanes of upper panel). Although the predicted molecular masses of the mutants are similar to those of the wild type proteins, the MA3-D282N band migrated more slowly than expected in the gelas discussed on page 84. CFTR was stably expressed in all cell lines and its levels were not altered by expression of the MA3 and MB2.1 mutants (Figure 3 A and B, last four lanes of middle panels). Finally, blots were probed with antitubulin antibody as a control for equal loading (Figure 3 A and B, bottom panels).

Figure 3 Inducible expression of MA3, MB2.1 and their putative dominant negative mutants in BHK cells

After 24 hour induction with ponasterone A, equal amounts of lysate from BHK cell lines stably transfected with different PP2C constructs were loaded on SDS-PAGE gels and transferred onto PVDF membranes. Western blots were probed using anti-HA monoclonal antibody (mAb) for HA tagged MA3, MB2.1 and putative mutants (upper), mAb 450 for CFTR (middle), and a monoclonal anti-tubulin A antibody as a loading control (bottom). Uninduced cells are shown to confirm inducible expression. (A) MA3 (A3) cell line and cell lines expressing the dominant negative mutants H62Q and D282N. Note the induction of PP2Cα constructs and the lack of effect on CFTR expression. (B) Inducible expression of MB2.1 phosphatase (B2.1) and the dominant negative mutants D286N and double mutant D286N,H62Q in stably-transfected BHK cell lines.


A.
MA3
A3
H62Q
D282N
Ponasterone A
+
-
+
-
+
-
anti-HA
47.5
32.5
anti-CFTR
175
KDa
anti-Tubulin
B.
MB2.1
B2.1
D286N
D286N
H62Q
Ponasterone A
+
-
+
-
+
-
anti-HA
83
47.5
anti-CFTR
175
KDa
anti-Tubulin

3.2 Activity of MA3, MB2.1 and mutants expressed in BHK cells

To verify that MA3 and MB2.1 are functional when expressed in the inducible BHK system, activities of both variants were tested using the *para*-nitrophenyl phosphate (*p*NPP) assay. *p*NPP is a chromogenic substrate for most phosphatases, including alkaline phosphatases and protein phosphatases. In this assay, there is the cleavage of pNPP to para-nitrophenol, yielding an intense yellow product that can be conveniently measured as absorbance at 405 nm using a spectrophotometer.

In most experiments, MA3 and MB2.1 were immunoprecipitated from cells that had been induced using ponasterone A for 24 hours. The immunocomplexes were incubated with *p*NPP in phosphatase buffer for 1 additional hour and absorbance at 405 nm was measured every 5 min.

Figure 4 A shows the *p*NPP phosphatase activity of MA3 immunocomplexes. To ensure that the heterologous MA3 was functional and had properties consistent with PP2C, the pNPP assay was repeated without Mn^{2+} or in the presence of calyculin A, a specific inhibitor of PP1 and PP2A. MA3 activity was abolished when Mn^{2+} was removed from the assay mix, consistent with the strict dependence of PP2C catalysis on metal ions. The phosphatase activity of MA3 immunocomplexes was not

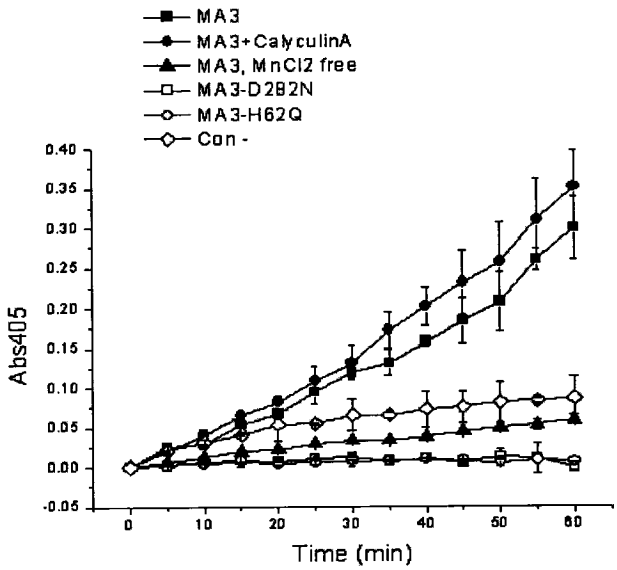
inhibited by calyculin A as expected. MB2.1 immunoprecipitates were shown to be a functional PP2C and have similar properties since it was also Mg^{2+}/Mn^{2+} -dependent and insensitive to inhibitors of PP1 and PP2A (Figure 4 B).

To test if site directed mutagenesis inactivates MA3 and MB2.1, the putative dominant negative mutants MA3-D282N, MA3-H62Q, MB2.1-D286N, and MB2.1- H62Q were induced and immunoprecipitated, then immunocomplexes were assayed using *p*NPP under the same conditions as for wild type enzymes. No phosphatase activity was detected using the dominant negative constructs (Figure 4 A and B). BHK/wt CFTR cells locking heterologous PP2C were used as the negative control in these experiments.

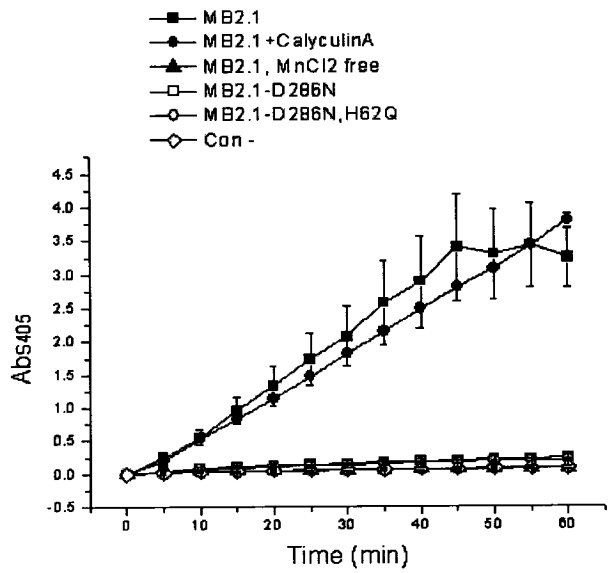
Figure 4 Protein phosphatase assays

After 24 h induction with ponasterone A, the tagged PP2Cα and -β isoforms MA3 and MB2.1 and their dominant negative mutants were immunoprecipitated from baby hamster kidney cells using anti-HA antibody. *p*NPP assays were performed on the immunocomplexes and absorbance was measured at 450 nm (see methods). (A) Immunoprecipitated MA3 had low phosphatase activity in the presence of Mn^{2+}, which was greatly reduced in Mn^{2+}-free solution. MA3 was not affected by calyculin A (20 nM) an inhibitor of PP1 and PP2A. Immunoprecipitated MA3-D282N and MA3-H62Q had no detectable phosphatase activity. BHK cells were used as the negative control (Con-). (B) Immunoprecipitated MB2.1 had more robust protein phosphatase 2C activity compared to MA3 (note the 10-fold difference in scale of the Y axis). MB2.1 activity was Mn^{2+} dependent and unaffected by the PP2A and PP1 inhibitor calyculin A. Phosphatase activity was abolished in putative dominant negative constructs of MB2.1 containing mutations at one (D286N) or two residues (D286N, H62Q).

A.


MA3
MA3+CalyculinA
MA3, MnCl2 free
MA3-D282N
MA3-H62Q
Con -
Abs405
Time (min)

B.


MB2.1
MB2.1+CalyculinA
MB2.1, MnCl2 free
MB2.1-D286N
MB2.1-D286N,H62Q
Con -
Abs405
Time (min)

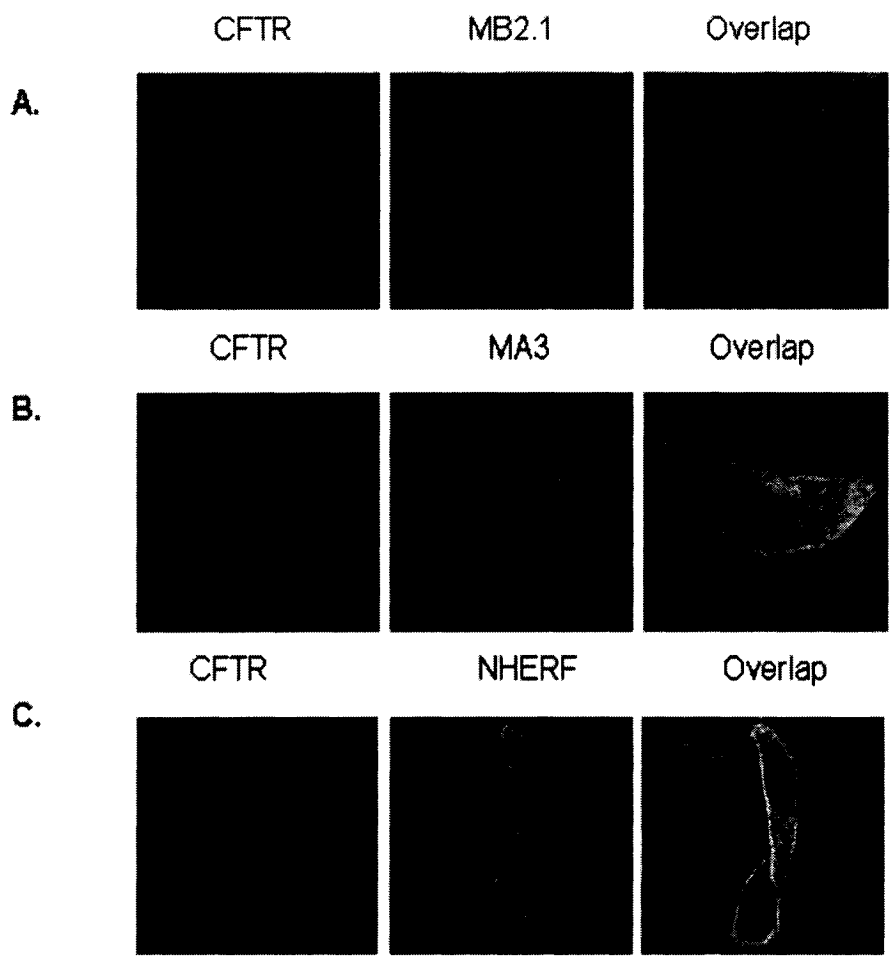
3.3 Localization of MA3, MB2.1 and CFTR in BHK inducible systems

Previously, immunoprecipitation and cross-linking experiments had indicated that PP2C and CFTR are in a protein complex at the cell membrane (Zhu et al., 1999). Furthermore, when HA-tagged MA3 and MB2.1 were transiently expressed in BHK cells, both were pulled down with CFTR that had been biotinylated at the cell surface (unpublished data). Confocal microscopy was used to examine whether MA3 and MB2.1are co-localized with CFTR and therefore capable of interacting with it in the intact cell. Ponasterone-induced cells were stimulated with forskolin (FSK), isobutyl methylxanthine (IBMX), and 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3', 5'-cyclic monophosphate sodium salt (8CPT-2-O-Me-cAMP) for 30 minutes, then fixed, permeabilized, and blocked in solutions containing paraformaldehyde and TritonX-100/BSA. Cells were then exposed to anti-HA monoclonal antibody against tagged MA3 and MB2.1, and to the anti-CFTR mAb 450, which is directed against the R domain. CFTR was visualized using goat anti-mouse IgG secondary antibody that had been conjugated with the fluorophores Cy5. MA3 and MB2.1 were visualized using goat anti-rat IgG secondary antibody that had been conjugated with the fluorophore Cy3.

After 24 h induction by ponasterone A, MB2.1 and MA3 were expressed throughout BHK cells, including the nucleus (Figure 5 panel A and panel B). CFTR immunofluorescence (red) was mainly localized on the cell membrane and endoplasmic reticulum (ER) as expected; whereas some cytoplasmic staining was also found that may be vesicular (Figure 5). MB2.1 and CFTR were not co-localized on the cell membrane or in the ER (Figure 5 panel A). MA3 and CFTR were both detected just below the cell membrane and in the ER, as indicated by the yellow overlay in Figure 5 (panel B). Na^+/H^+ exchanger regulatory factor (NHERF) is an adaptor protein that has been shown to modulate CFTR channel gating through its association with a C-termin consensus binding motif on CFTR (Raghuram et al., 2001). Colocalization of NHERF was used as a positive control for immunolocalization of PP2Cs. When the same protocols for MA3 and MB2.1 were used to stain HA-tagged NHERF and CFTR, overlapping membrane staining was obtained as indicated by yellow (Figure 5 panel C).

Figure 5 Localization of MA3, MB2.1 and CFTR

After 24 hour induction by ponasterone A, cells were fixed using paraformaldehyde and permeabilized with 0.1% Triton X-100, and immunostained for CFTR and MB2.1. Cells were incubated with anti-CFTR monoclonal antibody 450 (1:1000) and rat anti-HA tag antibody (1: 100). The monoclonal antibody signal was detected using a secondary Cy5-conjugated goat anti-mouse antibody (1:100, in red). Cy3-conjugated goat anti-rat secondary antibody was used to visualize MB2.1 (1:200, in green for MB2.1-HA, MA-HA, and NHERF-HA). Co-localization is shown as yellow colour when the images are overlaid as in the third column.


CFTR
MB2.1
Overlap
A.
CFTR
MA3
Overlap
B.
CFTR
NHERF
Overlap
C.

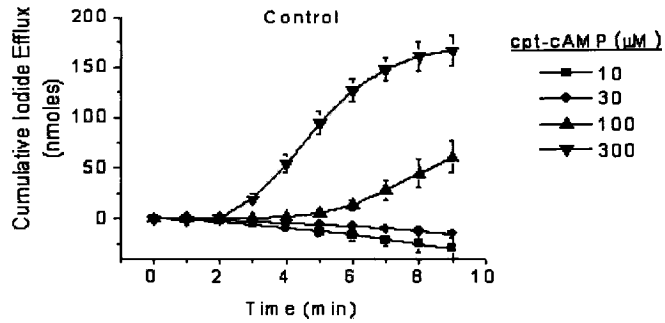
3.4 Effect of overexpressing wild-type and mutated MB2.1 on CFTR activity

Adding exogenous PP2C to excised patches reduces CFTR channel activity by >90% (Luo et al., 1998) and co-expression of PP2C with CFTR results in lower total Cl^- current (Travis et al., 1997). To assess if MB2.1 can modulate CFTR in intact cells, we examined its effect on chlorophenyl-thio-cAMP- (cpt-cAMP) stimulated iodide efflux from BHK cells, which stably express CFTR and either wild-type MB2.1 or a putative dominant negative mutant of MB2.1. No efflux could be detected with ~30 μM [cpt-cAMP], however efflux rate increased with [cpt-cAMP] as indicated by the increased slope of the cumulative efflux curves, which became steeper from the range 100 to 300 μM (Figure 6 A). cpt-cAMP-stimulated efflux was not altered at any concentration of cpt-cAMP or time point using cells that overexpress MB2.1 or the putative dominant negative mutants and the dependence on [cpt-cAMP] was also similar to that of BHK cells overexpressing only CFTR (Figure 6 B, C, D).

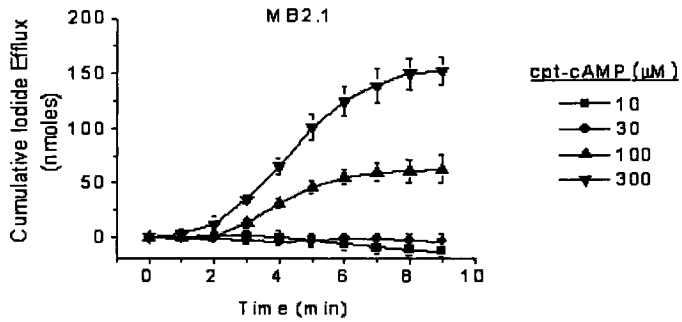
Figure 6 Chlorophenylthio-cAMP (cpt-cAMP)-stimulated iodide efflux is similar from BHK /wt CFTR cells overexpressing MB2.1, MB2.1- D286N, or MB2.1-D286N,H62Q

(A), control cells were pre-incubated with iodide buffer for 30 min at 37 °C, rinsed 8 times in efflux buffer, then stimulated with different concentrations of cpt-cAMP. Iodide was measured at 1 min intervals and plotted as cumulative efflux. (B), cumulative iodide efflux determined as in (A) using cells that over express MB2.1 after induction by 10 μM ponasterone A for 24 hours. (C), cumulative iodide effluxes measured as in (B), but using cells that have been induced to over-express MB2.1-D286N. (D), cumulative iodide effluxes measured as in (B), but using cells that over express MB2.1- D282N, H62Q. Values shown are the means of duplicate samples at each cpt-cAMP concentration.

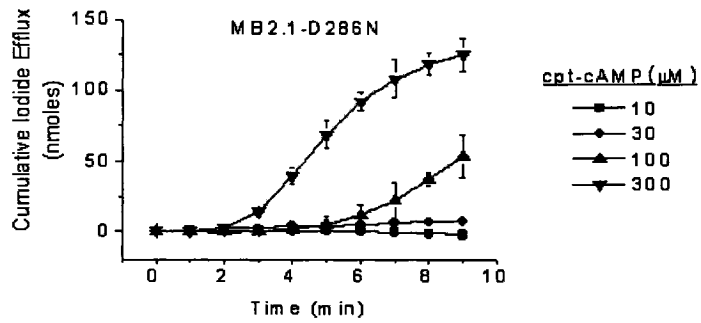
A.


Control
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (μM)
10
30
100
300

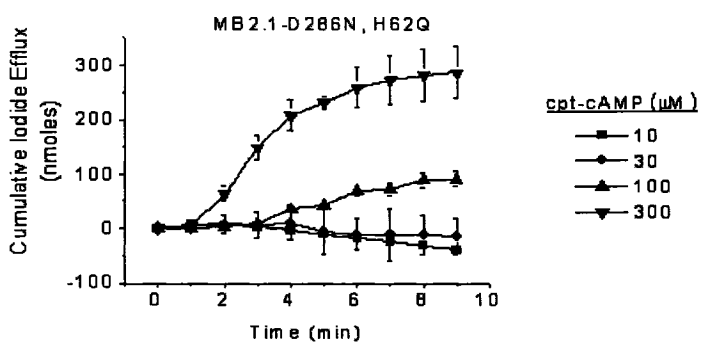
B.


MB2.1
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (μM)
10
30
100
300

C.


MB2.1-D286N
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (μM)
10
30
100
300

D.


MB2.1-D286N, H62Q
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (μM)
10
30
100
300

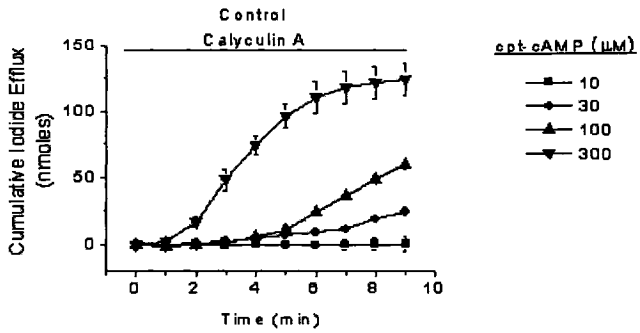
Previous studies have suggested that PP2A and PP2C are the two main phosphatases that regulate CFTR in epithelial cells and that they have different effects on CFTR channel activity (Luo et al., 1998). To help isolate the regulatory effect of MB2.1 on CFTR activity and improve its detection, PP2A was inhibited during iodide efflux experiments using calyculin A. Under these conditions, the cumulative efflux curves became steeper over the range 30-300 μM [cpt-cAMP], and no efflux was detectable with 10 μM [cpt-cAMP] (Figure 7 A). The increased sensitivity of activation by low cpt-cAMP concentration is consistent with the previous finding that PP2A is one of protein phosphatases that dephosphorylates CFTR. However, responsiveness to cpt-cAMP was not decreased noticeably by overexpression of MB2.1 or increased by overexpressing putative dominant negative mutants of MB2.1 even when tested in the presence of calyculin A, CFTR (Figure 7 B, C, and D).

In conclusion, inhibition of PP2A by calyculin A decreased CFTR dependence on cpt-cAMP, but overexpressing wild type or mutants of MB2.1 did not change CFTR responsiveness to cpt-cAMP.

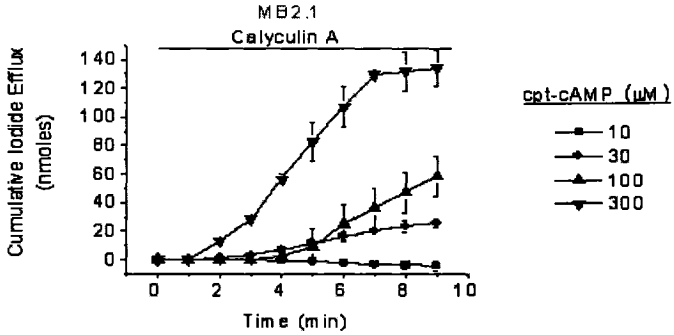
Figure 7 Chlorophenylthio-cAMP (cpt-cAMP) stimulates similar iodide effluxes from BHK /wt CFTR cells overexpressing MB2.1, MB2.1-D286N or MB2.1-D286N,H62Q in the presence of calyculin A

(A), BHK/wt CFTR cells were incubated with iodide buffer containing 20 nM for 30 min, at 37 °C. calyculin A (20 nM) was present throughout the entire experiment including rinsing steps. Cells were rinsed 8 times with efflux buffer, stimulated with different concentrations of cpt-cAMP, iodide was measured at 1 min intervals during exposure of the cells to cpt-cAMP. The results were plotted as cumulative iodide efflux. (B), cumulative iodide efflux determined as in (A) using cells that has been induced to over-express MB2.1 using 10 μM ponasterone A for 24 hours. (C), cumulative iodide effluxes determined as in (B) using cells that over express MB2.1- D286N. (D), cumulative iodide effluxes determined as in (B) using cells that over express MB2.1- D282N, H62Q. Values shown are the means of duplicate samples at each cpt-cAMP concentration.

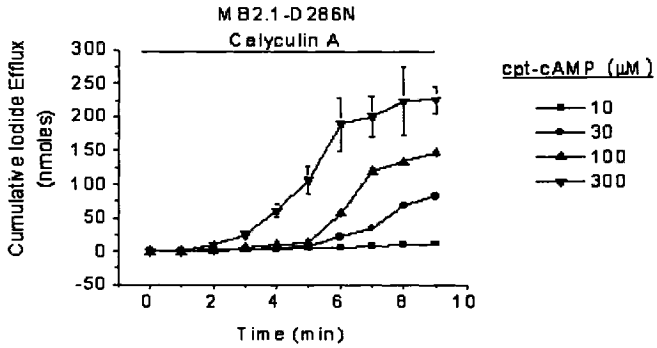
A.


Control
Calyculin A
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (µM)
10
30
100
300

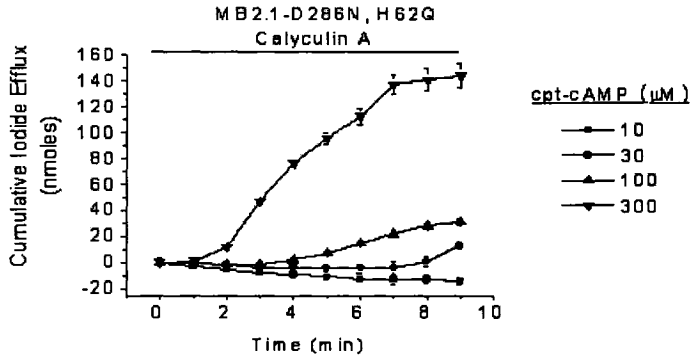
B.


MB2.1
Calyculin A
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (µM)
10
30
100
300

C.


MB2.1-D286N
Calyculin A
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (µM)
10
30
100
300

D.


MB2.1-D286N, H62Q
Calyculin A
Cumulative Iodide Efflux (nmoles)
Time (min)
cpt-cAMP (µM)
10
30
100
300

3.5 Effect of over-expressing wild-type and mutated MA3 on CFTR activity

To investigate the role of MA3 in regulating CFTR activity, the responsiveness of CFTR channels to cpt-cAMP was tested by measuring iodide effluxes from BHK cells that overexpress wild-type MA3 or its dominant negative mutants. Efflux rate increased with increasing [cpt-cAMP], as indicated by the increasing peak efflux within the range of 100 to 300 μM and no stimulated efflux was detected in the range 10 - 30 μM (Figure 8 A). The threshold concentration needed to activate iodide efflux was not altered by overexpressing MA3 or MA3-D282N, therefore these proteins did not change CFTR sensitivity to cpt-cAMP (Figure 8 B and C). Interestingly, simulation of iodide efflux from cells overexpressing MA3-H62Q was lower at all cpt-cAMP concentrations and at all time points when compared with cells expressing only CFTR, and MA3-H62Q cells needed higher [cpt-cAMP] to respond (Figure 8 D).

To further examine the effect of MA3 on CFTR activation by cpt-cAMP, further iodide efflux experiments were carried out in the presence of calyculin A to eliminate any possible downregulation by PP1 or PP2A. Efflux rate increased with [cpt-cAMP] again, as revealed by the peak of the efflux curve, but no efflux was detected at 10 μM. Interestingly, although MA3 overexpression did not alter the cpt-cAMP dependence of

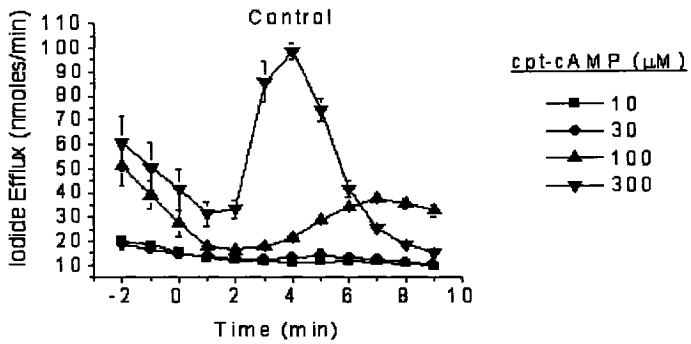
CFTR, iodide efflux was not detected at any concentration of cpt-cAMP when the cells overexpressed MA3-D282N or MA3-H62Q (Figure 9).

In summary, overexpression of MA3 did not affect the responsiveness of CFTR to [cpt-cAMP], however overexpressing the MA3 dominant negative mutant reduced iodide efflux, especially in the presence of calyculin A.

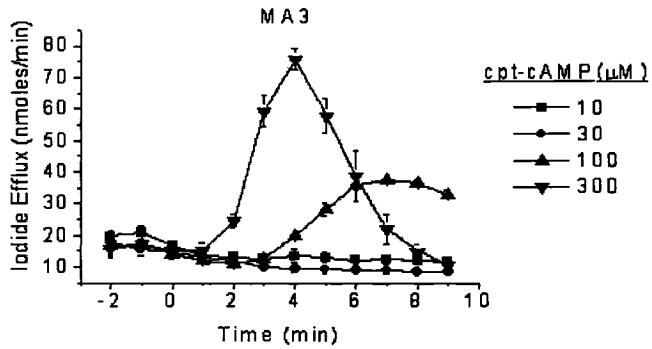
Figure 8 Chlorophenylthio-cAMP (cpt-cAMP)-stimulated iodide efflux from BHK /wt CFTR cells overexpressing MA3, MA3-D282N and MA2-H62Q.

Iodide effluxes were measured from BHK cells stably transfected with inducible MA3 constructs as described for MB2.1 constructs in Figure 7 Concentration dependence of cpt-cAMP stimulation is shown for control cells lacking heterologous phosphatase **(A)**, for comparison with cells induced to overexpress MA3 **(B)**, or the dominant negative constructs MA3-D282N **(C)** or MA3- H62Q **(D)**. Means ± s.e, n=5.

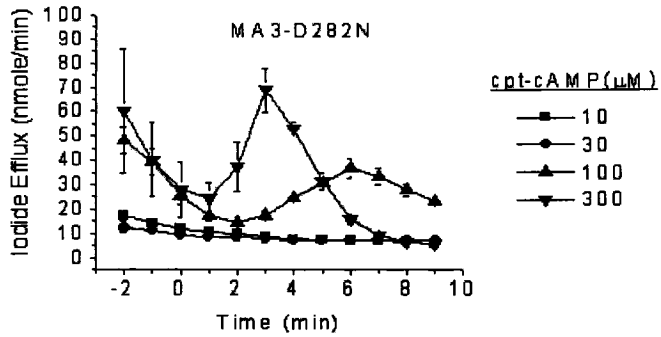
A.


Control
Iodide Efflux (nmoles/min)
Time (min)
cpt-cAMP (μM)
10
30
100
300

B.


MA3
Iodide Efflux (nmoles/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

C.


MA3-D282N
Iodide Efflux (nmole/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

D.

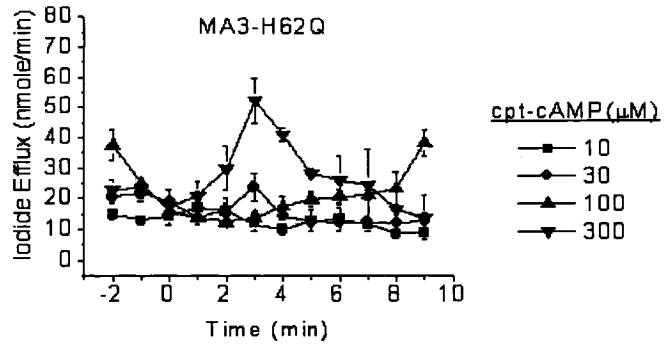
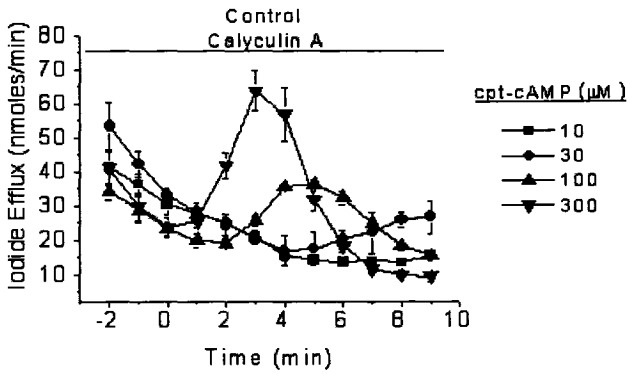

MA3-H62Q
Iodide Efflux (nmole/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

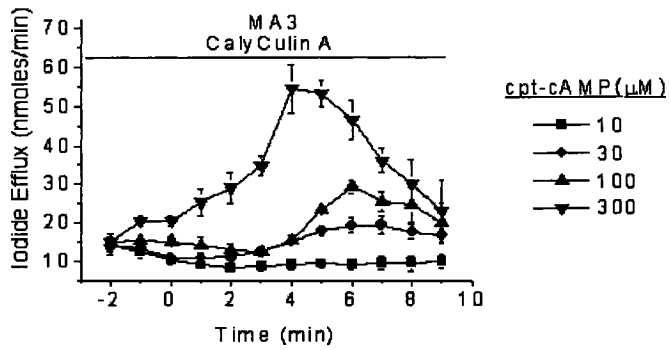
Figure 9 Chlorophenylthio-cAMP (cpt-cAMP)-stimulated iodide efflux from BHK /wt CFTR cells overexpressing MA3, MA3-D282N and MA2-H62Q in the presence of calyculin A

Iodide effluxes were measured from BHK cells at 1 min intervals as described in Figure 7. calyculin A (20 nM) was present during loading and throughout efflux measurements. Cells were stimulated at time 0 by different concentrations of cpt-cAMP as indicated. The cpt-cAMP concentration dependence of BHK/wt response is shown in (A), for comparison with cells induced to overexpress MA3 (B), the dominant-negative mutant MA3-D282N (C), or the double mutant MA3- H62Q (D). Means ± s.e, n=8.

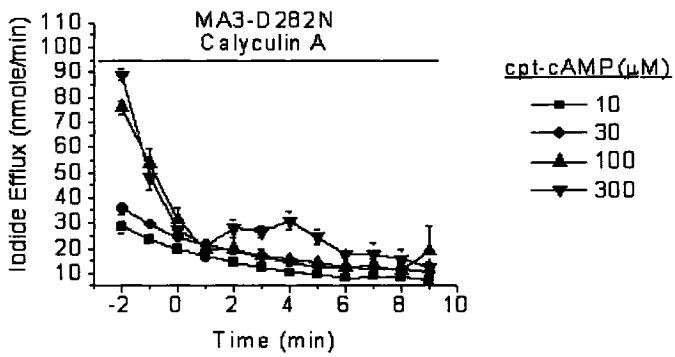
A.


Control
Calyculin A
Iodide Efflux (nmoles/min)
Time (min)
cpt-cAMP (μM)
10
30
100
300

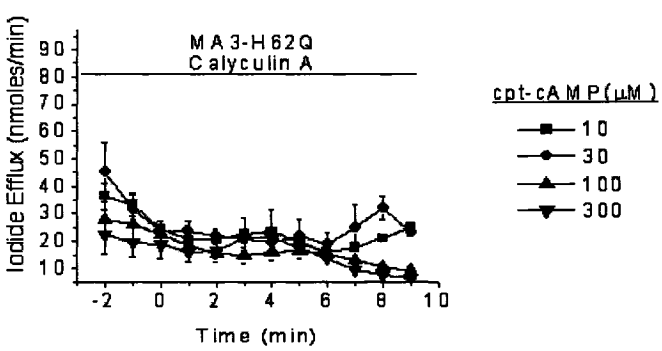
B.


MA3
CalyCulin A
Iodide Efflux (nmoles/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

C.


MA3-D282N
Calyculin A
Iodide Efflux (nmole/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

D.


MA3-H62Q
Calyculin A
Iodide Efflux (nmoles/min)
Time (min)
cpt-cAMP(μM)
10
30
100
300

3.6 Reduced iodide loading and increased iodide leakage in cells that overexpress MA3-H62Q

The unexpected reduction in stimulated iodide efflux could reflect either inhibition of CFTR activity or reduced iodide concentration in the cells. To distinguish between these two possibilities, intracellular [I^-] was determined immediately after the loading period or at the end of the experiment (after cpt-cAMP stimulation). Cells were collected and lysed, and [I^-] of cell lysates were measured. As shown in Table 1, [I^-] in control (BHK/wt CFTR), MA3, and D282N cells was lower after stimulation with 100 - 300 μM cpt-cAMP than with 10 - 30 μM, consistent with the larger integrated iodide efflux measured during experiments. In cells overexpressing MA3-H62Q, intracellular iodide concentration was lower at all cpt-cAMP concentrations. Moreover, the [I^-] within cells expressing MA3-H62Q and stimulated with 10 - 30 μM cpt-cAMP was similar to that in control cells (BHK/wt CFTR) following stimulation with 300 μM cpt-cAMP, and iodide levels in MA3-H62Q cells stimulated with 100 -300 μM cpt-cAMP were even lower (Table 1).

Cell pellet iodide levels were also compared after cpt-cAMP stimulation in the presence of calyculin A. Although no cpt-cAMP-stimulated efflux was detected when cells overexpressed MA3-D282N or

MA3-H62Q, intracellular [I^-] was lower at all cpt-cAMP concentrations, and [I^-] was similar to that in BHK/wt CFTR cells treated with 300 μM [cpt-cAMP], in which efflux was detected within 2 - 3 min (Table 2.).

We considered the possibility that small effluxes from MA3-H62Q cells might be due to reduced loading or excessive leakage during the initial wash. CFTR channel activity is inhibited by low temperature due to a reduction in open probability (P_o; (Mathews et al., 1998)). To increase [I^-] in cells at the beginning of the efflux period, we loaded cells for 30 min with iodide buffer containing calyculin A at 37 °C, then cooled them on ice for 10 min when removing extracellular iodide (by rinsing with cold efflux buffer containing calyculin A). When stimulation was initiated by adding 300 μM cpt-cAMP at 24 ° C, cpt-cAMP-stimulated iodide efflux was detected from cells overexpressing MA3-H62Q as expected. However, the responses were still smaller than from cells expressing only CFTR (Figure 10). Comparison of the [I^-] in cells indicated that intracellular levels were still reduced, and cpt-cAMP induced smaller effluxes(Table 3), apparently due to less efficient loading with iodide.

Table 1 Cell pellet iodide content (no calyculin A)

[cpt-cAMP] uM	Cell pellet iodide (nmoles)			
	Con.	MA3	MA3-D282N	MA3-H62Q
10	78 ±5.66	80 ±3.42	95 ±1.23	39 ±4.56 *
30	81 ±3.29	76 ±2.31	81 ±3.40	43 ±2.10 *
100	58 ±1.34	60 ±2.11	52 ±3.22	29 ±5.62 *
300	46 ±3.56	44 ±0.31	43 ±2.34	26 ±0.23 *

Cell pellet iodide is mean value ± s.e., n=4, * indicates difference from Control (Con.) value statistically significant at $p<0.05$.

Table 2 Cell pellet iodide content (with calyculin A)

[cpt-cAMP] uM	Cell pellet iodide (nmoles)			
	Con.	MA3	MA3-D282	MA3-H62Q
10	43±1.23	50 ±2.33	36 ±4.3	22 ±2.31 *
30	32 ±3.24	29 ±3.21	32 ±1.23	20 ±1.78 *
100	30 ±3.11	31 ±1.23	31 ±0.21	27 ±2.39
300	25 ±0.21	22 ±0.22	29 ±3.2	27 ±4.78

Cell pellet iodide is mean value ±s.e., n=4, * indicates difference from Control (Con.) value statistically significant at $p<0.05$.

Figure 10 Iodide efflux from cells overexpressing MA3-H62Q after rinsing at 4°C

To minimize iodide leakage during removal of extracellular iodide, cells were loaded with iodide buffer for 30 min at 37°C in the presence of 20 nM calyculin A, cooled on ice for 10 min, washed with cold efflux buffer containing 20 nM calyculin A, and then stimulated at time 0 while warming to room temperature. Iodide efflux was measured at 1 min intervals from control BHK/wt CFTR cells with, or without, stimulation and from dominant negative MA3-H62Q cells with or without stimulation. Means ± s.e, n=4.

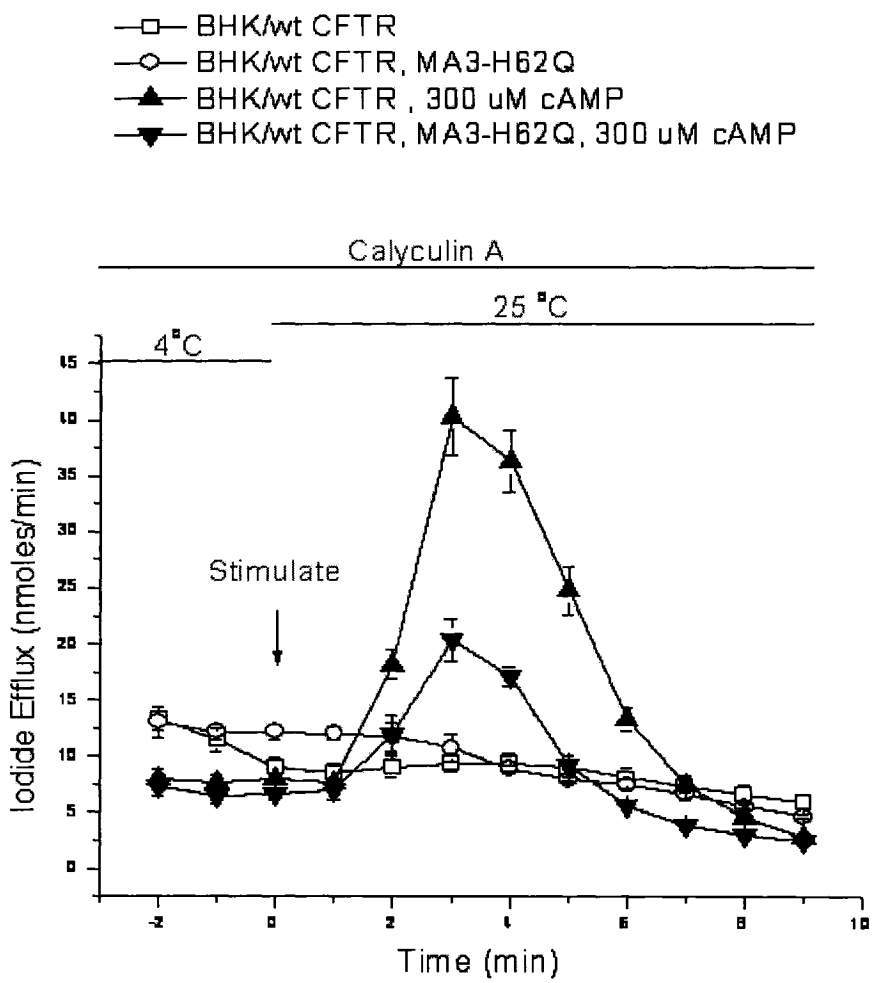

BHK/wt CFTR
BHK/wt CFTR, MA3-H62Q
BHK/wt CFTR , 300 uM cAMP
BHK/wt CFTR, MA3-H62Q, 300 uM cAMP
Calyculin A
25 °C
4°C
Stimulate
Iodide Efflux (nmoles/min)
Time (min)

Table 3 Cell pellet iodide content and protein concentration

	Cell pellet [I^-] nM	Cell pellet [Protein]μg/μl
BHK/wt CFTR	98±4.51	1.22±0.03
BHK/wt CFTR, MA3-H62Q	44±1.12 *	1.11±0.02
BHK/wt CFTR, 300 μM cpt-cAMP	35±3.23 *	1.13±0.04
BHK/wt CFTR, MA3-H62Q 300 μM cpt-cAMP	25±0.89 *	0.98±0.01

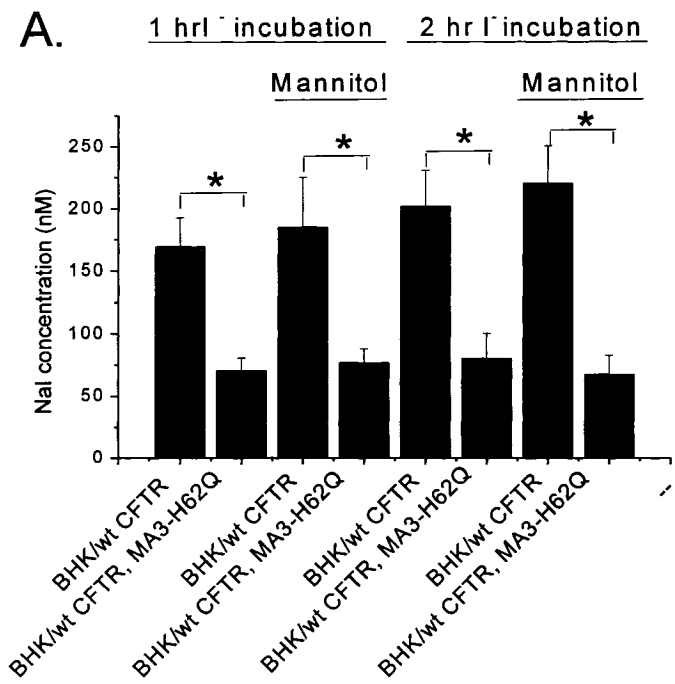
Cell pellet iodide and protein concentration are mean value ± s.e.,n=4,

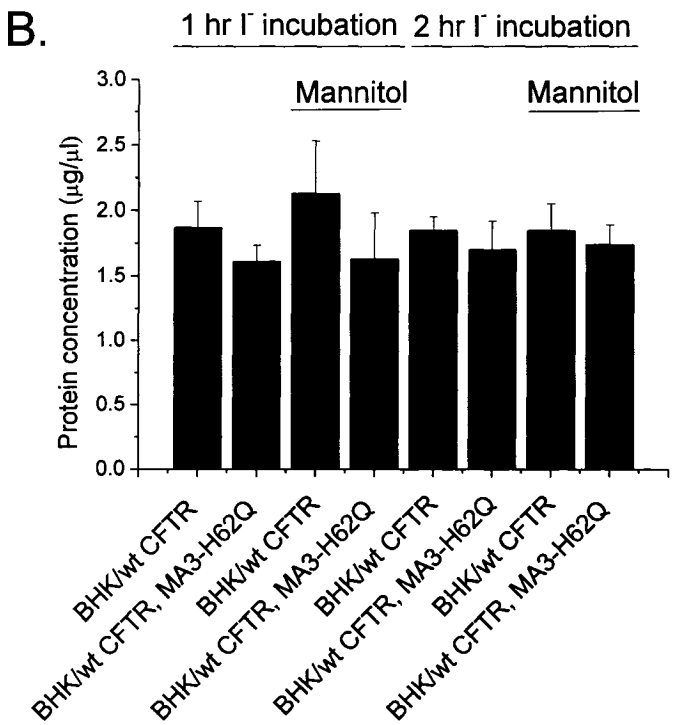
* indicates difference from BHK/wt CFTR value statistically significant at $p<0.05$.

Taken together, these results indicate that the iodide loading is less efficient in cells that overexpress MA3-H62Q and iodide leakage is elevated when they are rinsed to remove extracellular iodide buffer. This was further confirmed by measuring [I^-] in cells immediately after the loading period. Cells were incubated in iodide buffer containing calyculin A at 37 °C for 1 hr or 2 hr, washed with cold efflux buffer containing calyculin A, then lysed to measure iodide content. Long incubations in iodide loading buffer increased the [I^-] in BHK/wt CFTR cells, but not in MA3-H62Q cells. More importantly, the iodide concentration in MA3-H62Q cells was only half that in BHK/wt CFTR cells, making it impossible to compare MA3-H62Q and control cells. To exclude the possibility that low iodide concentration results from activation of some other anion channel secondary to cell swelling, mannitol was added to inhibit volume-regulatory channels. Iodide concentration in BHK/wt CFTR cells was increased by the presence of mannitol, however uptake was not enhanced in MA3-H62Q cells (Figure 11 A). Total protein concentrations were identical in the samples used for intracellular iodide measurements, therefore difference in [I^-] were not caused by variations in cell number (Figure 11 B).

Figure 11 Iodide loading of cells overexpressing MA3- H62Q is reduced compared to that of control BHK/wt CFTR cells

Iodide content was measured in lysates immediately after 1 or 2 hr iodide loading in the presence of calyculin A. **(A)** Intracellular iodide was consistently lower in the cell line expressingphosphatase dominant-negative phosphatase and was unaffected by the addition of mannitol to increase osmolarity. **(B)** Protein concentrations of cell lysates under the conditions used in (A), confirmed that similar numbers of cells were used for iodide assays under each condition.





NaI and protein concentration are mean value, n=4 , * $p<0.05$.

Chapter 4 Discussion

4.1 Inducible overexpression of PP2C isoforms in BHK cell lines

Protein phosphatase 2C (PP2C) is one of the four major protein serine/threonine phosphatase families (PP1, PP2A, PP2B and PP2C) in eukaryotes. There are at least nine distinct PP2C gene products (2Cα, 2C β, 2Cγ, 2Cδ, 2Cε, 2Cζ, 2Cη, Wip1 and Ca^{2+}/calmodulin-dependent protein kinase phosphatases) in mammalian cells, and most of these genes have multiple splice variants (Dai et al., 2006; Jin et al., 2004; Komaki et al., 2003). Dephosphorylation of CFTR by PP2C has been investigated during the last few years. Co-immunoprecipitation and crosslinking experiments indicated that CFTR and PP2C form a complex at the cell membrane (Zhu et al., 1999). Also, addition of PP2Cα from turkey gizzard reduced CFTR channel activity by more than 90% in excised patches (Luo et al., 1998). Co-expressing PP2Cα with CFTR in epithelial cells reduced the Cl^- current and increased the rate of channel deactivation (Travis et al., 1997). However, despite all these studies, the PP2C isoforms that dephosphorylate CFTR have not been identified. In this project, BHK cell lines were developed so that two mouse PP2Cs (splice variant #3 of the alpha isoform PP2Cα-3, or "MA3"; and splice variant #2 of the beta

isoform PP2Cβ-2, or "MB2.1") could be inducibly overexpressed in the presence of constitutive CFTR expression. I have focussed on these two PP2Cs because they were enriched in streptavidin pulldowns of CFTR that had been biotinylated on the cell surface (A. Evagelidis, unpublished data). In MA3 transfected cells, ponasterone A induced a band at 45 kDa as predicted from the molecular weight. By contrast, inducing cells that had been transfected with MB2.1 yielded two bands, one at ~45 kDa and the other at ~ 83 kDa. The latter band is probably caused by formation of a stable dimer. Heterologously expressed MA3 and MB2.1 had several characteristics of active PP2C enzymes. They both dephosphorylated a generic phosphatase substrate, their activities were dependent on mM levels of Mn^{2+}, and they were insensitive to potent PP2A and PP1 inhibitors. Furthermore, immunostaining revealed that MA3 and MB2.1 were present in both the cytosol and the nucleus, as previously reported (Das et al., 1996).

The effects of PP2C have been most intensively studied with respect to negative regulation of stress-activated protein kinase (SAPK) cascades and the control of cell cycle progression. When overexpressed in mammalian cells, human PP2Cα-2 inhibits stress-induced activation of p38 and JNK, but does not affect mitogen-induced activation of ERK

(Takekawa et al., 1998). Overexpressing PP2C β-1 in COS7 cells selectively suppresses the stress-induced activation of p38 and JNK but has no effect on the mitogen-induced activation of ERK(Hanada et al., 1998). In 293 cell clones, PP2Cα overexpression mediates cell cycle arrest in the G2/M phase followed by apoptosis (Ofek et al., 2003). An important role for PP2C isoforms in the regulation of receptors and channels also has been reported. PP2C α and Ca2+ form complex which is responsible for rapid dephosphorylation of Ca2+ channels and may contribute to regulation of synaptic transmission in neurons (Li et al., 2005). Furthermore, PP2C has been found to interact and dephosphorylate glutamate receptor 3 (Flajolet et al., 2003), and in *Arabidopsis thaliana*, PP2C has been shown to interact with the AKT3 potassium channel (Vranova et al., 2001) In this project, I developed the first inducible PP2C cell lines, and performed the first overexpression studies of MA3 (PP2Cα-3) or MB2.1 (PP2Cβ-2) after induction. These lines should be useful for studying MA3 and MB2.1 effects on CFTR, and the roles of these phosphatases in other cell signalling pathways.

4.2 Development of MA3 and MB2.1 dominant negative mutants

If cells express their own PP2C enzymes, heterologous overexpression may not have any further effect, thus inhibitors are very

useful when determining the physiological roles of phosphatases and kinases. Unfortunately, no pharmacological inhibitors of PP2C are available, although virtual screening has recently identified weak antagonists (70% inhibition at 100 μM) that might eventually be developed into useful inhibitors (Rogers et al., 2006). An alternative strategy is to use dominant negative mutants, which are widely used for studying protein functions in intact cells. A good example of the usefulness of dominant negative mutants is in studies of the Rab family of small GTPases. Endocytosis requires a balanced set of Rab proteins, and the correct regulation of the GDP-GTP cycle of these proteins. To study the function of particular Rabs at different steps of endocytosis, two Rab mutants are usually generated, a dominant negative mutant having reduced affinity for GTP, and a constitutively active mutant with reduced GTPase activity. Thus, in studies of Rab22a, cells expressing either wild type Rab22a or constitutively active mutants display decreased endocytosis whereas overexpression of a dominant negative mutant, which strongly affects the morphology of endosomes, does not inhibit endocytosis (Mesa et al., 2001). Another example is Rab5. Overexpression of constitutively active Rab5 mutants dramatically increases fluid phase and receptor-mediated endocytosis and leads to the

formation of enlarged early/sorting endosomes. Conversely, overexpression of a dominant negative Rab5 mutant reduces endocytic uptake and leads to the formation of a diffuse network of small endocytic vesicles (Li and Stahl, 1993).

In this project, MA3 mutants (MA3-D282N, and MA3-H62Q) and MB2.1 mutants (MB2.1-D286N, MB2.1-D286N, H62Q) were developed based on recent structure-function studies of PP2C residues carried out by John Denu and collaborators (Jackson et al., 2003). Mutants did not display *p*NPP phosphatase activity, consistent with the study of Jackson et al. (2003), and had similar cellular localization as the wild type protein (data not shown). Interestingly, the molecular weight of MA3-D282N appeared shifted according to Western blots, suggesting this mutant retains some secondary protein structure in SDS which differs from wild type protein, and which affects its mobility on SDS-PAGE gels.

Since most cells contain their own endogenous PP2C and no specific PP2C inhibitors are available, development of inducible PP2C dominant negative mutant cell lines was a promising approach in the present study. However, dominant negative mutants of PP2C have not been attempted previously, and we cannot exclude the possibility that they affect other cell signalling pathways and ion channel activities, which may

impact cell physiology. Surprisingly, overexpression of the wild-type or dominant negative MB2.1mutants did not significantly affect iodide efflux or alter cell appearance or proliferation rate, and results with the MA3 constructs were equivocal. Thus PP2C appears to play a minor role in for the cell signalling pathways regulating these processes.

4.3 Which phosphatase(s) dephosphorylate CFTR?

Previous studies suggested that multiple phosphatases are required to dephosphorylate CFTR. To further identify the phosphatase(s) that regulate CFTR, iodide efflux experiments were performed in this project as an assay of CFTR channel deactivation. Iodide efflux is a simple and widely used method for studying CFTR but has some disadvantages. The main limitation is that ion concentrations and electrical are not controlled and thus change during the course of the experiment, therefore effluxes can not be related quantitatively to channel activity, and the method is not well suited to measuring subtle regulation. Since iodide efflux is driven in part by the membrane potential, which depends on potassium channels and other conductances, changes in iodide efflux can also result indirectly from alterations in those pathways.

Although potent inhibitors of PP2A have little effect on chloride currents in secretory epithelia, electrophysiological evidence for PP2A

dephosphorylation of CFTR has been obtained in sweat ducts and cardiac cells(Hwang et al., 1993; Reddy and Quinton, 1996). PP2A interacts with CFTR by mass spectrometry, yeast hybrid, pull downs, and co-immunoprecipitations(Vastiau et al., 2005b; Thelin et al., 2005). Here we showed that CFTR indeed has higher sensitivity to the agonist cpt-cAMP when PP2A is inhibited by calyculin A, consistent with previous studies.

When transiently expressed in BHK cells, MB2.1 was pulled down with biotinylated CFTR using streptavidin beads, but there is no other functional evidence that MB2.1 is the isoform which regulates CFTR. Indeed, co-localization of MB2.1 and CFTR could not be detected by confocal microscopy after immunostaining, and CFTR activity was neither decreased by overexpressing MB2.1, nor increased by overexpressing dominant negative mutants of MB2.1 with, or without, calyculin A. Nevertheless these results should be confirmed using a more sensitive method such as patch clamping.

MA3 is another PP2C isoform that was pulled down with cell surface CFTR. MA3 and CFTR co-localization was detected at the cell membrane under confocal microscopy after immunostaining, which may reflect lower and/or transient interaction of MA3 with CFTR on cell membrane. However, overexpressing MA3 did not reduce CFTR

activation by cpt-cAMP. Since this negative result might be explained if cells already had high levels of endogenous PP2C, we tested the effect of overexpressing dominant negative MA3 variants. Unfortunately, low intracellular iodide levels made it impossible to compare effluxes from the mutant and control cell lines, when iodide efflux experiments were performed using cells that overexpress MA3-H62Q or MA3-D282N.

I used several protocols to determine if low iodide levels were caused by reduced loading or excessive leakage during the initial wash. To increase intracellular iodide levels at the beginning of the efflux period, I extended the loading period and inhibited CFTR channel activity when rinsing the cells to remove iodide. A previous study showed that CFTR channel activity is inhibited by low temperature(Mathews et al., 1998), therefore cells were cooled on ice. This increased intracellular iodide levels, but did not fully offset the reduction in iodide loading. Increasing the iodide incubation time and inhibiting volume-regulatory channels also did not increase cell iodide levels significantly.

These results suggest that overexpressing MA3-H62Q may affect other ion transporters involved in iodide uptake. SLC26A4 (or Pendrin) belongs to a relatively new gene family of anion exchangers capable of transporting a wide variety of monovalent and divalent anions. Different

transporters of the SLC26 protein family share the sulfate transporter domain (Bateman et al., 2004), which is most likely involved in the anion exchange mechanism, and a STAS (Sulphate Transporter and AntiSigma factor antagonist) domain, which binds of nucleotides and seems to mediate interactions with CFTR (Ko et al., 2004). SLC26A4 mediates iodide efflux in mammalian cells, such as human embryonic kidney 293 cells, COS-7 cells, Chinese hamster ovary cells and polarized Madin-Darby canine kidney (MDCK) cells (Taylor et al., 2002; Yoshida et al., 2002; Gillam et al., 2004), and since exchangers can operate in either direction, they might also mediate iodide fluxes during iodide loading studies.

Thus it is interesting to speculate that hyperphosphorylation of this exchanger may inhibit its activity, and reduce iodide loading. Alternatively, in cells expressing MA3-H62Q inhibiting PP2C may activate SLC26A directly or through CFTR, and induce iodide efflux. Further studies are needed to understand the mechanism by which MA3-H62Q inhibits iodide uptake.

Summary and Future Prospects

CFTR is an epithelial chloride channel which is tightly regulated by phosphorylation. Previous studies suggested that PP2A and PP2C are two major phosphatases that deactivate CFTR. In order to identify PP2C isoforms which regulate CFTR, I developed BHK cell lines which inducibly overexpress MA3 or MB2.1, the two PP2C isoforms pulled down with cell surface CFTR in preliminary experiments. I also mutated MA3 and MB2.1 to generate putative dominant negative mutants, and overexpressed these in BHK cells using an inducible expression system. BHK inducible cell lines overexpressing wild type and mutant MA3/MB2.1 allowed, for the first time, a determination of the effect of these two isoforms on CFTR activity and on other PP2C regulated cell signalling pathways in intact cells. Immunostaining and iodide efflux experiments did not provide evidence that MB2.1 regulates CFTR; however partial co-localization of MA3 and CFTR was detected using confocal microscopy. Definitive demonstration of the role of MA3 was precluded by the unexpected finding that expression of the MA3 dominant negative phosphatase inhibits iodide loading, which makes it impossible to investigate CFTR regulation using the iodide efflux technique.

To further explore the effect of MA3, MB2.1 and dominant negative mutants, other methods will be needed, such as patch clamp, for studies of CFTR regulation.

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