

BICARBONATE SECRETION, CYSTIC FIBROSIS AND CONGENITAL
CHLORIDE DIARRHEA: MOLECULAR MECHANISMS IN ION
TRANSPORT AND DISEASE

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To my family, Richard, Doris and Brian Dorwart, for all your love and support.

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CHLORIDE DIARRHEA: MOLECULAR MECHANISMS IN ION
TRANSPORT AND DISEASE

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2006

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Acknowledgements

Successful completion of a Ph.D. degree is a significant milestone in every student's professional and personal life. While every student's Ph.D. experience is different, there are some common elements which all students share, namely the support of others. During my time as a Ph.D. student I have interacted with numerous faculty members, post-docs, students and support staff who have all helped shape my Ph.D. experience. The scientific atmosphere here at UT Southwestern Medical Center is simply fantastic. It has been a tremendous place to study, with vast physical and intellectual resources which have allowed me to grow as a scientist and as a person. The list of people whom I need to thank for their help, guidance and support both here at UT Southwestern Medical Center and outside the confines of this institution is too long to ever fully document. So to everyone who has helped me along this journey, I am forever grateful and I sincerely say "Thank You!"

The person who has made the biggest impact on my Ph.D. experience is undoubtedly my mentor Philip J. Thomas. I cannot thank you enough for all that you have done for me over the past six years, from giving me the opportunity to study and work in your lab, to giving me the intellectual freedom to pursue my own scientific interests, for everything you have done for me I will forever be grateful. I would also like to thank you for putting together a group of scientists and support staff who are not only talented scientifically, but who are also

amazing people in their own right. Coming to work every day with such a wonderful group of people has made my experience as a student all that much better, so thank you Phil for cultivating such a wonderful laboratory environment for all of us to work in.

I have also had the distinct pleasure to work with some highly talented graduate students and post-docs during my graduate career that I will always be grateful for. The students in the Thomas' lab Patrick Thibodeau, Rhesa Stidham, Jonathan Moody, Karen Lewis, Michael Brinkman and Juan Mendoza have been scientific colleagues as well as friends. We have spent time together in the lab working on experiments, sharing ideas and helping one another interpret data which has enriched my experience here as a student, and I feel that I am a better scientist because of it. We have also spent some time together outside of lab, and whether we were going to a baseball game, making sushi or drinking "spikearitas," it was always enjoyable and you all have made such a positive impact on my life. Thank you so very much.

I have also had the great fortune to work with some highly talented post-docs who have helped me develop into the scientist that I am today. Chris Wigley taught me the importance of focus, and always being able to succinctly answer "what is the question you are asking?" Thank you to Chang-Wei Liu who showed me where hard work and dedication can get you in this world, and to Michael Corboy who always kept the lab atmosphere interesting. I am also thankful for

John Richardson, Balajee Somalinga and Li Zhu for their input at lab meetings as well as the culinary recipes that we have exchanged with one another!

Our laboratory would not have run nearly as efficiently as it has without the help of a talented support staff. My thanks goes to all of the people who have helped the lab run smoothly over the last six years, but an extra thanks needs to be given to our lab manager and fellow Canadian Linda Millen. Thank you Linda for all that you have done for me and the lab during my tenure here, without you things would not only have been chaotic, but the lab would not have been as much fun without your charming personality and sense of humor. Thanks needs to be given to Elizabeth Barney who has helped with cell culture experiments as well as helping to keep the day to day operations of lab running smoothly. I am also grateful to Jarod Watson who has kept our shelves stocked by ordering our required laboratory reagents.

Outside of the Thomas lab there are a number of people who need to be recognized for their support during my studies here at UT Southwestern Medical Center starting with my dissertation committee Johann Deisenhofer, Kevin Gardner, Shmuel Muallem and Stephen Sprang. All of you have made vital contributions to my research projects, and for that I will always be grateful. I would like to say a special thank you to Shmuel Muallem who allowed me to come into his lab and learn how to measure cellular bicarbonate transport which peaked my interest in transport physiology and for better or worse, has altered my

professional development and long term goals. I would also like to thank Kevin Gardner and Stephen Sprang for leading the Molecular Biophysics program which I have had the pleasure of being part of during my time as a student.

I would like to thank our collaborators starting with Shmuel Muallem and Shigeru Ko, Meeyoung Park, Nikolay Shcheynikov, and Weizhong Zeng who are current or past members of the Muallem laboratory. I would also like to thank Jennifer Baker in Julie Forman-Kay's laboratory at the University of Toronto for their NMR work on the SLC26A3 STAS domain and CFTR R-domain. In addition, I would like to thank Carlos Amezcua who collected a number of Aquifex STAS HSQC spectra for me here at UT Southwestern. I would like to thank the UT Southwestern Biopolymers facility who worked with me to obtain a variety of mass spectrometry and N-terminal sequencing data and to Helen Yin's laboratory for their technical advice on my immunofluorescence experiments. A special thank you needs to be given to the Deisenhofer laboratory and to Andrew Ferguson in particular, for his help in our attempts to crystallize a STAS domain.

One of the wonderful things about my Ph.D. experience has been how many wonderful people I have met along the way. People like Andrew Ferguson who I met here on campus at a Molecular Biophysics Discussion group, and has now become one of my closest friends. Thank you very much for being such a wonderful friend and for your advice, both professional and personal, during my time as a student. I would like to thank my friends Debbie Ferguson, Carolyn

Cummins, Dan Adamson and Scott Enke for their friendship and support throughout my Ph.D. work.

For anyone who knows me, they know that I love the game of hockey. While the vast majority of my time here in Dallas has been spent on my studies, I have taken the opportunity to play and coach some hockey here in Dallas. I have met a number of wonderful people through the game of hockey during my time here in Dallas, too many to single out by name so collectively I say thank you for all of your friendship and support.

Last, but certainly not least, I would like to thank my family for all their love and support over the past seven years. For those who know me well, know that I love my family, and for me, family always has been and always will be the most important entity in my life. I have had the great fortune in my life to be born into a very loving and supportive family who has always been there for me when I needed them. I would like to thank my parents Richard and Doris Dorwart for all of your love and support throughout my time here as a graduate student, but also throughout my entire life. Without your love and support I would not have been able to complete this dissertation, and surely would not be the person that I am today. Whether I needed a few extra dollars at the end of the month to make ends meet, an airline ticket home to see you and Brian for the weekend, or simply listening to me on the phone and giving me the advice and encouragement that I needed, you were always there for me and I will always be truly grateful. In

addition to my parents I would also like to thank my brother Brian Dorwart. Simply put, you are the best brother anyone could ever ask for, and you have supported me in countless ways over the past seven years. Thank you for all of your love and support, not just over my graduate student career, but throughout my entire life.

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CHLORIDE DIARRHEA: MOLECULAR MECHANISMS IN ION
TRANSPORT AND DISEASE

Publication No. _____

Michael Richard Dorwart, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, December 2006

Philip J. Thomas, Ph.D.

Cystic Fibrosis (CF) and Congenital Chloride Diarrhea (CLD) are two genetic diseases which without treatment can be fatal. Cystic Fibrosis is a disease which disrupts normal fluid secretion in multiple organs and tissues, and is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Congenital Chloride Diarrhea is a disease caused by mutations in

the SLC26A3 gene, which cause patients to suffer from watery stool and dehydration. The proteins encoded by the CFTR and SLC26A3 genes have been demonstrated to be a chloride channel and a chloride-bicarbonate exchanger respectively. They have also been shown to reciprocally activate one another's transport activity. Understanding how these proteins transport chloride and bicarbonate across the apical membrane of epithelial cells, and how they mutually activate one another's transport activity is critical for our understanding of CF and CLD.

The SLC26A3 protein is a membrane protein predicted to contain 12 transmembrane spanning α -helices, and a C-terminal STAS domain which is homologous to the bacterial anti-sigma factor antagonists. The STAS domain is required for proper SLC26A3 chloride-bicarbonate exchange function, and it is also required for the reciprocal activation of SLC26A3 and CFTR activities. Here we investigate the molecular interaction between the STAS domain of SLC26A3 and the R-domain of CFTR, as well as the molecular mechanism(s) by which four CLD causing mutations (Δ Y526/7, I544N, I675/6ins and G702Tins) residing in the SLC26A3 STAS domain lead to disease. The STAS domain and R-domain have been demonstrated to directly bind to one another, and this interaction is modulated by phosphorylation of the R-domain. Functional, biochemical and cell biological experiments performed on wild type and mutant SLC26A3 proteins suggest that the CLD mutations cause transporter misfolding and/or

mistrafficking. Biochemical and biophysical studies performed on the purified STAS domains suggest that these CLD causing mutations have differential effects on the STAS domain's structure. Our data taken together suggest that the CLD causing mutations cause disease by at least two distinct molecular mechanisms, ultimately leading to loss of functional protein at the plasma membrane.

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Prior Publications

Michael Dorwart, Nikolay Shcheynikov, Jennifer Baker, Michael Brinkman, Julie Forman-Kay, Shmuel Muallem, Philip Thomas. The effect of CLD causing mutations in the STAS domain on SLC26A3 function. *In Preparation*

Youxue Wang, Abigail A. Soyombo, Nikolay Shcheynikov, Weizhong Zeng, **Michael Dorwart**, Christopher R. Marino, Philip J. Thomas and Shmuel Muallem. Slc26a6 regulates CFTR activity *in vivo* to determine pancreatic duct HCO₃⁻ secretion: Relevance to Cystic Fibrosis (2006) *EMBO J.*, In Press.

Nikolay Shcheynikov, Youxue Wang, Meeyoung Park, Shigeru B.H. Ko, **Michael Dorwart**, Satoru Naruse, Philip J. Thomas, and Shmuel Muallem. Coupling Modes and Stoichiometry of Cl⁻/HCO₃⁻ Exchange by slc26a3 and slc26a6 (2006) *J Gen Physiol.* May;127(5):511-24.

Michael Dorwart, Patrick Thibodeau and Philip Thomas. Cystic fibrosis: recent structural insights (2004) *J. Cystic Fibros* Aug;3 Suppl 2:91-4.

Shigeru B.H. Ko, Weizhong Zeng, **Michael R. Dorwart**, Xiang Luo, Kil Hwan Kim, Linda Millen, Hidemi Goto, Satoru Naruse, Abigail Soyombo, Philip J. Thomas & Shmuel Muallem. Gating of CFTR by the STAS domain of SLC26 transporters (2004) *Nat Cell Biol.* Apr;6(4):343-50.

Nikolay Shcheynikov[‡], Kil Hwan Kim, Kyung-mi Kim, **Michael R. Dorwart**, Shigeru B. H. Ko, Hidemi Goto, Satoru Naruse, Philip J. Thomas, and Shmuel Muallem. Dynamic Control of Cystic Fibrosis Transmembrane Conductance Regulator Cl⁻/HCO₃⁻ Selectivity by External Cl⁻. (2004) *J. Biol Chem.* May 21;279(21):21857-65.

Hal A Lewis, Sean G Buchanan, Stephen K Burley, Kris Connors, Mark Dickey, **Michael Dorwart**, Richard Fowler, Xia Gao, William B Guggino, Wayne A Hendrickson, John F Hunt, Margaret C Kearins, Don Lorimer, Peter C Maloney, Kai W Post, Kanagalaghatta R Rajashankar, Marc E Rutter, J Michael Sauder, Stephanie Shriver, Patrick H Thibodeau, Philip J Thomas, Marie Zhang, Xun Zhao, and Spencer Emtage. Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator (2004) *EMBO J.* Jan 28;23(2):282-93.

Yong-Hwan Lee, **Michael R. Dorwart**, Karsten R. O. Hazlett, Ranjit K. Deka, Michael V. Norgard, Justin D. Radolf, and Charles A. Hasemann. The Crystal Structure of Zn(II)-Free *Treponema pallidum* TroA, a Periplasmic Metal-Binding Protein, Reveals a Closed Conformation (2002) *J Bacteriol.* April; 184(8): 2300–2304.

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List of Abbreviations

ABC	ATP Binding Cassette
ADP	Adensine 5'-diphosphate
AE1	Anion exchanger 1
ATP	Adensine 5'-triphosphate
AUC	Analytical ultra centrifugation
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
cAMP	Cyclic adensine 5'-monophosphate
CAP70	CFTR associated protein 70
CD	Circular Dichroism
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CLD	Congenital Chloride Diarrhea
ΔF508	Deletion of Phe 508 in CFTR which causes CF
ΔIVS STAS	Deletion of the IVS in the SLC26A3 STAS domain
ΔSTAS	Deletion of the STAS domain in SLC26A3
DLS	Dynamic Light Scattering
DMEM	Delbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DRA	Down regulated in adenocarcinoma

DTDST	Diastrophic Dysplasia sulfate transporter
DTT	Dithiothreitol
E3KARP	Na/H exchanger isoform 3 kinase A regulatory protein
EndoH	Endoglycosidase H
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tags
FITC	Fluorescein isothiocyanate
GDP-γS	Guanosine 5'-triphosphate with a sulfur atom replacing the γ- phosphate
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
HRP	Horseradish peroxidase
HSQC	Heteronuclear single quantum correlation
IPTG	Isopropyl β-D-thiogalactopyranoside
IVS	Intervening sequence
LB	Luria-Bertoni broth
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MBP	Maltose binding protein
NAD⁺/NADH	Nicotine adenine dinucleotide
NBD	Nucleotide binding domain

NHERF	Na/H exchanger isoform 3 regulatory factor
NMR	Nuclear magnetic resonance
O.D.	Optical density
PBS	Phosphate buffered saline
PDS	The gene responsible for Pendred Syndrome (also known as SLC26A4)
PDZ	PDS-95/disklarge/ZO-1
pH_i	Intracellular pH
PKA	Protein kinase A
PNGaseF	Peptide N-glycosidase F
PVDF	Polyvinylidene fluoride
rcf	relative centrifugal force
RIPA	Radio-immunoprecipitation assay
RMSD	Root Mean Square Deviation
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SLC4	Solute carrier family 4
SLC26	Solute carrier family 4
STAS	Sulfate transporter and anti-sigma factor antagonist
Sultr	Sulfate transporter
TBS	Tris buffered saline

TMD	Transmembrane domain
TRITC	Tetramethylrhodamine isothiocyanate
TROSY-HSQC	Transverse Relaxation-Optimized Spectroscopy
Ulp1	Ubiquitin like protease 1
UV	Ultraviolet

Chapter One

Introduction

All living organisms are composed of one or more cells and the physical boundaries of each cell are defined by the plasma membrane. The plasma membrane separates the intracellular environment, or cytosol, from the extracellular environment. This separation allows a different composition of molecules to be established in the cytosol than in the extracellular environment. In eukaryotic cells, intracellular membrane bound organelles exist that have a similar membrane composition to that of the plasma membrane. These additional intracellular membrane compartments allow for further separation of ions and molecules resulting in the specialization of each organelle's function.

Biological membranes of cells are composed of various lipid molecules which have hydrophilic "head groups" and hydrophobic "tails." These lipid molecules assemble into a lipid bilayer in which the polar head groups are facing the aqueous environment while the hydrophobic tails associate on the inside of the bilayer, forming an aqueous impermeable barrier. It is this hydrophobic barrier which prevents polar and charged molecules from freely passing through the membrane, and is the physical basis by which the cell can separate the extracellular environment from the inside of the cell.

In addition to lipid molecules, cholesterol and proteins are also found in cellular membranes. As a consequence of forming a lipid membrane, methods for moving charged and polar molecules across the membrane had to be developed by the cell. Through the course of evolution proteins, the primary structural and functional machines of the cell, evolved to satisfy this requirement. The movement of ions across the membrane is critical for cellular function, as it allows for the concentration of nutrients in the cell as well as excretion of metabolic waste products. Establishing ion concentration gradients across biological membranes is also critical for life as it is one of the fundamental energy sources in biological systems.

There are two main classes of membrane proteins which move ions and molecules across biological membranes: channels and transporters. Ion channels and transporters differ both in architecture and in function. Ion channels essentially form a selective pore or hole in the membrane that is opened and closed by a gate. When the gate is open, the ion specific to that class of channel can flow down its electrochemical gradient until equilibrium is established or the gate is closed. Transporters differ in the sense that they can move molecules against their electrochemical gradient by coupling the movement of a second molecule down its electrochemical gradient, or by utilizing another energy source such as ATP. Transporters are thought to function by binding their target

molecules and undergoing a conformational change which alters access to the extracellular and cytosolic environments.

Ion channels and transporters play a number of important roles in the human body. Ion channels in neurons are responsible for the rapid propagation of electrical signals across these cells, and when coupled with chemical signals between neurons form the basis of communication in our nervous system. Ion channels are also important for ion uptake in our intestine, which in turn forms the ionic gradient which causes water absorption in the colon. Ion transporters also play an important role in nutrient uptake, coupling sodium transport down its electrochemical gradient to the import of various sugars or amino acids into the cell.

Bicarbonate plays an important role in a number of physiologically important processes. Bicarbonate is used by our bodies to buffer the pH of our cells as well as secreted fluids. It plays a role in buffering the mucus lining of our stomach to protect it from the low pH of stomach acid, and it helps to regulate the pH and viscosity of the mucus lining in our lungs. Bicarbonate transporters in the pancreas are responsible for secreting high concentrations of bicarbonate which is responsible for helping to neutralize the acidic chyme passing from the stomach into the small intestine, as well as to keep the zymogen digestive enzymes secreted by the pancreas from prematurely activating before they reach the small intestine. Bicarbonate also plays a central role in our respiratory system.

Bicarbonate is in equilibrium with carbon dioxide which is a waste product of cellular respiration. This carbon dioxide waste can diffuse across the plasma membrane of the cell and into the bloodstream where most of it diffuses into erythrocytes. In the erythrocyte the enzyme carbonic anhydrase catalyzes the hydration of carbon dioxide to carbonic acid, which can then dissociate into bicarbonate and a proton. The bicarbonate ion is then efficiently transported back into the bloodstream via the chloride/bicarbonate exchanger 1 (AE1). The bicarbonate ion, which is more soluble in plasma than carbon dioxide, can then travel in the bloodstream to the lungs where the partial pressure of carbon dioxide is lower than it is in the blood. This results in the diffusion of carbon dioxide out of the blood and into the lungs, and drives bicarbonate transport back into the erythrocyte by mass action through AE1, where it is converted into carbon dioxide by carbonic anhydrase. The carbon dioxide in the erythrocyte then freely diffuses out of the cell and into the lungs to be exhaled, and thereby a major metabolic waste product of our bodies is efficiently removed.

With the number of physiologically important processes that ion channels and transporters play in the body, it should come as no surprise that when these processes are impaired, severe physiological consequences sometimes arise. Cystic fibrosis is a disease that is caused by mutations in a chloride channel named the cystic fibrosis transmembrane conductance regulator (CFTR). This disease affects the proper function of secretory epithelial cells and often results in

pancreatic and lung disease. In addition, mutations in the solute carrier 26 family member A3 (SLC26A3), which is a chloride-bicarbonate transporter, cause a disease named congenital chloride diarrhea. The studies described in this dissertation will focus on the structural and functional characterization of four congenital chloride diarrhea causing mutations found in the C-terminal domain of SLC26A3, as well biochemical studies of the intracellular domains of CFTR. Chapter II presents a review of the literature of CFTR structure, function and regulation as well as the function of SLC26A3 and other SLC26A family members. Chapter III describes experiments that addressed the biochemical properties of the first and second nucleotide binding domains of CFTR, along with a discussion of the three dimensional structure of the first nucleotide binding domain of CFTR. Chapter IV describes the expression and characterization of the biochemical, biophysical and functional properties of the C-terminal STAS domain of SLC26A3, while chapter V will discuss the molecular pathology of four congenital chloride diarrhea causing mutations impact these properties of the STAS domain as well as the properties of the full length transporter. Chapter VI presents structural data collected on the SLC26A3 STAS domain as well as a bacterial homolog of the STAS domain. In conclusion, chapter VII suggests future experiments that will extend and expand upon the work described in the previous chapters of this dissertation.

Chapter Two

Literature Review

Introduction:

The cell is the basic unit of life, and all organisms are comprised of one or more cells. A common feature among all cells regardless of the organism or the cell's specialized function is the plasma membrane. The plasma membrane is comprised of lipid molecules, which are amphipathic molecules that have a hydrophilic "head group" and a "hydrophobic tail." The amphipathic nature of these molecules allows them to form lipid bilayers where the "hydrophobic tails" orient themselves on the inside of the bilayer while the hydrophilic "head groups" face the aqueous environment (Figure 2-1). The plasma membrane of the cell extends around the cell and forms a water-impermeable barrier that separates the inside of the cell from the extracellular environment. This separation allows different concentrations of molecules to be established in the cytosol of the cell compared to the external environment, a process which is essential for life.

The hydrophobic core of the lipid bilayer prohibits most polar and charged molecules from passing through it unaided. The cell has evolved protein molecules that reside in the lipid bilayer to facilitate the movements of ions and other solutes across biological membranes. These membrane proteins catalyze

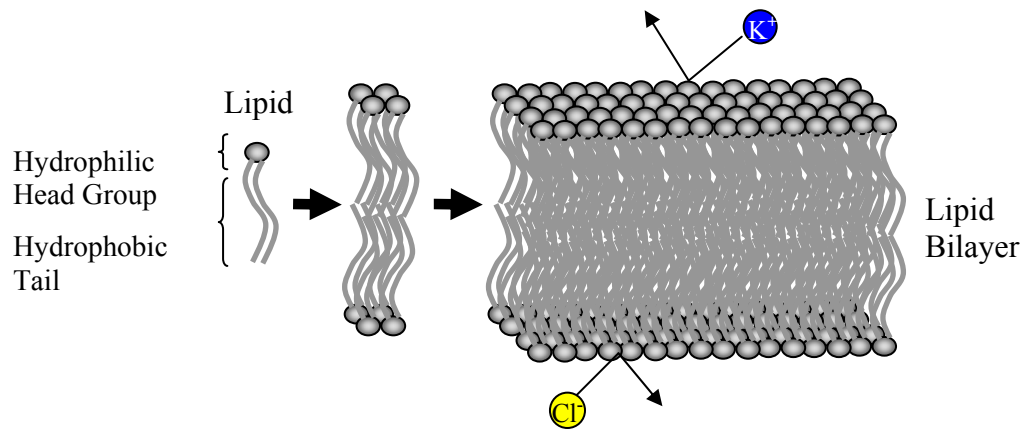


FIGURE 2-1: Lipid schematic and bilayer assembly. A cartoon model of a lipid molecule is shown depicting the hydrophilic head group and hydrophobic tail. Hydrophobic interactions between lipid tails and repulsive forces between the lipid tails and the aqueous environment result in the assembly of bilayers.

the movement of various solutes across cellular membranes, and are responsible for the cell's ability to concentrate nutrients in the cytosol and expel waste products into the extracellular environment.

Membrane proteins that move solutes across biological membranes can be divided into one of two broad classes of proteins: channels or transporters. Both channels and transporters have distinct functions in the cell as well as solute transport properties. Ion channels are comprised of a selective pore which typically allows for the transport of a specific ion across the membrane, and a gate that regulates the opening and closing of the pore. When the gate is open, the ion specific to the channel can flow down its electrochemical gradient until the gradient no longer exists and equilibrium is reached, or the gate is closed. Ion

channels are considered “passive transporters” since they use existing electrochemical gradients to move their solute across the membrane. In contrast to channels, transporter proteins can move a wide variety of molecules across biological membranes such as ions, sugars, amino acids and proteins. Transporter proteins are thought to function by altering access of the solute binding sites to the cytosol and extracellular environment by undergoing a conformational change. Transporters come in four main varieties: pumps, uniporters, symporters and exchangers. Pumps utilize chemical energy from molecules such as ATP to transport solutes against their electrochemical gradient. One distinguishing feature between pumps and the other transporters are that pumps are enzymes and can catalyze the hydrolysis of ATP to ADP and P_i , thereby utilizing the energy of ATP binding and hydrolysis for the vectorial transport of substrate. As a result pumps are often referred to as “primary active transporters,” as they are responsible for establishing electrochemical gradients that are used by “secondary active transporters” to move other solutes across membranes (see below). Uniporters on the other hand transport a single solute down its electrochemical gradient similar to ion channels, thus are considered “passive transporters.” Symporters and exchangers move two or more substrates across the membrane during each transport cycle. Symporters move their substrates in the same direction while exchangers move their solutes in opposite directions across the membrane. Depending upon the concentration gradients of the solutes,

symporters and exchangers can be “passive transporters” moving their solutes down their electrochemical gradients, or they can couple the movement of one solute down its electrochemical gradient to another solute which is transported up its electrochemical gradient. In the latter example, the transporter is releasing some of the potential energy stored in the electrochemical gradient by moving one solute “downhill,” while coupling that released energy to the movement of a second solute “uphill” against its electrochemical gradient. Since the transporter is using the energy stored in the electrochemical gradient to actively concentrate a solute, this membrane protein is considered to be a “secondary active transporter.”

The ATP binding cassette (ABC) transporter family is an example of “primary active transporters” which use the chemical energy stored in ATP to concentrate solutes against their chemical gradient, and will be reviewed in the following section of this chapter. Subsequent to the review of ABC transporters, the solute carrier family 26A (SLC26A), which are ion exchangers, will be extensively reviewed. The final section of this literature review will be on the structure and function of the SLC26A C-terminally conserved STAS domains, and their ability to regulate the function of a unique member of the ABC transporter family, the cystic fibrosis transmembrane conductance regulator (CFTR).

ABC Transporter Introduction:

ATP binding cassette (ABC) transporters move solutes across biological membranes by utilizing the energy of ATP binding and hydrolysis to pump a solute up its chemical gradient (Higgins, 1992; Schneider and Hunke, 1998). ABC transporters are found in all three kingdoms of life, and thus likely represent an ancient mechanism for solutes transport (Higgins, 1992). These transporters have a number of biological functions that include but are not limited to nutrient uptake, protein secretion, lipid transport, antibiotic resistance, and antigen presentation (Dean et al., 2001; Frelet and Klein, 2006; Higgins, 1992; Schneider and Hunke, 1998). In humans, ABC transporter dysfunction can lead to a variety of diseases such as Cystic Fibrosis, Tangier and Stargardt disease and Adrenoleukodystrophy.

ABC transporters can be recognized by their domain structure and conserved sequence motifs. Typically ABC transporters are composed of two transmembrane domains (TMDs), each containing 6 or more transmembrane α -helices, and two cytoplasmic nucleotide binding domains (NBDs) (Gottesman and Ambudkar, 2001; Higgins, 1992; Schneider and Hunke, 1998). The NBDs contain the highly conserved Walker A (GXXGXGKS/T) and extended Walker B ($\Phi\Phi\Phi\Phi$ DEP) motifs where X represents any amino acid and Φ is a hydrophobic amino acid, and a third highly conserved motif called the signature sequence (LSGGQ) (Higgins, 1992). The Walker A and B motifs are known to play a role

in ATP binding and hydrolysis in other proteins (Higgins et al., 1985), while the signature motif is unique to ABC transporters (Walker et al., 1982).

The architecture of ABC transporters often differ in prokaryotes and eukaryotes. In prokaryotes the TMDs and NBDs are often encoded by different genes while in eukaryotic organisms the TMDs and NBDs are often fused together as a single polypeptide (Figure 2-2) (Higgins, 1992). However, other domain organizations do exist with some ABC transporters existing as a single TMD fused with a single NBD, creating a “half transporter” or having the two TMDs fused together on a single gene and the two NBDs fused together on a separate gene (Figure 2-2). In some ABC transporters, additional regulatory domains exist which provide additional functionality for these transporters. The mechanism by which ABC transporters moves solutes across biological membranes, and the roles that the individual domains play are not completely understood. However, ABC transporters are predicted to function by coupling the energy of ATP binding and hydrolysis in the NBDs to substrate translocation through the TMDs. Recent findings have begun to elucidate the molecular mechanism(s) by which ABC transporters function, and the role that the individual domains play during the transport process.

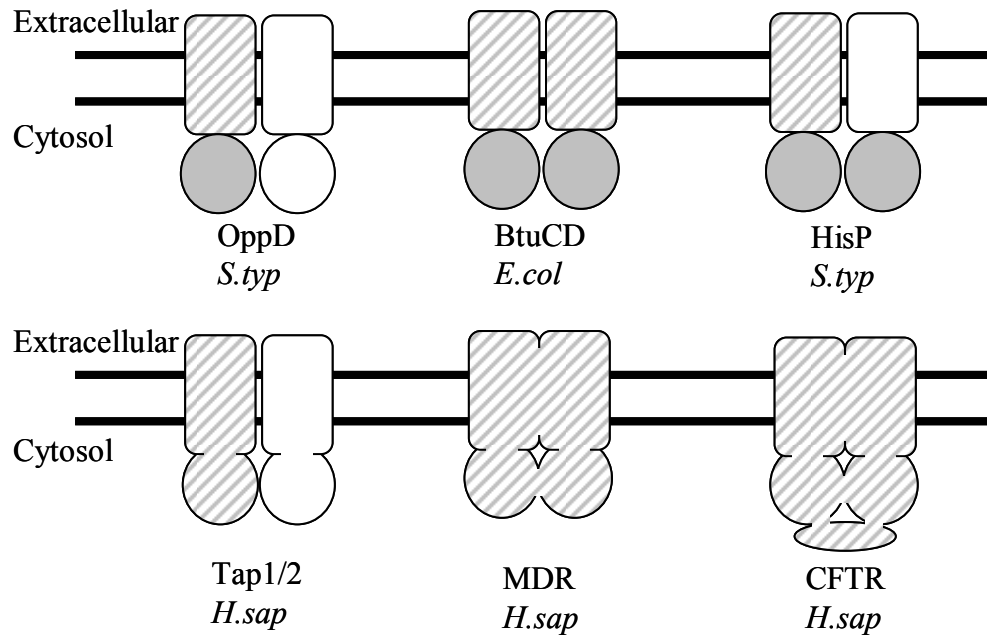


FIGURE 2-2: ABC transporter structure. ABC transporters typically contain four domains, two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). The genetic structure of these domains vary as they can exist as independent polypeptide chains that can assemble into active transporters, or they can be fused together in multiple ways to form the basic four domain structure. Six examples of ABC transporter domain architectures are shown with independent or fused domains, and identical or different NBDs or TMDs.

ABC Transporter NBD Structure and Function:

The NBDs of ABC transporters are approximately 200 amino acids in length and contain the conserved Walker A and B motifs along with the signature sequence which are thought to be required for ATP binding and hydrolysis (Higgins, 1992; Higgins et al., 1985; Walker et al., 1982). Indeed, many recombinantly expressed and purified NBDs have ATPase activity which supports the idea that the ATP binding and hydrolysis function of ABC transporters resides

in the NBDs (Morbach et al., 1993; Nikaido et al., 1997). Prior to 1998, no high-resolution structures of ABC transporters NBDs existed, and mutational studies provided the main source of information regarding these domains. The highly conserved Walker A lysine residue is thought to play a role in coordinating the γ -ATP phosphate. Thus mutation studies of transporters in which this lysine is mutated support this hypothesis, and typically result in proteins which have reduced ATP hydrolysis activities (Aparicio et al., 1996; Delepelaire, 1994; Lewis et al., 2004; Schneider et al., 1994). The crystal structures of the F₁-ATPase and the RecA protein suggested that the Walker B aspartate residue may be involved in hydrolysis of ATP (Abrahams et al., 1994; Story and Steitz, 1992; Story et al., 1992). Mutations of the Walker B aspartate residue also resulted in proteins that have abolished ATPase activity and nucleotide binding (Koronakis et al., 1995; Panagiotidis et al., 1993; Shyamala et al., 1991). Mutations in the ABC transporter signature sequence are also known to cause a loss of ATPase activity, suggesting that this sequence forms part of the ATP binding site (Bakos et al., 1997; Bliss et al., 1996; Browne et al., 1996; Hoof et al., 1994; Panagiotidis et al., 1993; Schmees et al., 1999).

The first crystal structure of an ABC transporter NBD was solved in 1998 (Hung et al., 1998). The HisP structure revealed a mixed α/β protein which can be divided into two sub-domains, one with a core Rossmann-like fold and a second sub-domain comprised of α -helices (Figure 2-3). The HisP NBD was crystallized

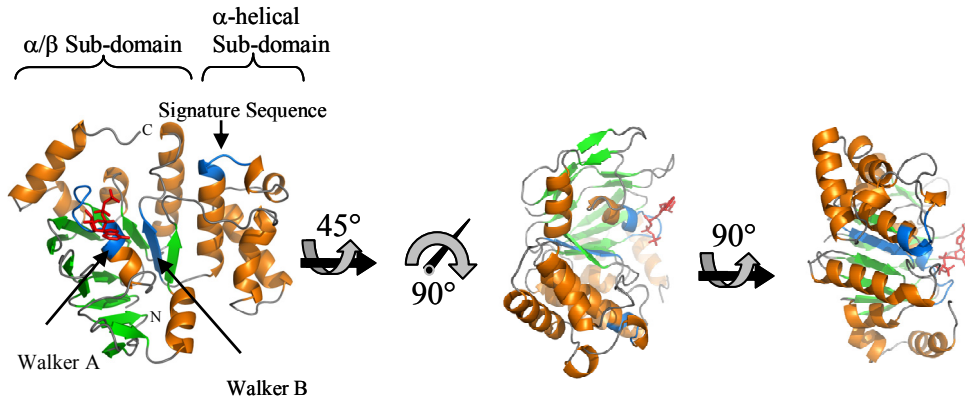


FIGURE 2-3: The crystal structure of HisP. The crystal structure of HisP (PDB: 1B0U) is shown in three different orientations. The α -helices and β -strands are colored orange and green respectively while the ATP molecule is colored red. The blue colored secondary structural elements represent the highly conserved Walker A and B motifs and the ABC signature sequence which are labeled appropriately along with the N and C-termini. Notice that HisP contains a mixed α/β and an α -helical subdomain, which can be easily observed in the orientation on the left.

in the presence of ATP which allowed the molecular interactions between the HisP residues and the ATP molecule to be better understood. As predicted, the Walker A lysine was found to help coordinate the γ -phosphate of the ATP molecule, while additionally conserved residues in the Walker A motif help coordinate the β -phosphate of the ATP molecule. The highly conserved Walker B aspartate was found to coordinate a water molecule that interacts with the γ -phosphate of ATP, and it was suggested that the position of this water would likely be the position of a Mg^{2+} ion in a hydrolytically competent complex based upon the RecA and F_1 -ATPase structures (Hung et al., 1998). The conserved glutamate residue just after the Walker B aspartate also interacts with a water

molecule which is in position to facilitate the hydrolysis of the β - γ phosphate bond, consistent with it being the “attacking water” for the ATP hydrolysis reaction, suggesting that this glutamate residue may serve as the activating residue. Interestingly, the signature sequence of HisP was not found to be close to the ATP binding site, and there was no obvious structural requirement for this sequence which could explain the high sequence conservation.

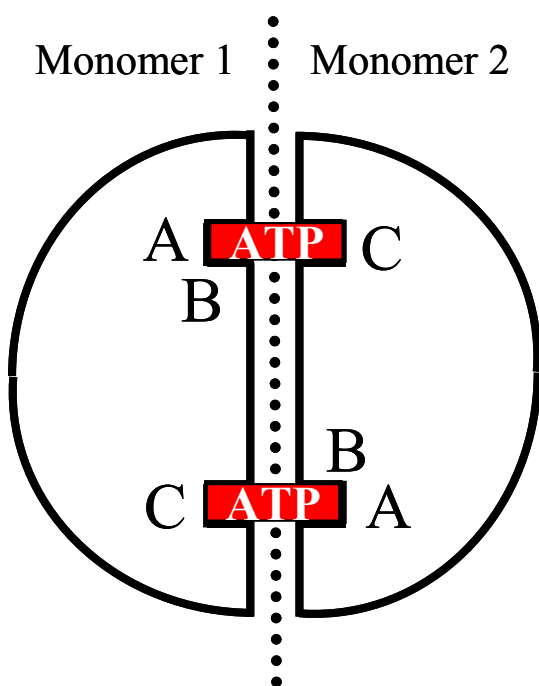


FIGURE 2-4: A cartoon schematic of an ABC transporter NBD dimer. A cartoon of two ABC transporter NBDs forming the proposed “head-to-tail” dimer. Notice that the ATP binding pocket is composed of the highly conserved Walker A and B motifs of one monomer, and the signature sequence (labeled C) of the opposing monomer.

Shortly after the publication of HisP, a mini-review of ABC transporters was published by Jones and George who suggested a functional role for the signature sequence (Jones and George, 1999). They proposed that ABC transporter NBDs form symmetrical head-to-tail dimers, where the Walker A and B motifs of one monomer are in close proximity to the signature sequence of the opposing monomer (Figure 2-4). Their hypothesis for an NBD dimer was based on the fact that the

conservation of the Walker A and B motifs was most likely due to their involvement in ATP binding and hydrolysis, and logic suggested that the conservation of the signature sequence would also be due to its involvement in ATP binding and hydrolysis. In addition, Jones and George proposed this model based upon another symmetry-related HisP dimer not discussed in the original publication that had two residues close to the signature sequence interacting with the ATP γ -phosphate. Models created from the HisP coordinates suggested that an NBD dimer could exist without significant steric clashes of backbone and side chain atoms which further supported their model for dimerization. This model also explained the previously reported data suggesting cooperativity between the two NBDs and their ATPase activities (Davidson et al., 1996; Liu et al., 1997).

ABC Transporter NBD Dimerization:

The first structural evidence to support the “head-to-tail” hypothesis came from the crystal structure of the DNA repair enzyme Rad50, a homolog of ABC transporter NBDs (Hopfner et al., 2000). The Rad50 structure showed a symmetrical dimer similar to that which was proposed by Jones and George a year earlier. However, subsequent to the publication of the Rad50 structure, four separate NBD crystal structures were published, none of which crystallized in a “head-to-tail” fashion (Diederichs et al., 2000; Gaudet and Wiley, 2001; Karpowich et al., 2001; Yuan et al., 2001). All of these structures revealed very

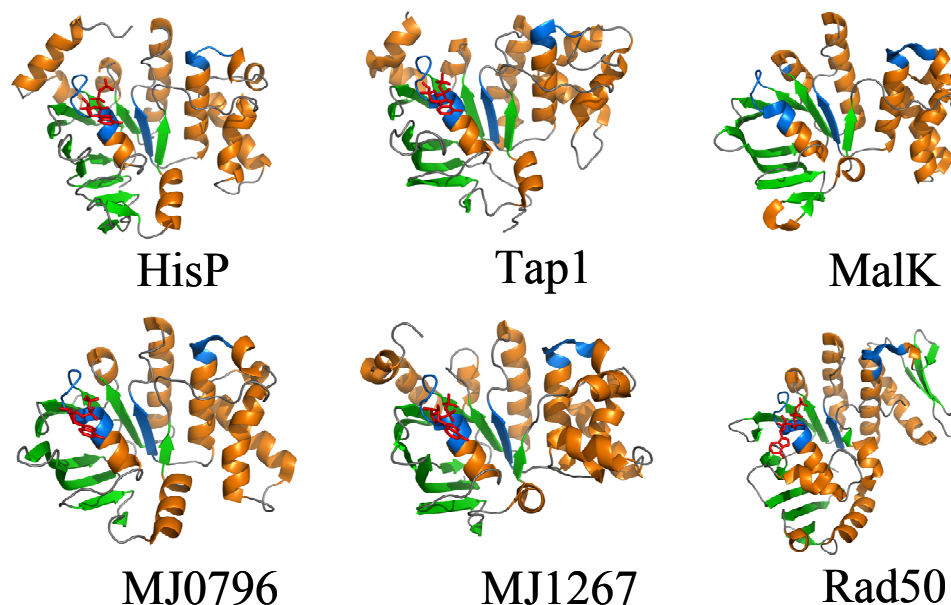


FIGURE 2-5: Examples of the ABC transporter NBD fold. Five crystal structures are shown of ABC transporter NBDs along with one NBD-like fold from the DNA repair enzyme Rad50. The PDB files shown in the figure are as follows: HisP 1B0U, Tap1 1JJ7, MalK 1G29, MJ0796 1F3O, MJ1267 1G6H, Rad50 1F2U.

similar overall structures to HisP (Figure 2-5), but it wasn't until the publication of the BtuCD and MJ0796 structures that the “head-to-tail” dimer was observed in ABC transporter NBDs (Locher et al., 2002; Smith et al., 2002) (Figure 2-6).

Both the BtuD and MJ0796 structures were “trapped” in a dimer state, BtuD by vanadate and the transmembrane domains of BtuC, while MJ0796 was trapped in a dimer by the mutation E171Q (Locher et al., 2002; Moody et al., 2002). The subunits in the vanadate trapped BtuD dimer are not as close together as those in the ATP MJ0796 dimer, suggesting that the BtuD dimer may represent a transition state of the NBD ATP hydrolysis cycle. The E171Q mutation in

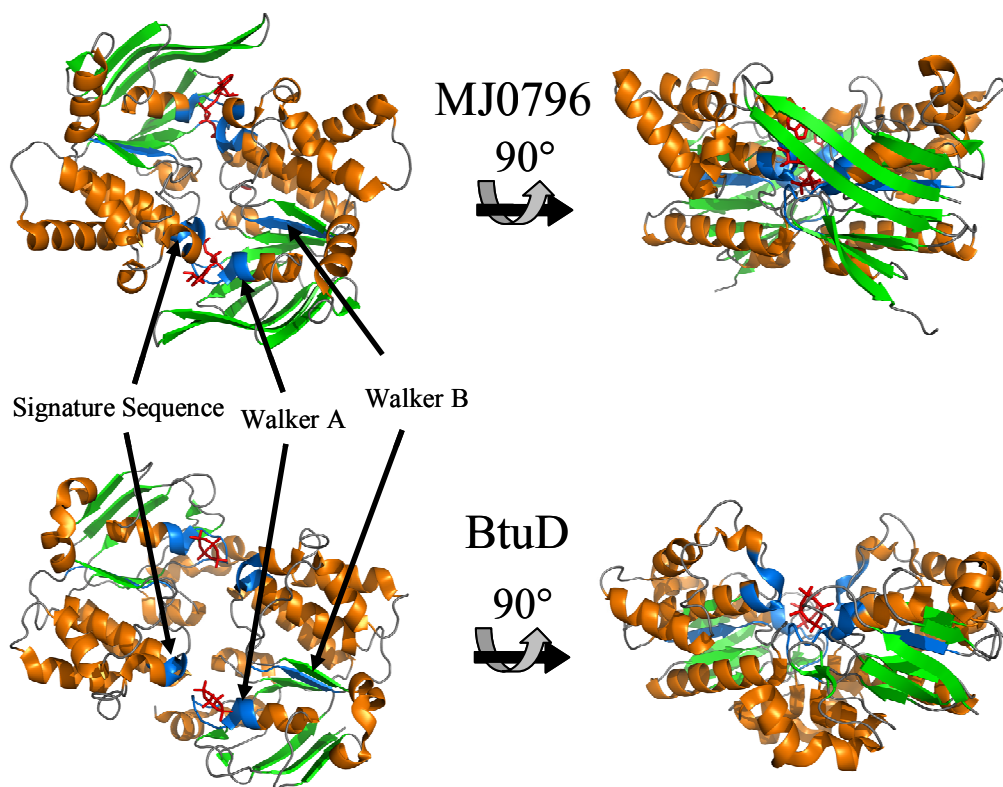


FIGURE 2-6: Crystal structures of ABC transporter NBD dimers. The crystal structures of E171Q MJ0796 (PDB: 1L2T) and BtuD (PDB: 1L7V) are shown with their α -helices and β -strands are colored orange and green respectively, while the ATP or vanadate molecules are colored red. The blue colored secondary structural elements represent the highly conserved Walker A and B motifs and the ABC signature sequence in the NBDs.

MJ0796 abolishes ATPase activity and allows for stable dimers to form in the presence of ATP as measured by gel filtration and analytical ultracentrifugation, whereas wild type dimers were not observable by these techniques (Moody et al., 2002). Since these initial studies, several other groups have observed the “head-to-tail” dimers in crystal structures including another full length ABC transporter as well as in isolated NBDs. These studies suggest that this dimeric arrangement

of the NBDs is functionally important for the transport activity of ABC transporters (Chang, 2003; Chen et al., 2003; Horn et al., 2003; Reyes and Chang, 2005; Verdon et al., 2003b; Zaitseva et al., 2005).

ABC Transporter Transmembrane Domains:

The transmembrane domains of ABC transporters provide the permeation pathway for solute transport across the membrane. ABC transporters typically have two transmembrane domains, each consisting of six transmembrane spanning α -helices (Higgins, 1992). However, the TMDs are not as well conserved across the ABC transporter superfamily as are the NBDs, thus variations in the number of TM spans do exist which may stem from the wide variety of solutes that ABC transporters move across membranes. The crystal structures of BtuCD from *E. coli* and MsbA from *E. coli*, *S. typhimurium* and *V. cholera* provide a molecular picture of the α -helical organization and a potential permeation pathway through the TMDs (Locher et al., 2002; Reyes and Chang, 2005) (Figure 2-7).

MsbA is a lipid flipase which transports lipid A and lipopolysaccharide (LPS) to the outer membrane (Karow and Georgopoulos, 1993; Polissi and Georgopoulos, 1996; Reuter et al., 2003; Zhou et al., 1998). Three different crystal structures have been solved for the MsbA protein and show varied structural details in the orientation of the TMDs and NBDs (Chang, 2003; Chang

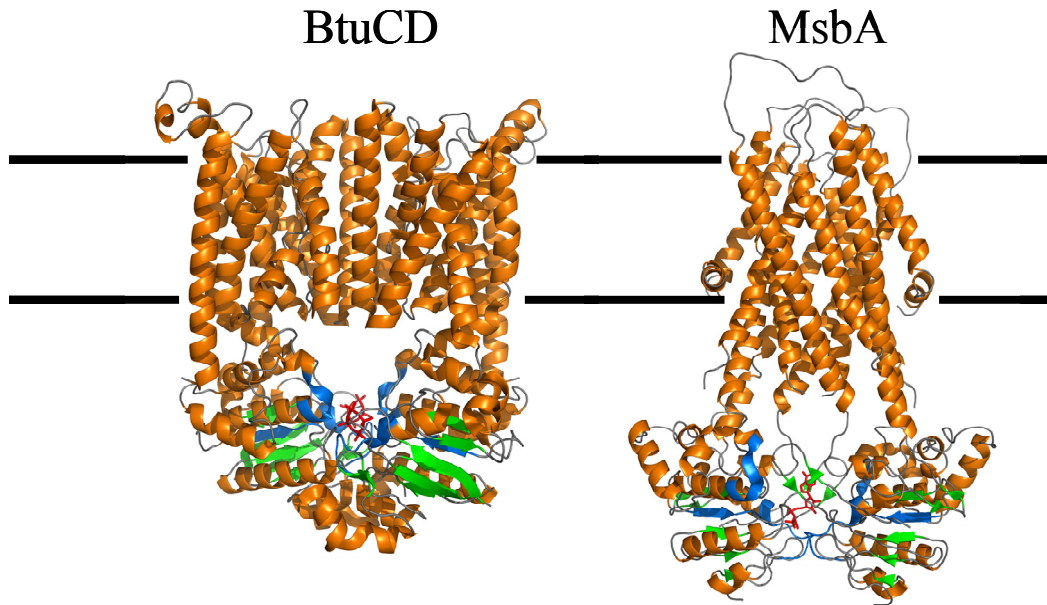


FIGURE 2-7: The crystal structures of complete ABC transporters. The crystal structures of the ABC Transporters BtuCD (PDB: 1L7V) and MsbA (PDB: 1Z2R) are shown. The α -helices and β -strands are colored orange and green respectively, while the ATP molecule is colored red. The blue colored secondary structural elements represent the highly conserved Walker A and B motifs and the ABC signature sequence in the NBDs. The horizontal lines represent the lipid bilayer in which the TMDs are imbedded. The TMD-NBD interface is composed of specific cytoplasmic loops of the TMDs and the α -helical sub-domain of the NBDs.

and Roth, 2001; Reyes and Chang, 2005). Each MsbA molecule has six transmembrane α -helices, which in the *E. coli* structure have portions that are not continuous α -helix. This is possibly due to the transmembrane spans being partially unfolded in the *E. coli* structure, which is in contrast to the *V. cholera* or *S. typhimurium* structures that have continuous transmembrane α -helices. Consistent throughout the three structures is TM span six which lines the inner cavity of the transporter and is thought to play a role in binding lipid A and

transporting it from the inner to outer leaflet of the membrane (Chang, 2003; Chang and Roth, 2001; Reyes and Chang, 2005). Indeed, when the MsbA monomers are superimposed upon one another, TM spans five and six show significant conformational changes in the post-hydrolysis *S. typhimurium* structure, consistent with these α -helices contributing to the transport cycle (Reyes and Chang, 2005).

BtuCD is a vitamin B₁₂ transporter and unlike most ABC transporters has ten transmembrane spanning α -helices instead of the canonical six. The BtuCD structure shows the TMDs closely packed with one another, and a cavity that is formed between the two BtuD monomers by TM spans five and ten (Figure 2-7). Although the BtuCD structure was crystallized in the absence of vitamin B₁₂, this cavity is of sufficient size to accommodate a vitamin B₁₂ molecule and is suspected to be the permeation pathway (Locher et al., 2002). The intracellular loop between TM spans four and five form the putative gate that separates the cytosol from the periplasmicly filled cavity (Locher et al., 2002).

The BtuCD and the *V. cholera* and *S. typhimurium* MsbA structures show intradomain contacts between the NBDs and the TMDs (Chang, 2003; Locher et al., 2002; Reyes and Chang, 2005). Much of the interaction between the TMDs and NBDs is formed by intracellular loops of the TMDs which interact with the α -helical sub-domain of the NBDs, as well as the groove formed between the α -helical and α/β core sub-domains. Previously, the α -helical sub-domain of the

NBDs had been postulated to be involved in providing specificity between NBDs and TMDs as well as coupling conformational changes in the NBDs from ATP binding and hydrolysis to conformational changes in the TMDs which ultimately result in substrate transport (Hyde et al., 1990).

Cystic Fibrosis Introduction:

Cystic Fibrosis (CF) is an autosomal recessive disease which causes dysfunction of epithelial cells in several organ systems. The loss of proper pulmonary function is the most common cause of death in CF patients. The loss of function in these organs arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes an ABC transporter (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). ABC transporters typically use the energy of ATP binding and hydrolysis to move solutes across biological membranes (Higgins, 1992; Schneider and Hunke, 1998). However, the CFTR protein is unusual among the ABC transporters in that it is not a transporter, rather it is an ATP gated chloride channel (Rich et al., 1990). Mutations in the CFTR gene that give rise to CF do so in a number of ways and as a result, CF causing mutations can be categorized into one of five classes. Class I mutations affect CFTR biosynthesis while class II mutations affect protein maturation (Kulczycki et al., 2003; Rowntree and Harris, 2003). Class III and IV mutations affect CFTR channel gating and conductance

respectively, while class V mutations lower the expression of the CFTR protein (Kulczycki et al., 2003; Rowntree and Harris, 2003). The most common CF causing mutation is the deletion of phenylalanine at position 508 ($\Delta F508$) in CFTR. This mutation is a class II mutation and accounts for approximately 70% of the CF alleles and roughly 90% of CF patients have at least one copy of this mutation (Kerem et al., 1989; Riordan et al., 1989).

The CFTR protein is found in multiple tissues and organs and as a result the loss of normal CFTR function impacts a wide variety of bodily functions (Gadsby et al., 2006; Rowntree and Harris, 2003). The CFTR protein plays a role in the exocrine pancreatic function which is responsible for secreting a number of enzymes that aid in the digestion of consumed foodstuffs (Ahmed et al., 2003; Choi et al., 2001a; Choi et al., 2001b; Soleimani and Ulrich, 2000). The loss of proper CFTR function in the pancreatic duct leads to blockage of the duct and retention of the digestive enzymes, which in turn digest the acini and islets cells and results in pancreatic insufficiency. Pancreatic insufficiency can result in malnutrition and death if left untreated. A genotype-phenotype correlation exists between mutations in CFTR and pancreatic insufficiency, while no such correlation exists for airway disease (Kulczycki et al., 2003; Rowntree and Harris, 2003; Zielenski, 2000). Airway disease in CF patients is complicated and not completely understood. The loss of normal CFTR function leads to viscous mucus accumulation in the lungs that is difficult for patients to clear (Moskowitz

et al., 2005). This leads to chronic bacterial infection by the *Pseudomonas aeruginosa* bacterium which causes inflammation and ultimately leads to lung failure (Moskowitz et al., 2005; Pier, 2002). The reason for the increase in viscosity of the airway mucus in CF patients is not well understood, and two hypotheses have been put forth to explain the increase in mucus viscosity. One hypothesis suggests that the loss of proper CFTR function leads to improper hydration of the mucus in the lung due to a reduced airway surface liquid layer (Guggino, 1999; Guggino, 2001; Song et al., 2003; Tarran et al., 2001; Zabner et al., 1998), while the second hypothesis suggests that the loss of proper CFTR function results in increased salt concentration in the airway surface liquid layer that inhibits the potency of the antimicrobial defensin molecules and causes mucins to precipitate out of solution (Bhaskar et al., 1991; Coakley et al., 2003; Devor et al., 1999; Lee et al., 1998). Regardless of the cause of the increased mucus viscosity, combating the *Pseudomonas aeruginosa* infections presents one of the greatest challenges to CF patients and physicians alike.

With the high prevalence of the $\Delta F508$ mutation in the CF population, and its highly deleterious effects on the patient's lung and pancreatic functions, it has been intensely studied. Many studies have focused on trying to understand what is wrong with the $\Delta F508$ protein and how can it be corrected. The $\Delta F508$ mutation has been shown to inhibit protein trafficking and maturation to the plasma membrane (Cheng et al., 1990; Ward and Kopito, 1994). If cells

expressing $\Delta F508$ CFTR protein are grown at a reduced temperature, some of the mutant protein can properly traffic to the plasma membrane (Sato et al., 1995). Growing cells expressing the $\Delta F508$ CFTR protein in the presence of osmolytes such as glycerol and myoinositol have also been demonstrated to help correct the trafficking defect of the mutant CFTR protein (Sato et al., 1995; Zhang et al., 2003). Once at the plasma membrane, the $\Delta F508$ CFTR protein does have some chloride channel activity (Cheng et al., 1995; Hwang et al., 1997; Sato et al., 1995; Zhang et al., 2003). This suggests, and gives hope, that if a therapeutic agent can be found to allow the proper trafficking of the $\Delta F508$ CFTR protein to the plasma membrane, it may restore proper epithelial function and help to reduce or eliminate the pancreatic insufficiency and airway disease that afflicts CF patients.

CFTR Structure and Function:

When the CFTR gene was cloned it was found to encode a 1480 amino acid protein which contained two putative transmembrane domains, two nucleotide binding domains and a unique regulatory or R-domain (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) (Figure 2-8). The domain structure of the protein along with the highly conserved Walker A and B motifs and the signature sequence suggested that the CFTR protein was a member of the ABC transporter superfamily. The cloning of an ABC transporter was

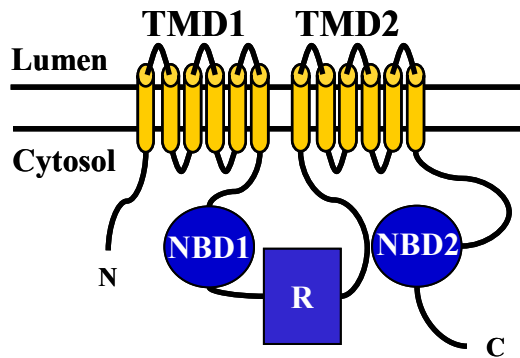


FIGURE 2-8: The domain structure of CFTR. A cartoon model of CFTR is shown with the NBDs and R-domain in the cytoplasm, and the two transmembrane domains consisting of six α -helices imbedded in the membrane.

unexpected, as it was previously known that CF patients had epithelia that were deficient in chloride transport and thus the assumption was that the protein responsible for CF was a chloride channel (Quinton, 1983). It was also known at the time that the chloride channel activity of secretory epithelial was cAMP protein kinase A (PKA) dependant, and the regulatory

domain of CFTR contained numerous PKA phosphorylation consensus sites (Riordan et al., 1989). As a result, the CFTR gene product was suspected of regulating chloride channel activity and hence the name cystic fibrosis transmembrane conductance regulator (Riordan et al., 1989).

Although the CFTR protein is a member of the ABC transporter family, it was discovered to have chloride channel properties itself (Anderson et al., 1991b; Kartner et al., 1991) which were regulated by PKA phosphorylation (Anderson et al., 1991a; Berger et al., 1991; Cheng et al., 1991). ATP is required for proper channel gating, and chloride conductance is observed only when ATP can bind to both NBD1 and NBD2 (Anderson et al., 1991a; Anderson and Welsh, 1992; Berger et al., 1991; Cheng et al., 1991). More recently it has been demonstrated

that ATP binding to NBD1 and NBD2 is required for opening of the CFTR channel, while hydrolysis of ATP at NBD2 closes the channel (Basso et al., 2003; Berger et al., 2005; Vergani et al., 2005; Vergani et al., 2003).

In addition to chloride channel activity CFTR has also been suggested to transport bicarbonate (Gray et al., 1990; Linsdell et al., 1997; Poulsen et al., 1994). This data is consistent with the observation that bicarbonate transport is altered in CF patients (Choi et al., 2001b; Johansen et al., 1968; Kopelman et al., 1988). Recently, CFTR was demonstrated to transport bicarbonate only in the absence of extracellular chloride (Shcheynikov et al., 2004). It was demonstrated that CFTR undergoes a conformational change in the absence of chloride which presumably alters the pore region of CFTR and allows for bicarbonate transport (Shcheynikov et al., 2004) (Figure 2-9). However, when chloride is present the CFTR pore is more selective for chloride than bicarbonate, suggesting that under many physiological conditions CFTR does not transport the majority of bicarbonate in secretory epithelial cells (Shcheynikov et al., 2004).

High-resolution structural information regarding CFTR is only available for NBD1 (Lewis et al., 2004; Lewis et al., 2005). The first structures reported for CFTR NBD1 were of mouse sequence, and revealed a number of important findings. Firstly, the domain structure of the mouse CFTR NBD1 was highly similar to other NBDs whose crystal structures had been previously solved (Chang, 2003; Chang and Roth, 2001; Chen et al., 2003; Diederichs et al., 2000;

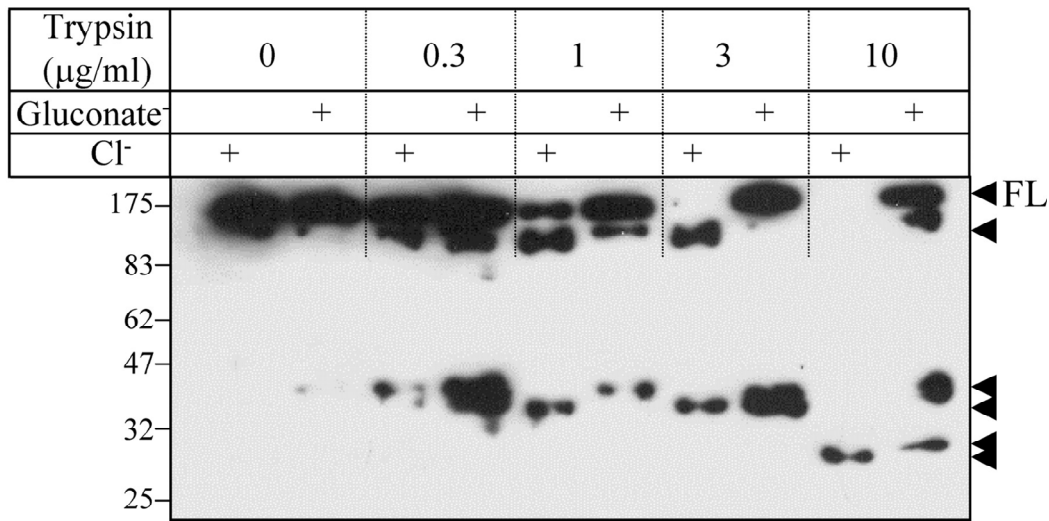


FIGURE 2-9: CFTR conformation is altered by Cl^- ions. Cell lysates from Calu-3 cells were made using buffers where the major anion was Cl^- or gluconate, as indicated in the figure. The lysates were subjected to digestion by various concentrations of trypsin for 10 min on ice and separated by SDS-PAGE. The digests were probed with a mixture of anti-CFTR antibodies to maximize detection of CFTR fragments. Similar results were obtained in four experiments. Arrowheads on the right mark CFTR fragments while *FL* indicates full-length. Molecular weight markers are measured in kDa. Adapted by permission from ASBMB: Shcheynikov *et al.* J Biol Chem (2004) 279(21):21857-65.

Gaudet and Wiley, 2001; Horn *et al.*, 2003; Hung *et al.*, 1998; Karpowich *et al.*, 2001; Locher *et al.*, 2002; Reyes and Chang, 2005; Schmitt *et al.*, 2003; Smith *et al.*, 2002; Verdon *et al.*, 2003a; Yuan *et al.*, 2001) (Figure 2-10). The structure also revealed that there was a large unstructured insertion between the first and second β -strands of the domain which had not been identified previously (Figure 2-11). As a result, the N-terminus of CFTR NBD1 is earlier in sequence than previously predicted (Lewis *et al.*, 2004). In addition to the location of the N-terminus, the C-terminus was also found to be further in sequence than predicted

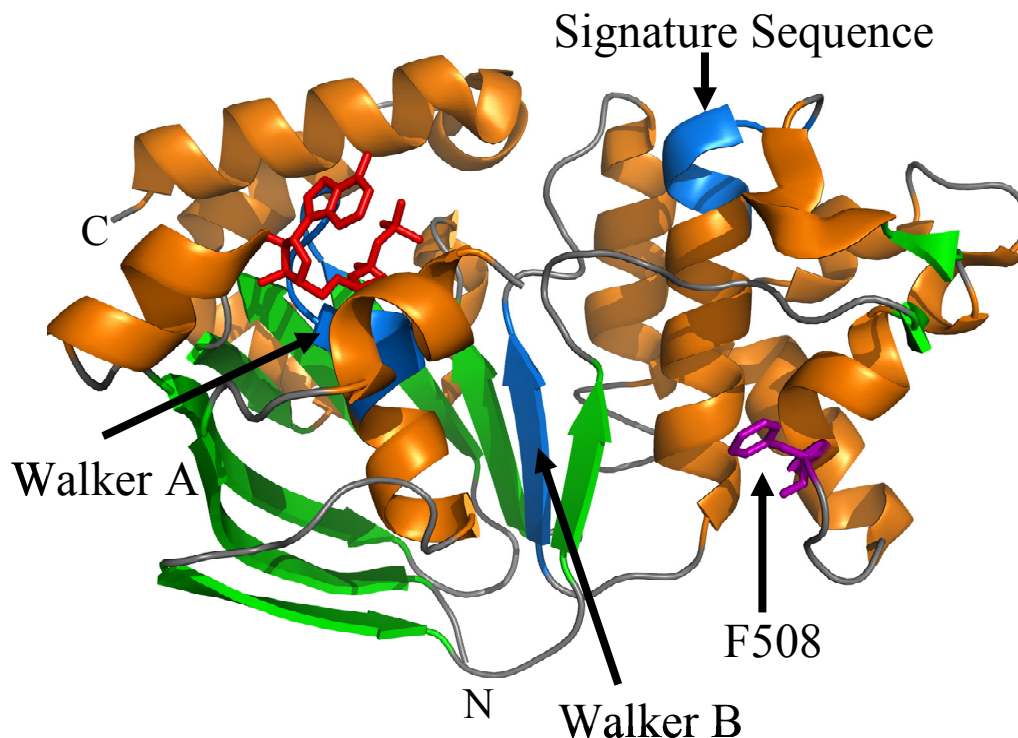


FIGURE 2-10: The crystal structure of mouse CFTR NBD1. The crystal structure of mouse CFTR NBD1 (PDB: 1R0X) is shown with its α -helices and β -strands colored orange and green respectively, while the ATP molecule is colored red. The blue colored secondary structural elements represent the highly conserved Walker A and B motifs and the ABC signature sequence. The F508 side chain is shown in stick figure representation and is colored purple.

(Lewis et al., 2004). The CFTR NBD1 structure also revealed that the F508 residue was on the surface of the domain near the end of the first α -helix in the α -helical sub-domain. Subsequently, the structure of human CFTR NBD1 was solved with and without the F508 residue, but with a number of solubilizing mutations (Lewis et al., 2005). The loss of the F508 residue did not grossly change the structure of the domain, consistent with previous suggestions that the

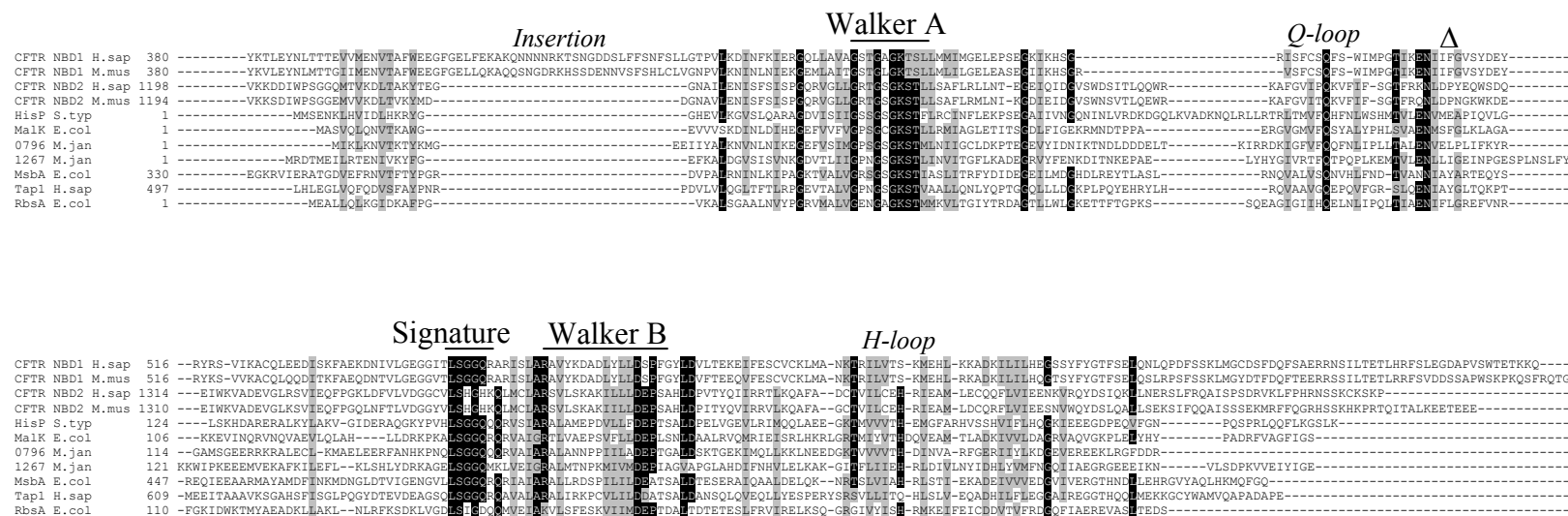


FIGURE 2-11: An ABC transporter multiple sequence alignment. A multiple sequence alignment of ABC transporter nucleotide binding domains was constructed using the T-coffee and Boxshade programs, and highlights the N-terminal insertion in CFTR NBD1 which was missed in previous alignments. The highly conserved Walker A and B motifs along with the signature sequence are denoted with a bar above the sequence and are labeled accordingly. The conserved Gln and His are labeled the Q and H-loops respectively, and the most common CF causing mutation, ΔF508, is denoted with a Δ. The names of the proteins and the numerical position of the first amino acid in the alignment are labeled on the left along with the species the protein sequence is from (H.sap-*Homo sapiens*, M.mus-*Mus musculus*, S.typ-*Salmonella typhimurium*, M.jan-*Methanococcus jannaschii*, E.col-*Escherichia coli*).

loss of F508 impacts the folding pathway of the protein and not the native state stability (Qu et al., 1997; Qu and Thomas, 1996; Strickland et al., 1997).

Functional data suggests that CFTR NBD1 and NBD2 form a heterodimer similar to those observed in previous crystal structures (Aleksandrov et al., 2002; Basso et al., 2003; Berger et al., 2005; Vergani et al., 2005; Vergani et al., 2003). This is consistent with the fact that critical residues involved in ATP hydrolysis are absent from both NBD1 and NBD2. The lack of a canonical signature motif in NBD2 and the lack of the conserved histidine and aspartate in the H-loop and Walker B motif respectively in NBD1 suggest that homodimers would not support ATPase activity (Figure 2-12). Consistent with this notion are biochemical data that suggest that NBD1 alone does not support ATP hydrolysis (Lewis et al., 2004; Yike et al., 1996). When NBD1 and NBD2 are mixed with one another the ATPase activity of the mixture is approximately three fold above the ATPase activity of NBD1 or NBD2 alone, consistent with the heterodimers forming a hydrolytically competent active site (Kidd et al., 2004).

In contrast to the NBDs of CFTR, the TMDs are functionally less well understood. Numerous mutational studies have been performed on residues in the TMDs in an attempt to define the pore region of CFTR. The results from many of those studies suggest that transmembrane spans 1, 5, 6 and 12 line the pore of CFTR (Cheung and Akabas, 1996; Cheung and Akabas, 1997; Mansoura et al., 1998; McDonough et al., 1994; Zhang et al., 2000; Zhang et al., 2002). Even with

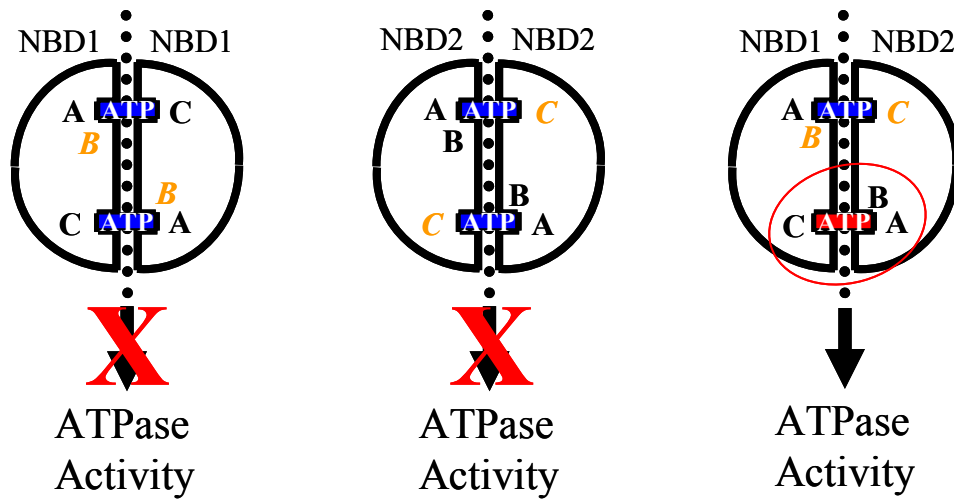


FIGURE 2-12: CFTR NBD sequence variations and their potential impact on NBD ATPase activity. Cartoon models of the three possible CFTR NBD dimers are shown with their Walker A, Walker B and signature sequences labeled A, B and C respectively. Canonical sequences are labeled in black while motifs that deviate from the consensus sequence are labeled in orange. The only dimer arrangement which allows for an ATP binding site that contains canonical Walker A and B motifs and a canonical signature sequences is an NBD1-NBD2 heterodimer. Presumably this is the only arrangement which would allow for ATPase activity.

the mutational data available a complete picture of the chloride permeation pathway through CFTR is not apparent, and a complete understanding of the CFTR pore and transport mechanism will likely require high resolution crystallographic studies of the TMDs.

The R-domain is unique to CFTR and contains multiple PKA phosphorylation consensus sites (Riordan et al., 1989). Eight serine residues are phosphorylated by PKA *in vitro* while five residues have been demonstrated to be phosphorylated *in vivo* (Cheng et al., 1991; Neville et al., 1997; Picciotto et al., 1992; Townsend et al., 1996). The R-domain has been shown to be highly

unstructured in solution by CD and limited proteolysis experiments, regardless of the phosphorylation state of the domain (Ostedgaard et al., 2000). The molecular mechanism by which the R-domain regulates CFTR activity is not well understood. Current models suggest that the R-domain activates CFTR by binding to unknown sites after PKA phosphorylation which results in a conformational change of CFTR that allows channel gating to occur in the presence of ATP (Chappe et al., 2005; Ostedgaard et al., 2001). In this sense the R-domain of CFTR is operating like the clutch of a car, where phosphorylation of the domain allows the clutch to engage and channel activity to occur. In the absence of phosphorylation the clutch of the car is disengaged, and channel activity is not observed.

In addition to the regulation of CFTR channel activity, the R-domain has also been shown to be involved in regulating the activity of members of the SLC26A family of proteins (Ko et al., 2002; Ko et al., 2004). In a reciprocal fashion, the intracellular domains of the SLC26A proteins activate CFTR's chloride channel activity, and this activity is mediated through the R-domain of CFTR (Ko et al., 2002; Ko et al., 2004). This novel regulatory interaction will be reviewed in the following section along with an extensive discussion on the SLC26A3 protein and additional SLC26A family members.

Introduction to SLC26A Transporters:

The solute carrier 26 A (SLC26A) family of proteins is a recently described family of anion transporters that are expressed in the apical or basolateral membrane of epithelial cells. These membrane proteins transport a wide variety of anions across cellular membranes that include chloride, bicarbonate, hydroxide, formate, oxalate, sulfate, and iodide (Jiang et al., 2002; Karniski et al., 1998; Moseley et al., 1999; Satoh et al., 1998; Scott and Karniski, 2000; Shcheynikov et al., 2006; Soleimani et al., 2001; Xie et al., 2002). This family of proteins currently has eleven members, ten of which have been shown to be expressed in humans and one, SLC26A10, which is thought to be a pseudogene (Mount and Romero, 2004). The individual SLC26A family members have various tissue expression patterns with some members being expressed in very specific cells of the body, such as SLC26A5, while others like SLC26A6 are expressed in many tissues throughout the body (Dawson and Markovich, 2005; Haila et al., 2001; Hoglund et al., 1996b; Jacob et al., 2002; Knauf et al., 2001; Lohi et al., 2000; Lohi et al., 2002a; Royaux et al., 2000; Satoh et al., 1998; Soleimani et al., 2001; Toure et al., 2001; Waldegger et al., 2001; Xie et al., 2002). Mutations in SLC26A2, SLC26A3, SLC26A4 and SLC26A5 have all been implicated in different human diseases, highlighting the important role the SLC26A transporters play in the human body (Everett et al., 1997; Hastbacka et al., 1994; Hastbacka et al., 1996a; Hastbacka et al., 1996b; Hoglund et al.,

1996b; Liu et al., 2003; Superti-Furga et al., 1996a; Superti-Furga et al., 1996b; Superti-Furga et al., 1999).

Because SLC26A transporters are newly described, little is known about them. The transport properties and biological functions are known for many of the SLC26A family members. A number of functional roles have been described for the SLC26A transporters which include thyroid hormone and cartilage synthesis, gastric acid secretion and neutralization, as well as skeletal development and the transport of various ions in the kidney (Mount and Romero, 2004). With the wide variety of anions transported and the various biological functions the SLC26A play in the body, it is not unexpected that the sequence identity of SLC26A homologs range from 21-43% (Mount and Romero, 2004). The SLC26A transporters typically are between 700-800 amino acids in length, although exceptions exist as SLC26A8 is 970 amino acids long. The N-terminal two-thirds of the protein are largely hydrophobic and presumably comprise the transmembrane spans of the transporter and form the ion permeation pathway. Various models have been put forth as to the number of transmembrane spans each SLC26A protein contains, but the emerging consensus is that most SLC26A transporters likely have twelve transmembrane spanning α -helices with their N and C-termini located in the cytosol (Lohi et al., 2002a; Lohi et al., 2003; Moseley et al., 1999; Navaratnam et al., 2005; Taylor et al., 2002; Toure et al., 2001; Zheng et al., 2001) (Figure 2-13). The C-terminal third of the SLC26A

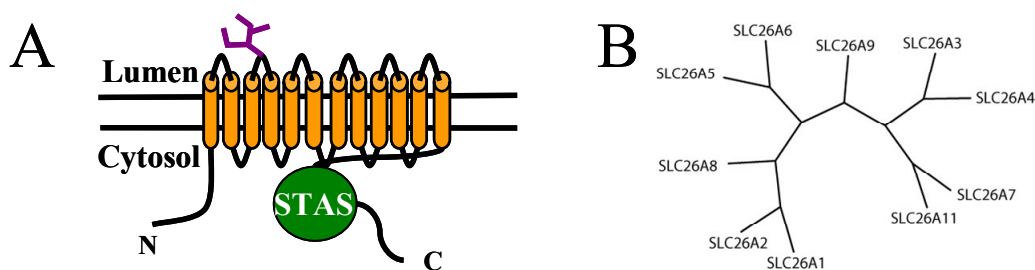


FIGURE 2-13: SLC26A domain structure and evolutionary relationships. (A) A cartoon model of the predicted domain structure of the SLC26A protein showing a TMD containing twelve α -helices and a cytosolic STAS domain. (B) A phylogenetic tree showing the evolutionary relationships between the eleven human SLC26A family members. The phylogenetic tree was made using the Molphy program and the STAS domains of the SLC26A proteins without the intervening sequence.

proteins is comprised of a conserved STAS domain (Aravind and Koonin, 2000).

The name STAS stands for sulfate transporters and anti-sigma factor antagonists, as these domains are homologous to the bacterial anti-sigma factor antagonists that regulate bacterial sporulation (Aravind and Koonin, 2000). A detailed description of the STAS domains and anti-sigma factor antagonists will be described in the last section of this literature review.

While the transport function of many of the SLC26A proteins is known, the regulatory mechanisms that control their function remain largely unknown. The SLC26A3, 6, 7, 8 and 9 proteins all contain a PDZ binding motif at their C-termini (Lohi et al., 2002a; Mount and Romero, 2004). The PDZ binding sequence of SLC26A3 have been demonstrated to bind to the PDZ domain-containing proteins E3KARP and CAP70, which have also been shown to bind to CFTR (Ko et al., 2004; Lamprecht et al., 2002; Rossmann et al., 2005; Sun et al.,

2000; Wang et al., 2002a). Indeed, functional regulation between CFTR and SLC26A3, SLC26A4 and SLC26A6 have been previously observed (Ko et al., 2002; Ko et al., 2004), suggesting a functional complex between the CFTR protein and some members of the SLC26A protein family.

SLC26A1:

SLC26A1, also known as the sulfate anion transporter or Sat-1, was first cloned from rat liver and demonstrated to be a glycoprotein with sulfate transporting properties (Bissig et al., 1994; Karniski et al., 1998). Subsequently, the SLC26A1 gene has been cloned from human, mouse and rainbow trout and shown to transport sulfate in exchange for chloride (Bissig et al., 1994; Katoh et al., 2006; Lee et al., 2003). Additional functional studies have shown that SLC26A1 can support sulfate transport as well as minimal amounts of chloride and oxalate (Bissig et al., 1994; Lee et al., 2003; Regeer et al., 2003).

The biological function of SLC26A1 is not entirely clear, however it has been suggested that SLC26A1 plays a role in sulfate transport in liver canalicular cells (Meier et al., 1985; Meier et al., 1987). In addition, it has recently been shown that SLC26A1 can mediate sulfate-bicarbonate exchange, further supporting the proposed role of SLC26A1 in sulfur transport in the liver (Quondamatteo et al., 2006). In addition to the liver, SLC26A1 is highly expressed in the kidney and expressed to lower levels in the pancreas and testes

(Regeer et al., 2003). This led Regeer and colleagues to suggest a role for SLC26A1 in sulfate reabsorption in the kidney. SLC26A1 has been demonstrated to be localized to the basolateral membrane of various epithelial cells (Karniski et al., 1998; Quondamatteo et al., 2006; Regeer and Markovich, 2004). In addition, a dilucine motif in the C-terminal domain of SLC26A1 has been suggested to be the basolateral targeting sequence, and when mutated the protein appears to be retained intracellularly (Regeer and Markovich, 2004). When the C-terminal 70 amino acids were placed on the Tac protein, which is targeted to the apical membrane, Tac traffics to the basolateral membrane. In addition, when the dilucine motif is removed by deleting the last 30 amino acids of the fusion protein, Tac regained apical membrane targeting. However, it is still possible that the targeting sequence is not the dilucine motif and resides elsewhere in C-terminal portion of SLC26A1.

SLC26A2:

SLC26A2, also known as DTDST, was discovered by positional cloning of the gene responsible for diastrophic dysplasia (Hastbacka et al., 1994). Dystrophic dysplasia is an autosomal recessive disorder that is characterized by dwarfism as well as bone and joint malformations. In addition to diastrophic dysplasia, three additional chondrodysplasias (achondrogenesis, atelosteogenesis type II and autosomal recessive multiple epiphyseal dysplasia) have also been

linked to mutations in the SLC26A2 gene (Hastbacka et al., 1994; Hastbacka et al., 1996a; Hastbacka et al., 1996b; Superti-Furga et al., 1996a; Superti-Furga et al., 1996b; Superti-Furga et al., 1999). The SLC26A2 gene product is a sulfate transporter that also likely transports chloride and bicarbonate (Satoh et al., 1998). Consistent with the chondrodysplasia phenotype, mutant SLC26A2 was found to be highly expressed in cartilage, where it may play a key role in the production of sulfated proteoglycans necessary for proper cartilage function (Forlino et al., 2005; Satoh et al., 1998; Superti-Furga et al., 1996c). SLC26A2 transcript has also been found in a variety of other tissues including the placenta, small intestine, colon and testis (Haila et al., 2001; Satoh et al., 1998) where it may play a role in sulfating other proteins such as mucins. The SLC26A2 protein is expressed in the apical membrane of epithelial cells, which is in contrast to its closest paralog SLC26A1, which is expressed in the basolateral membrane (Haila et al., 2001). A number of chondrodysplasia causing mutations in the SLC26A2 gene have been studied, and a genotype-phenotype correlation exists between the residual sulfate transport activity and the severity of the disease (Karniski, 2001; Karniski, 2004; Macias-Gomez et al., 2004; Maeda et al., 2006; Rossi and Superti-Furga, 2001).

SLC26A3:

The SLC26A3 gene was first identified as a gene that was down regulated in human colon adenomas and adenocarcinomas and hence named DRA (Schweinfest et al., 1993). SLC26A3 has not been conclusively proven to be a tumor suppressor gene, but it has been suggested to play a role in growth suppression and individuals with mutations in the SLC26A3 gene do have a slightly elevated risk of intestinal cancers (Antalis et al., 1998; Chapman et al., 2002; Hemminki et al., 1998). SLC26A3 transcript has been identified in the cecum, colon and prostate, while mouse SLC26A3 protein has been identified in the pancreas, small intestine and parotid ducts (Byeon et al., 1996; Greeley et al., 2001; Hoglund et al., 1996b; Ko et al., 2004; Silberg et al., 1995). The high level of SLC26A3 transcript observed in the colon is consistent with the proposed tumor suppressor role and potentially an anion transport role as well.

Functional studies on SLC26A3 have demonstrated that it is an anion exchanger which transports sulfate, oxalate, chloride, hydroxide and bicarbonate (Byeon et al., 1998; Chernova et al., 2003; Ko et al., 2004; Lamprecht et al., 2002; Moseley et al., 1999; Silberg et al., 1995). SLC26A3 has also been demonstrated to have a chloride:bicarbonate stoichiometry of 2:1 (Shcheynikov et al., 2006). Consistent with SLC26A3's chloride base exchange, mutations in the SLC26A3 gene were identified in patients who suffer from congenital chloride diarrhea (CLD) (Hoglund et al., 1996a). This disease is characterized by watery

diarrhea with low pH which results in dehydration and death if left untreated (Holmberg, 1986). Butyrate has been shown to be effective in relieving the diarrhea suffered by patients, while increased salt and water uptake are part of the traditional clinical management of the disease (Canani et al., 2004). Unlike the chondrodysplasias caused by mutations in SLC26A2, no genotype-phenotype correlation exists for CLD, and distinct clinical outcomes are possible depending upon the age of diagnosis and clinical intervention (Hoglund et al., 2001). Similar to CF patients, males who suffer from CLD often also suffer from infertility, which is consistent with the observation that the SLC26A3 gene is transcribed in the prostate (Hihnala et al., 2006b; Hoglund et al., 1996a; Hoglund et al., 2006).

The SLC26A3 protein has been demonstrated to be a membrane glycoprotein that resides in the apical membrane of epithelial cells (Byeon et al., 1996; Ko et al., 2004). A PDZ binding sequence resides on the C-terminus of SLC26A3 which has been demonstrated to bind to the PDZ domain containing proteins CAP70 and E3KARP (Lamprecht et al., 2002; Rossmann et al., 2005). CAP70 and E3KARP have also been demonstrated to interact with CFTR's C-terminal PDZ domain, likely co-localizing CFTR and SLC26A3 in the apical membrane (Lamprecht et al., 2002; Sun et al., 2000; Wang et al., 2002b). In addition, some CF patients have abnormal bicarbonate secretion and CFTR is not likely to be the primary bicarbonate transporting protein (Choi et al., 2001b; Greeley et al., 2001; Poulsen et al., 1994; Shcheynikov et al., 2004; Shumaker et

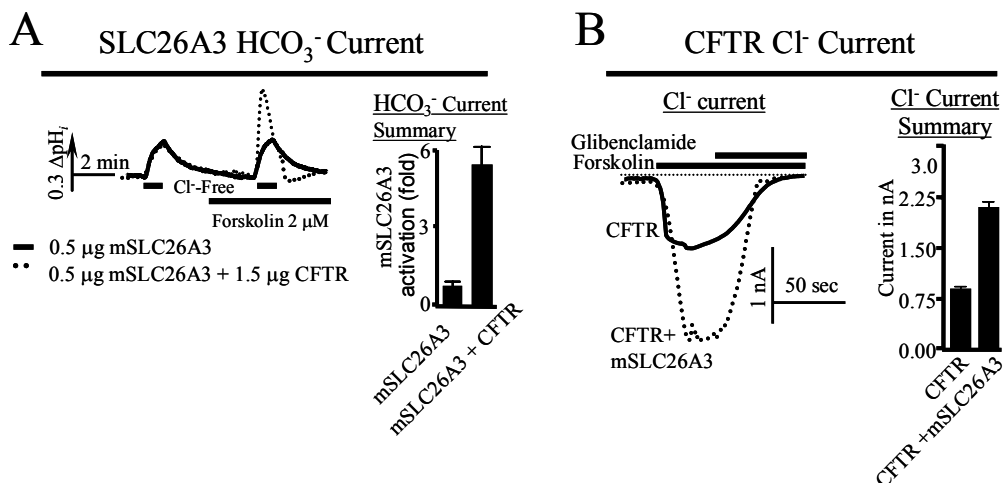


FIGURE 2-14: **Reciprocal activation of CFTR and SLC26A3.** (A) pH_i measurements were made on HEK 293 cells transfected with mouse SLC26A3 alone or co-transfected with CFTR. pH_i changes were measured upon removal of extracellular Cl^- in media buffered with HCO_3^- in the presence or absence of forskolin stimulation. (B) Cl^- current measurements were recorded from HEK 293 cells transfected with CFTR or co-transfected with CFTR and mouse SLC26A3 in the presence or absence of forskolin stimulation. Data collected by S.B. Ko.

al., 1999). Since CFTR was not likely the major bicarbonate transporting protein of secretory epithelia, it was possible that CFTR was regulating a bicarbonate transporter. Members of the SLC4 family of bicarbonate transporters (the anion exchangers AE1-4) as well as members of the SLC26A family of anion transporters were tested for their ability to be activated by CFTR. It was found that the SLC4 family of proteins was not activated by CFTR, however, members of the SLC26A family of transporters were markedly activated by CFTR (Ko et al., 2002) (Figure 2-14). In addition, CFTR's chloride channel activity was activated by the presence of SLC26A3 indicating that these two proteins reciprocally activated each other's ion transport activity (Ko et al., 2002) (Figure

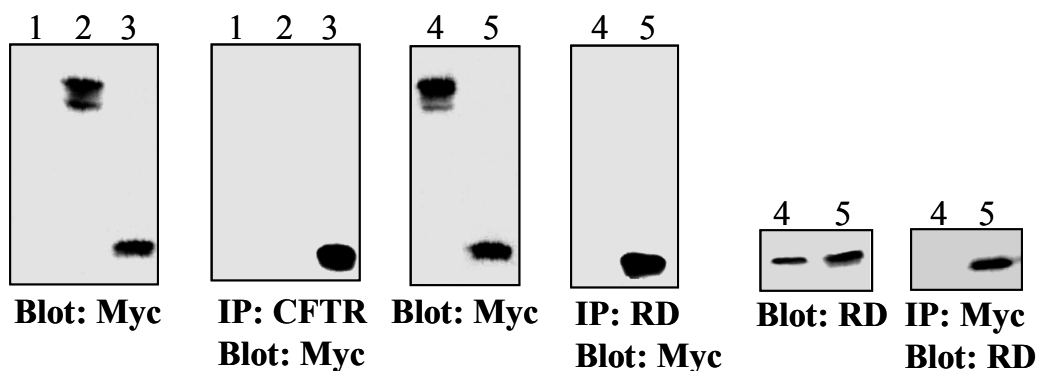


FIGURE 2-15: The STAS domain of SLC26A3 and the R-domain of CFTR are required for co-immunoprecipitation. Western blots and co-immunoprecipitation experiments of CFTR, CFTR R-domain (RD), mouse SLC26A3 with the STAS domain deleted (Δ STAS), and mouse SLC26A3 STAS domain (STAS) are presented with the blotting and immunoprecipitation antibodies indicated below the blots. The Δ STAS and STAS domain constructs are myc-tagged while the R-domain is HA tagged. The proteins transfected in each lane are as follows: lane 1) CFTR, 2) CFTR + Δ STAS, 3) CFTR + STAS, 4) RD + Δ STAS, 5) RD + STAS. Adapted by permission from Macmillan Publishers Ltd: Ko *et al.* Nat Cell Biol. (2004) 6(4):343-50.

2-14). The interaction between SLC26A3 and CFTR has been studied in detail which has revealed an approximately four fold enhancement of SLC26A3 exchange activity, and roughly a three fold activation of CFTR's chloride exchange activity (Ko *et al.*, 2004) (Figure 2-14). This functional activation suggested a possible physical association between SLC26A3 and CFTR. Co-immunoprecipitation experiments demonstrated a physical association between SLC26A3 and CFTR (Ko *et al.*, 2004) (Figure 2-15). The PDZ domains were found to be important for this interaction but not required as increased expression of mutant CFTR and SLC26A3 proteins that did not contain the PDZ binding

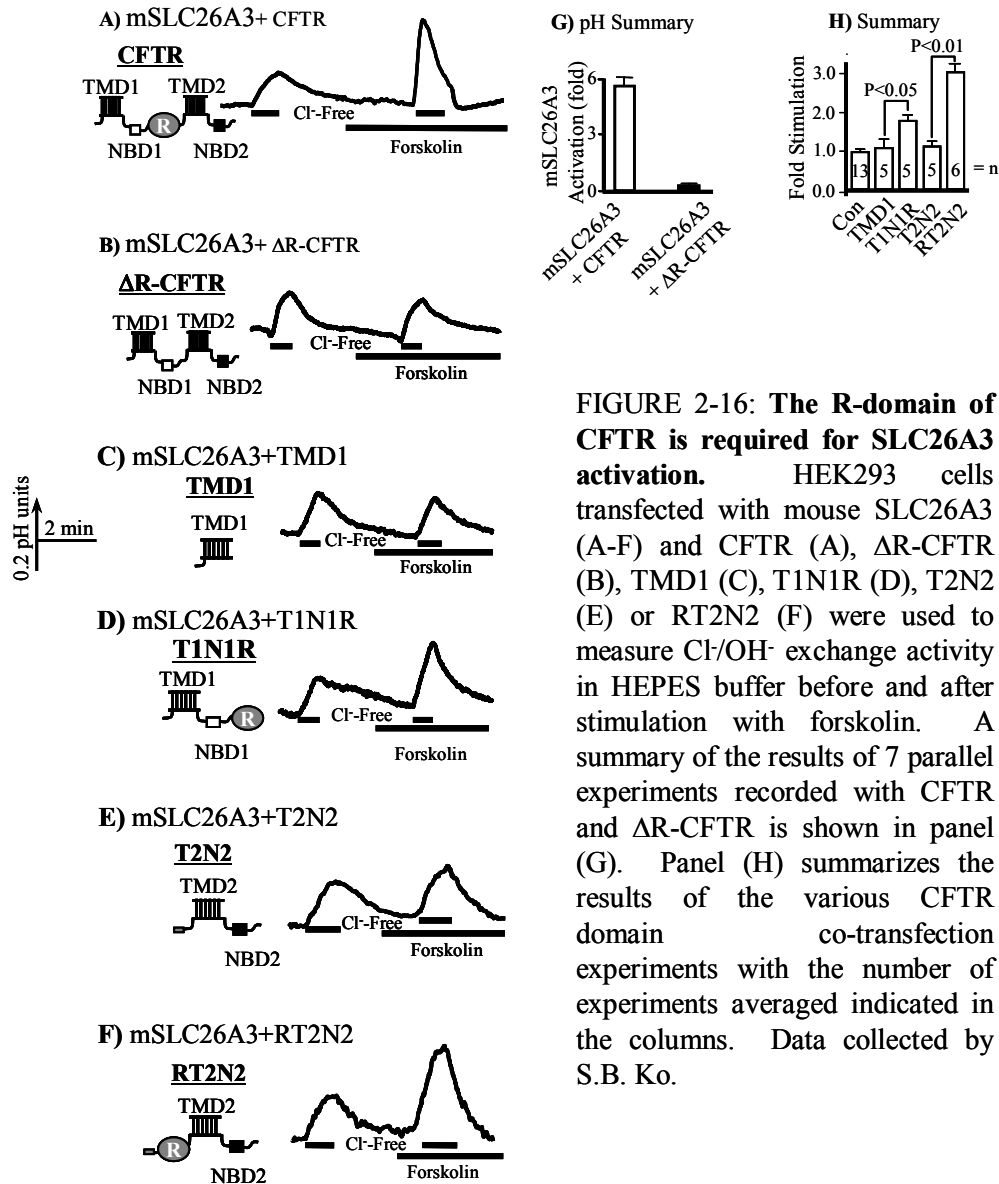


FIGURE 2-16: The R-domain of CFTR is required for SLC26A3 activation.

HEK293 cells transfected with mouse SLC26A3 (A-F) and CFTR (A), ΔR-CFTR (B), TMD1 (C), T1N1R (D), T2N2 (E) or RT2N2 (F) were used to measure Cl⁻/OH⁻ exchange activity in HEPES buffer before and after stimulation with forskolin. A summary of the results of 7 parallel experiments recorded with CFTR and ΔR-CFTR is shown in panel (G). Panel (H) summarizes the results of the various CFTR domain co-transfection experiments with the number of experiments averaged indicated in the columns. Data collected by S.B. Ko.

sequences preserved the co-immunoprecipitation interaction, whereas in the absence of over expression no interaction was detected (Ko et al., 2004). This interaction required the STAS domain of SLC26A3 and the R-domain of CFTR (Figure 2-15), suggesting that these domains play a role in the reciprocal

activation previously observed (Ko et al., 2004). Deletion studies of CFTR confirmed that the R-domain was required for SLC26A3 activation (Figure 2-16), while the STAS domain alone was sufficient to stimulate CFTR's chloride channel activity in cell culture models as well as in mouse parotid duct cells (Ko et al., 2004) (Figure 2-17). This data prompted further investigation of the STAS domain which is the subject of chapters four, five and six of this dissertation.

SLC26A4:

Positional cloning of the gene responsible for Pendred syndrome identified the fourth member of the SLC26A family (Coyle et al., 1996; Sheffield et al., 1996). Mutations in SLC26A4, also known as pendrin or PDS, is known to cause Pendred syndrome, which is the most common form of syndromic hearing loss, as well as DFNB4, which is a non-syndromic form of deafness (Campbell et al., 2001; Coyle et al., 1996; Fraser, 1965; Li et al., 1998; Prasad et al., 2004). In addition to deafness, patients with Pendred syndrome can also present with goiter suggesting a SLC26A4 function in the thyroid (Fraser, 1965). Consistent with clinical observations, SLC26A4 transcript and protein has been identified in the thyroid, kidney and cochlea (Everett et al., 1997; Royaux et al., 2000). SLC26A4 has been demonstrated to transport a wide variety of monovalent anions including chloride, hydroxide, bicarbonate, nitrate, bromide, iodide and formate, but not divalent anions such as sulfate (Royaux et al., 2000; Scott and Karniski, 2000;

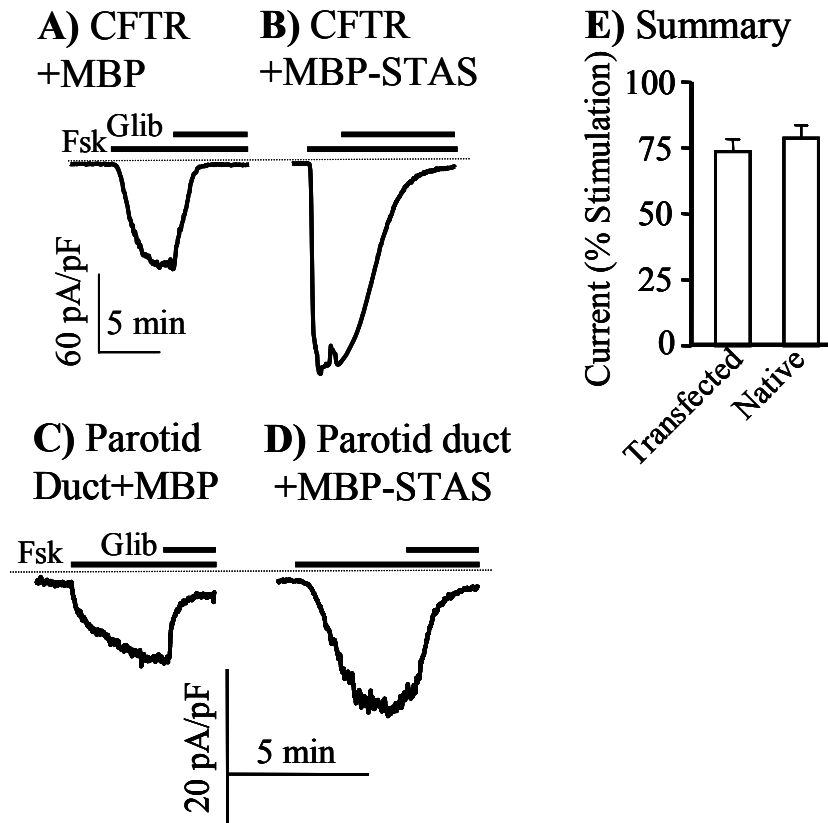


FIGURE 2-17: MBP-STAS activates CFTR in HEK 293 cells and in parotid duct cells. (A) and (B) show Cl^- current recordings from HEK 293 cells transiently transfected with CFTR and infused with either MBP or MBP-STAS and treated with forskolin (Fsk) or glibenclamide (Glib). (C) and (D) show Cl^- current recordings from mouse Parotid duct cells infused with MBP (panel C) or MBP-STAS (panel D) and treated with forskolin (Fsk) and glibenclamide (Glib). (E) A summary of the Cl^- current stimulation of the parotid duct cells and the transiently transfected HEK 293 cells. Adapted by permission from Macmillan Publishers Ltd: Ko *et al.* Nat Cell Biol. (2004) 6(4):343-50.

Soleimani *et al.*, 2001). Over 90 mutations have been identified in the SLC26A4 gene to date (www.medicine.uiowa.edu/pendredandbor), some of which have been biochemically characterized. Many of these mutations have been characterized using functional and cell biological techniques indicating that loss

of iodide efflux and ER retention are major causes of Pendred syndrome (Rotman-Pikielny et al., 2002; Taylor et al., 2002). A genotype-phenotype correlation has been suggested for DFNB4 and Pendred syndrome, however, other modifying genes and or environmental factors likely play a role in determining the severity of the disease (Campbell et al., 2001; Masmoudi et al., 2000; Scott and Karniski, 2000; Taylor et al., 2002).

SLC26A5:

The fifth member of the SLC26A protein family was found when Zheng and co-workers identified a gene highly expressed in outer hair cells of the cochlea which they named Prestin (Zheng et al., 2000). Zheng and colleagues were looking for the protein responsible for outer hair cell electromotility in response to changes in membrane potential, which is thought to be the basis of cochlear amplification (Ashmore, 1987; Dallos, 1992). Unlike other members of the SLC26A family of proteins, SLC26A5 does not seem to be an anion exchanger; rather it shows voltage-dependent charge movement across the membrane that can be measured in the form of nonlinear capacitance (Santos-Sacchi, 1991; Santos-Sacchi and Huang, 1998; Zheng et al., 2000). It was later shown that unlike voltage-gated ion channels, the charge movement across the membrane was not mediated by acidic or basic residues in the protein, but rather intracellular chloride and bicarbonate ions served as the voltage sensor (Oliver et

al., 2001). The C-terminal domain of SLC26A5 has been demonstrated to play a role in this nonlinear capacitance as well as protein trafficking (Bai et al., 2006; Zheng et al., 2005). A recent report suggests that the nonlinear capacitance of SLC26A5 cannot fully account for the previously published data, and the authors suggest that an electrogenic anion transport model for SLC26A5 can better describe the previously observed data (Muallem and Ashmore, 2006). However, conclusive data showing anion transport activity has not yet been published.

Unlike other SLC26A family members SLC26A5 has been proposed to function as an oligomer. The N-terminal portion of SLC26A5 as well as cystine residues in the transmembrane domain have been identified as sites of homomultimerization (Navaratnam et al., 2005; Zheng et al., 2005). Additional studies on other SLC26A family members may also identify physiologically relevant oligomers, or this oligomerization may continue to be a unique property of the SLC26A5 protein. As in SLC26A4, mutations in SLC26A5 have also been linked to non-syndromic forms of hearing loss (Liu et al., 2003). This is consistent with the deafness phenotype of the SLC26A5 knock out mouse and the chromosomal mapping of the SLC26A5 gene to the non-syndromic hearing loss loci DFNB14 (Lieberman et al., 2002; Zheng et al., 2000).

SLC26A6:

The SLC26A6 gene was identified through homology searches of the human genome using the sequences of other SLC26A family members as probes (Lohi et al., 2000). SLC26A6 transcript was found in a number of human tissues including skeletal muscle, kidney, pancreas, small intestine, stomach and heart (Lohi et al., 2000; Waldegger et al., 2001; Wang et al., 2002b). The SLC26A6 protein localizes to the apical membrane of epithelial cells (Lohi et al., 2000; Waldegger et al., 2001; Wang et al., 2002b), and has been demonstrated to be a chloride, formate and bicarbonate exchanger in the kidney, as well as a chloride bicarbonate exchanger in the heart, stomach and small intestine (Alvarez et al., 2004; Knauf et al., 2001; Petrovic et al., 2003a; Petrovic et al., 2003b; Petrovic et al., 2002; Simpson et al., 2005). In addition to monovalent anions such as chloride, formate and bicarbonate, SLC26A6 has also been demonstrated to transport the divalent anions oxalate and to a lesser extent sulfate (Jiang et al., 2002). Interestingly, SLC26A6 has an overlapping expression pattern with SLC26A3 in the pancreas. Careful examination of the transport stoichiometries of SLC26A6 and SLC26A3 revealed that both proteins are electrogenic transporters, in the case of SLC26A6 exchanging a single chloride ion for two bicarbonate ions while SLC26A3 transports two chloride ions for a single bicarbonate ion (Shcheynikov et al., 2006). These transport stoichiometries are consistent with the model of pancreatic bicarbonate secretion previously proposed

by Ko and colleagues in which SLC26A6 secretes large quantities of bicarbonate in the proximal pancreatic duct, while SLC26A3 absorbs the remaining chloride in the distal pancreatic duct and increases the bicarbonate concentration in the lumen to approximately 140 mM (Ko et al., 2004).

Like SLC26A3, SLC26A6 also has a functional PDZ binding sequences at its C-terminus which can interact with the PDZ domain containing proteins E3KARP and NHERF, which have previously been shown to interact with CFTR (Ahn et al., 2001; Lohi et al., 2002a; Sun et al., 2000). Consistent with this observation, SLC26A6 has been demonstrated to be functionally regulated by CFTR in both cell culture models and in animal tissues (Ko et al., 2002; Simpson et al., 2005). In addition to the functional regulation by CFTR, SLC26A6 has also been demonstrated to interact physically and functionally with carbonic anhydrase II, forming a bicarbonate metabolon that greatly enhances bicarbonate transport across epithelial membranes (Alvarez et al., 2005). A third regulatory mechanism of SLC26A6 has recently been identified, in which prostaglandin E₂ stimulates SLC26A6 bicarbonate secretion in the duodenum (Tuo et al., 2006).

SLC26A7:

SLC26A7 was identified by searching the EST database for SLC26A2 homologs (Vincourt et al., 2002). The SLC26A7 protein showed a tissue distribution which included expression in the kidney, high endothelial venules,

placenta, testis and the stomach (Barone et al., 2004; Dudas et al., 2006; Kujala et al., 2005; Petrovic et al., 2004; Petrovic et al., 2003a; Vincourt et al., 2002). Like other SLC26A family members, the SLC26A7 protein has been demonstrated to be a transporter of chloride, sulfate, oxalate and bicarbonate (Dudas et al., 2006; Kujala et al., 2005; Lohi et al., 2002a; Petrovic et al., 2004; Petrovic et al., 2003a). However, a more recent study presents data that contradicts the suggestion that SLC26A7 is a transporter. Instead, Kim and co-workers present data that suggests that SLC26A7 is a chloride channel that is regulated by pH_i (Kim et al., 2005).

Most published studies in the literature suggest that the physiological role of SLC26A7 is to absorb chloride and secrete bicarbonate in epithelial cells (Barone et al., 2004; Petrovic et al., 2003a; Petrovic et al., 2003b). An alternative hypothesis has recently been suggested by Kim and colleagues that the role of SLC26A7 is to electrically couple the chloride entry and exit pathways of acid secreting cells to allow for efficient acid secretion (Kim et al., 2005). Similar to SLC26A1, the SLC26A7 protein is targeted to the basolateral membrane of outer medullary collecting duct cells of the kidney where it is thought to help facilitate bicarbonate absorption by the medullary collecting duct (Petrovic et al., 2004). SLC26A7 has also been shown to be expressed in the basolateral membrane of parietal cells of the stomach (Petrovic et al., 2003a), where it is thought to play a role in maintaining intracellular chloride and bicarbonate levels that are critical

for proper gastric acid secretion. The mechanisms by which SLC26A7 is regulated are now beginning to be uncovered. Hypertonicity and water deprivation have been shown to play a role in regulating the protein levels of SLC26A7 (Barone et al., 2004; Petrovic et al., 2004). In addition, a vasopressin analog has been reported to regulate the SLC26A7 post-transcriptionally, while MAPK has been suggested to be involved in the targeting and trafficking of SLC26A7 from intracellular endosomes to the basolateral membrane of the outer medullary collecting duct cells (Petrovic et al., 2006; Xu et al., 2006). One potential regulatory mechanism that has been described, but not yet in detail, is the alternatively spliced form of SLC26A7, the SLC26A7.2 protein, which has an altered C-terminus (Vincourt et al., 2002). The SLC26A7.2 isoform lacks the PDZ binding sequence that the canonical SLC26A7 protein contains, suggesting that PDZ domain-containing proteins may also differentially regulate the various isoforms of SLC26A7.

SLC26A8, SLC26A9 and SLC26A11:

The eighth, ninth and eleventh members of the SLC26A family of proteins were all identified through homology searches of the human genome (Lohi et al., 2000; Lohi et al., 2002a; Vincourt et al., 2003). These proteins have been more recently identified and no human diseases have been linked to these genes at the

time of writing of this dissertation. SLC26A10 is thought to be a pseudogene and thus will not be further discussed (Mount and Romero, 2004).

Little information is known about SLC26A8, however, it does have the ability to transport chloride, sulfate and oxalate like other members of the SLC26A family (Lohi et al., 2002a; Toure et al., 2001). It was independently identified through a yeast two-hybrid screen as a protein which interacted with the RhoGTPase *MgcRacGAP*, and was found to be exclusively expressed in the testes (Toure et al., 2001). This led to the hypothesis that SLC26A8 might be involved in male infertility, which was subsequently tested and shown that mutations in SLC26A8 do not cause an increase in male infertility (Makela et al., 2005).

SLC26A9 expression has been identified in the lung and the stomach where it presumably plays a role in bicarbonate secretion and mucus pH regulation (Lohi et al., 2002a; Xu et al., 2005). The SLC26A9 protein has been demonstrated to be expressed on the apical membrane of stomach epithelial cells and its bicarbonate transport activity is inhibited by ammonium (Xu et al., 2005). This finding led to the hypothesis that the bacterium *Helicobacter pylori*, the causative agent in stomach ulcers, may be inhibiting the activity of SLC26A9 by secreting ammonia in the stomach. It was hypothesized that the loss of SLC26A9 activity may cause the mucus lining to have a lower buffering capacity which could result in increased acid damage to the epithelial cells of the stomach. This hypothesis was tested in mice where it was found that mice infected with the *H.*

pylori bacteria had a thinner tightly adherent mucus layer which was not as well buffered as that in the control animals (Henriksnas et al., 2006). In addition, the SLC26A9 transcript was upregulated in the stomach cells of infected mice over the control animals, consistent with the epithelial cells trying to compensate for the loss of proper bicarbonate secretion due to ammonium inhibition of SLC26A9 (Henriksnas et al., 2006).

SLC26A11 was cloned from an EST database by searching for human homologs of the *S. cerevisiae* Sul2 protein (Vincourt et al., 2003). Vincourt and coworkers reported SLC26A11 expression in the kidney, placenta, brain and high endothelial venules as well as sulfate transport activity (Vincourt et al., 2003). One potential difference between SLC26A11 and other SLC26A family members is that the N-terminus of SLC26A11 was reported to be extracellularly exposed (Vincourt et al., 2003), whereas for all other SLC26A family members data indicate, or it is predicted, that both the N and C-termini are intracellularly located (Lohi et al., 2002a; Lohi et al., 2003; Moseley et al., 1999; Mount and Romero, 2004; Navaratnam et al., 2005; Taylor et al., 2002; Toure et al., 2001; Zheng et al., 2001). Further investigation of the topology of SLC26A11 as well as that of other members of the SLC26A family will help to reconcile this potential discrepancy as well as further our understanding of the structure and function of these proteins.

STAS Domains:

The recently described solute carrier family 26A (SLC26A) comprises ten human genes (Lohi et al., 2000; Mount and Romero, 2004). These genes encode putative membrane proteins that contain a C-terminally conserved domain which is one of the hallmarks of this protein family. (Lohi et al., 2002a; Lohi et al., 2003; Moseley et al., 1999; Navaratnam et al., 2005; Taylor et al., 2002; Toure et al., 2001; Zheng et al., 2001). These C-terminally conserved domains have been shown to be homologous to the bacterial SpoIIAA anti-sigma factor antagonists (Aravind and Koonin, 2000) (Figure 2-18). As a result, the C-terminally conserved domain of the SLC26A was named STAS for sulfate transporters and anti-sigma factor antagonists. The function of the STAS domain in the SLC26A transporters is not well understood. However, the function of the STAS domain homologs, the SpoIIAA proteins, has been well established.

The SpoIIAA proteins function as regulators of gene transcription during bacterial spore formation (Errington, 2003). The regulatory function of SpoIIAA is achieved through a somewhat complex four component signaling circuit that includes the anti-sigma factor kinase SpoIIAB, a phosphatase SpoIIE and a RNA polymerase sigma factor F (σ^F) (Figure 2-19). Under normal growth conditions the SpoIIAB protein binds to σ^F , effectively sequestering it from solution and preventing it from binding to the RNA polymerase and activating specific gene transcription (Duncan and Losick, 1993). The SpoIIAA protein can also bind to

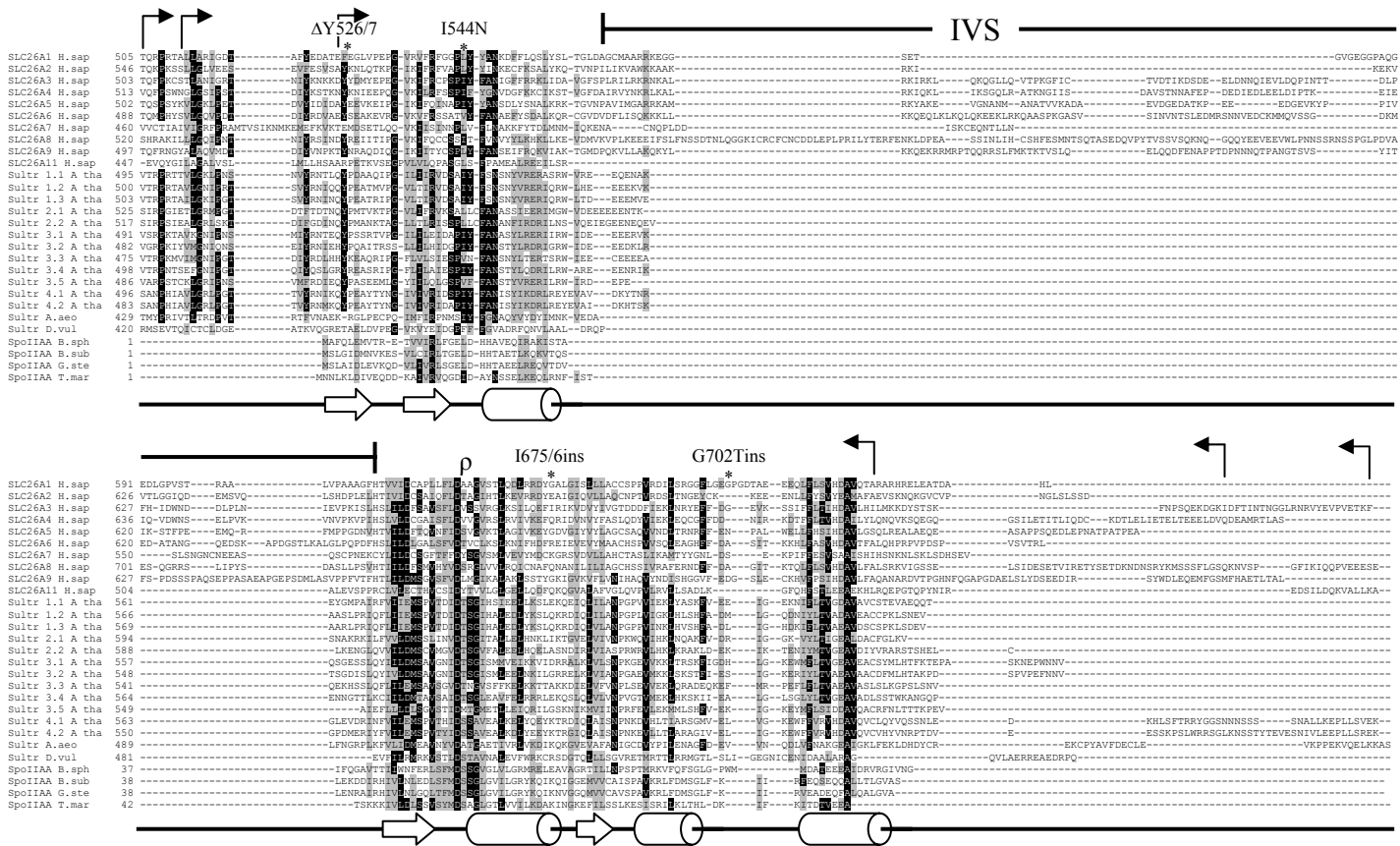


FIGURE 2-18: STAS domain alignment. A multiple sequence alignment of the ten SLC26A STAS domains from *Homo sapiens* along with twelve sulfate transporters (Sultr) from *Arabidopsis thaliana*, two predicted sulfate transporters from *Desulfovibrio vulgaris* Hildenborough (gi:46448107) and *Aquifex aeolicus* VF5 (gi:15606490), and four SpoIIAA proteins from *Bacillus sphaericus*, *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Thermotoga maritima*. The multiple sequence alignment was generated using T-coffee and manually edited while the program Boxshade was used to color the alignment. The arrows and tubes found below the alignment represent the consensus of the α -helices and β -strands found in the three dimensional structures of the SpoIIAA proteins. The line above the alignment denotes the intervening sequence (IVS) which is defined as the large sequence insertion in the STAS domains not present in the SpoIIAA proteins. The “*” indicates the position of known CLD causing mutations found in the SLC26A3 STAS domain, and the ρ symbol denotes the site of phosphorylation in the SpoIIAA proteins.

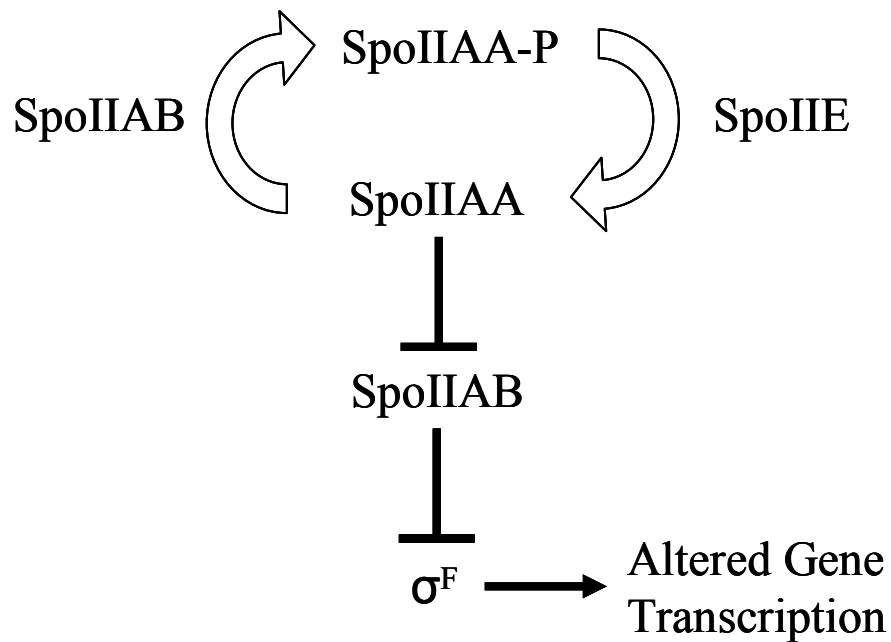


FIGURE 2-19: **σ^F regulation by SpoIIAA, SpoIIAB and SpoIIE.** The regulatory network that controls the transcription of the genes under σ^F control is schematically diagramed. SpoIIAB inhibits σ^F activity by physically sequestering it from the RNA polymerase, while the unphosphorylated SpoIIAA releases this inhibition. This activity of SpoIIAA can be inhibited by phosphorylation by the kinase activity of SpoIIAB. SpoIIAA can be reactivated by the phosphatase SpoIIE which removes the phosphate from the highly conserved Ser58 on SpoIIAA.

SpoIIAB, which physically displaces σ^F from SpoIIAB due to overlapping binding sites (Duncan et al., 1996; Garsin et al., 1998; Ho et al., 2003; Masuda et al., 2004). However, SpoIIAA is a substrate for the kinase activity of SpoIIAB, and is phosphorylated at a highly conserved serine residue, which dissociates the SpoIIAA•SpoIIAB complex due to electrostatic repulsion between the SpoIIAA phospho-serine and the β -phosphate of ADP bound to SpoIIAB (Clarkson et al.,

2003; Kovacs et al., 1998; Kovacs et al., 2001; Magnin et al., 1996; Masuda et al., 2004; Seavers et al., 2001a). Therefore, under normal growth conditions the vast majority of SpoIIAA protein is in the phosphorylated state. This allows formation of stable SpoIIAB• σ^F complexes that prevent the transcription of the genes under σ^F control.

Under sporulating conditions, transcription and translation of the SpoIIE phosphatase is induced (Guzman et al., 1988). SpoIIE can dephosphorylate SpoIIAA-P, allowing it to bind to SpoIIAB and disrupt the SpoIIAB• σ^F complex (Duncan et al., 1996; Magnin et al., 1997). In the presence of SpoIIE, the SpoIIAA protein is not typically phosphorylated because the SpoIIE phosphatase activity out-competes the SpoIIAB kinase activity due to the low turnover rate of SpoIIAB (Magnin et al., 1997; Najafi et al., 1997). The low turnover rate of SpoIIAB results in a SpoIIAB(ADP) complex with which unphosphorylated SpoIIAA forms a stable complex (Lee et al., 2000; Magnin et al., 1997). The SpoIIAB(ADP)SpoIIAA complex is refractory to nucleotide exchange due to SpoIIAA binding, which physically traps the nucleotide in the binding pocket (Lee et al., 2000; Masuda et al., 2004). Thus, σ^F is free to bind to the RNA polymerase and specifically alter gene transcription (Duncan et al., 1996; Lee et al., 2000).

A great deal of structural work has been done on this signaling circuit and has resulted in several crystal and NMR structures of the SpoIIAA protein alone

or in complex with SpoIIAB (Clarkson et al., 2001; Clarkson et al., 2003; Kovacs et al., 1998; Kovacs et al., 2001; Masuda et al., 2004; Seavers et al., 2001a; Seavers et al., 2001b). The SpoIIAA structures solved to date show a mixed α/β protein containing a central four stranded β -sheet and four surrounding α -helices (Clarkson et al., 2001; Kovacs et al., 1998; Kovacs et al., 2001; Masuda et al., 2004; Seavers et al., 2001a; Seavers et al., 2001b). Since the SpoIIAA proteins were identified as homologues of the SLC26A transporter C-terminal domains, researchers studying this family of transporters have used the SpoIIAA protein as a structural model to aid in interpreting experimental results (Rouached et al., 2005; Shibagaki and Grossman, 2004; Shibagaki and Grossman, 2006).

The Sultr sulfate transporters from *Arabidopsis thaliana* are members of the SLC26A family of proteins. They too contain a C-terminally conserved STAS domain which has been shown to be important for proper protein trafficking and sulfate transport activity of the Sultr1.2 protein (Shibagaki and Grossman, 2004). The highly conserved serine residue in the SpoIIAA proteins is a threonine residue in Sultr1.2. When this residue is mutated to a serine, aspartate, alanine or deleted altogether, transport activity is decreased, suggesting that phosphorylation of this residue may be critical for function (Rouached et al., 2005). A pair of cystine residues which are predicted to be in a loop between the final two α -helices in the Sultr1.2 STAS domain have also been demonstrated to be important for function. Mutation of these two residues to serines reduce the

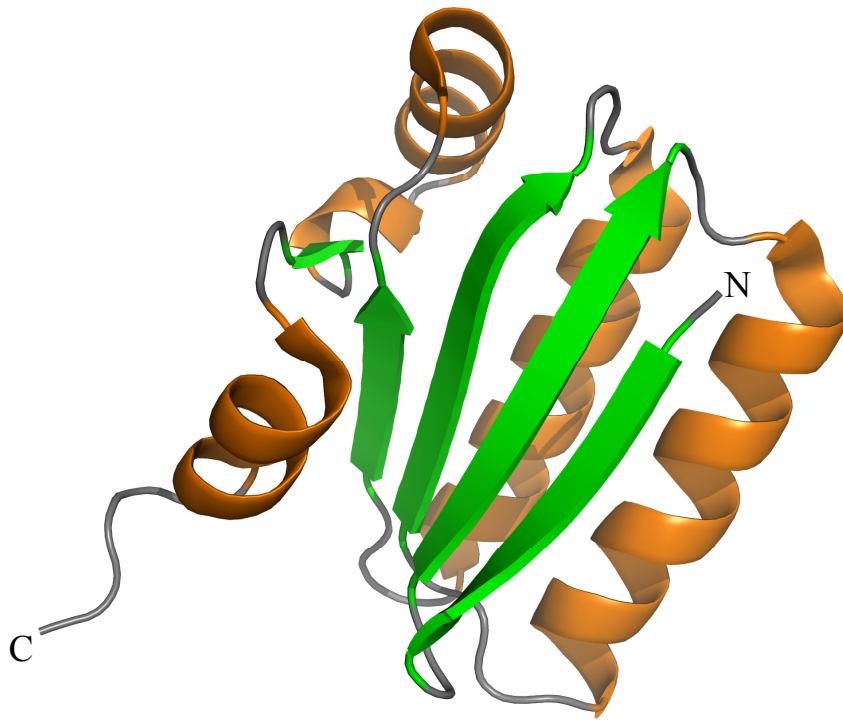


FIGURE 2-20: SpoIIAA crystal structure. The crystal structure of the SpoIIAA protein from *Bacillus sphaericus* (PDB: 1H4Y) is shown with the α -helices and β -sheets colored orange and green respectively. The N and C-termini are labeled accordingly.

sulfate transport activity of the transporter, while C-terminal truncations that remove them are no longer able to sustain growth, suggesting that these residues are critical for the structure and function of the STAS domain (Rouached et al., 2005). Shibagaki and Grossman also found a number of residues in the Sultr1.2 STAS domain which caused a loss of sulfate transport activity which map to the “top” face of the SpoIIAA crystal structure as shown in figure 2-20 (Shibagaki and Grossman, 2006).

In addition to the studies on the Sultr1.2 STAS domain, the STAS domains of the human SLC26A family of transporters have also been implicated in protein trafficking and functional regulation. The SLC26A1 and SLC26A5 STAS domains have been suggested to be involved in proper protein trafficking consistent with the results from the Sultr1.2 studies (Regeer and Markovich, 2004; Shibagaki and Grossman, 2004; Zheng et al., 2005). In addition, the SLC26A5 STAS domain has also been demonstrated to be critical for proper voltage sensing (Bai et al., 2006; Zheng et al., 2005). The STAS domains of SLC26A3 and SLC26A6 have also been found to participate in protein-protein interactions with the R-domain of CFTR and carbonic anhydrase II (Alvarez et al., 2005; Ko et al., 2004). When taken together, the data on the STAS domains suggest that it plays an important role in the proper function and regulation of these transporters. To further explore the functional role of the STAS domain, I studied the impact that four CLD causing mutations, which reside in the STAS domain of SLC26A3, had on transporter structure and function.

Chapter Three:

Biochemical and Biophysical Characterization of CFTR NBD1 and NBD2

Introduction:

Cystic Fibrosis (CF) is an autosomal recessive disease which causes dysfunction of epithelial cells in several organ systems. The loss of proper pancreatic and pulmonary function is the most common cause of death in CF patients. The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the gene mutated in Cystic Fibrosis (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989), is a member of the ABC transporter superfamily of proteins which typically transport solutes across membranes in an ATP dependant fashion (Higgins, 1992; Schneider and Hunke, 1998). The protein encoded by the CFTR gene is a 1,480 amino acid membrane protein comprised of two transmembrane spanning domains (TMDs), two nucleotide binding domains (NBDs), and a regulatory domain (R-domain) (Gregory et al., 1990; Riordan et al., 1989). The first NBD is the site of the most common CF causing mutation, an in-frame deletion of Phe508 ($\Delta F508$), which accounts for approximately 70% of all CF alleles and at least one copy of this mutant allele is found in 90% of CF patients (Kerem et al., 1989; Riordan et al., 1989). Unlike most ABC

transporters, CFTR is a cAMP regulated Cl⁻ channel where phosphorylation of multiple sites in the R-domain by PKA leads to channel activity in the presence of ATP (Cheng et al., 1991; Townsend et al., 1996). ATP is required for proper channel gating, and Cl⁻ conductance is observed only when ATP can bind to both NBD1 and NBD2 (Anderson et al., 1991a; Anderson and Welsh, 1992). The mechanism by which the NBDs bring about channel activity has been under investigation since the CFTR gene was cloned in 1989, but only recently have we begun to understand the molecular details of how ATP binding to these domains brings about Cl⁻ channel activity.

The NBDs of ABC transporters are highly conserved domains containing Walker A and B motifs that are involved in ATP binding and hydrolysis (Walker et al., 1982), and a C-consensus or signature sequence which is unique to the ABC transporter superfamily (Figure 3-1) (Bianchet et al., 1997; Higgins, 1992; Walker et al., 1982). Mutations in these three conserved sequences can lead to loss of normal channel activity, indicating that these positions are critical for proper channel function (Anderson and Welsh, 1992; Carson et al., 1995; Cotten and Welsh, 1998; Gregory et al., 1991; Melin et al., 2004; Smit et al., 1993; Wilkinson et al., 1996). In addition, conserved His and Gln residues in the NBD are important for ATP binding and hydrolysis, and have been named the H and Q-loops respectively (Hopfner et al., 2000; Karpowich et al., 2001).

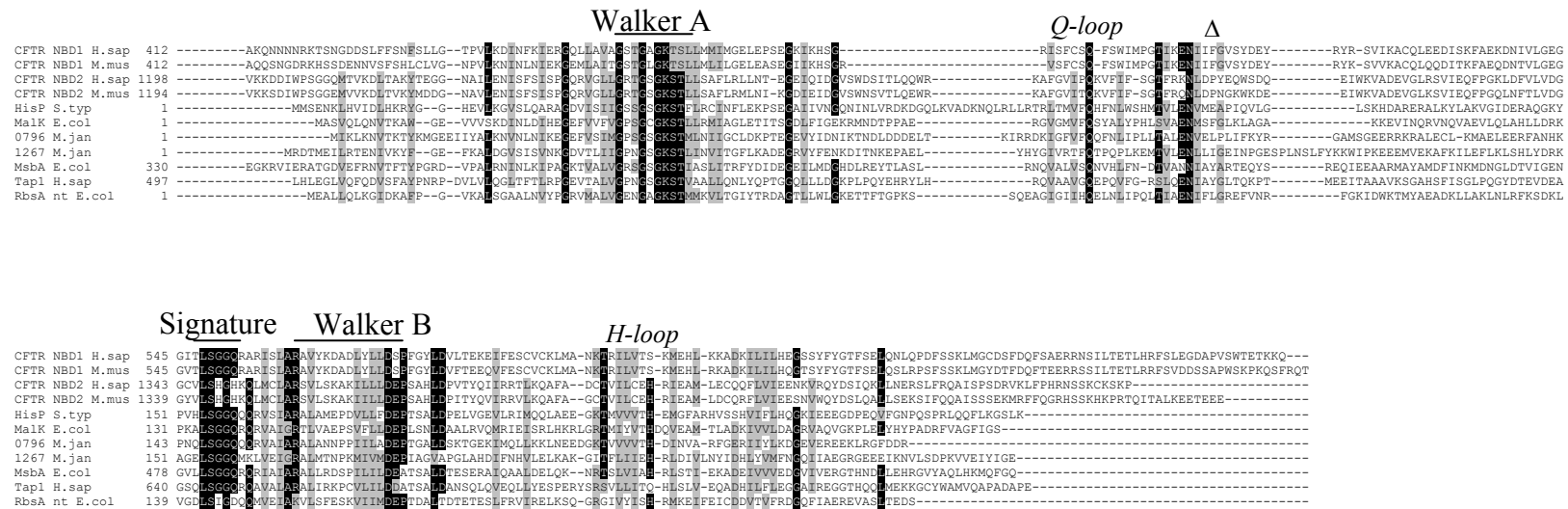


FIGURE 3-1: Nucleotide binding domain multiple sequence alignment. A multiple sequence alignment of ABC transporter nucleotide binding domains was constructed using the T-coffee and Boxshade programs. The highly conserved Walker A and B motifs along with the signature sequence are denoted with a bar above the sequence and are labeled accordingly. The conserved Gln and His are labeled as the Q and H-loops respectively, and the most common CF causing mutation, Δ F508, is also labeled. The names of the proteins and the first amino acid in the alignment are labeled to the left of the alignment along with the species the protein sequence is from (H.sap-*Homo sapiens*, M.mus-*Mus musculus*, S.typ-*Salmonella typhimurium*, M.jan-*Methanococcus jannaschii*, E.col-*Escherichia coli*)

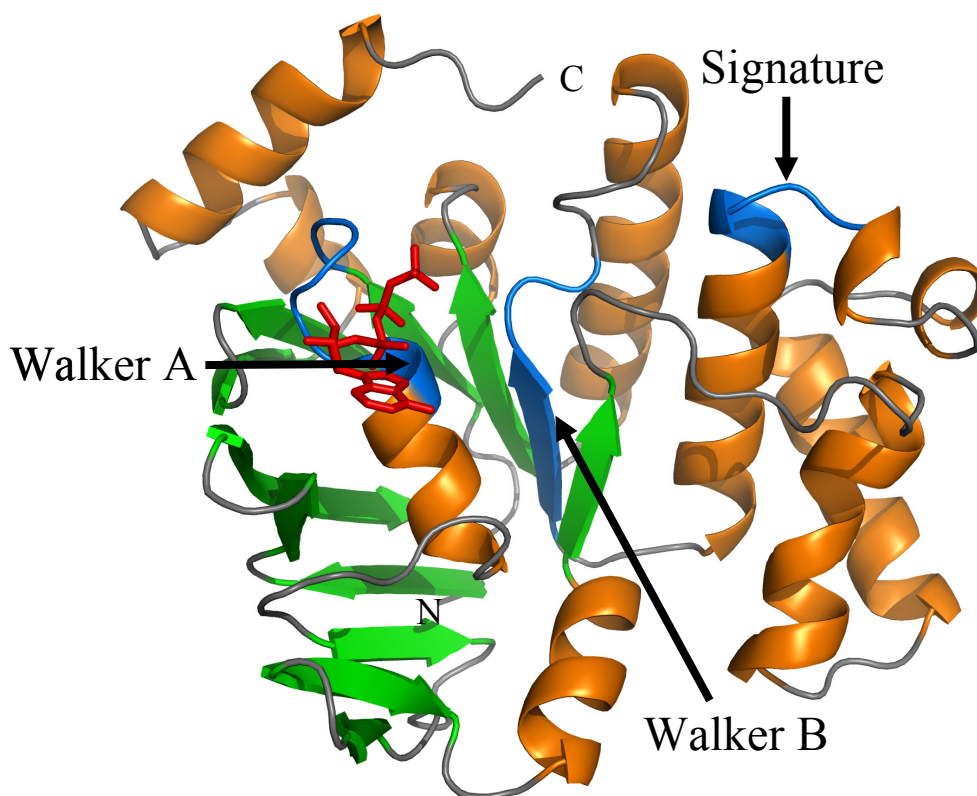


FIGURE 3-2: The crystal structure of HisP: The crystal structure of HisP (PDB: 1B0U) is shown as a ribbon diagram with α -helices and β -strands colored orange and green respectively. The conserved Walker A and B motifs along with the signature sequence are colored blue and labeled accordingly. The molecule of ATP bound to the Walker A and B motifs is colored red and the N and C-termini of the protein are labeled appropriately with an N and C.

The first high resolution crystal structure solved of an NBD was HisP, and it revealed a mixed α/β protein with two sub-domains (Hung et al., 1998) (Figure 3-2). The HisP structure revealed the close proximity of the Walker A and B motifs in the α/β sub-domain, while the signature sequence resides in the α -helical sub-domain. A molecule of ATP crystallized in the active site of the NBD

bound to the Walker A and B motifs as expected. The highly conserved signature sequence is over 20 Å away from the ATP molecule, suggesting that it does not play a role in ATP binding. As a result, the signature sequence was postulated to be critical for the stability of the HisP molecule while the α -helical sub-domain was suggested to be imbedded in the membrane (Hung et al., 1998).

A different role for the signature sequence was put forth by Jones and George who suggested it played a role in ATP binding and hydrolysis (Jones and George, 1999). They postulated that the NBDs dimerized in a “head-to-tail” fashion which would put the signature sequence of one monomer in close proximity of the Walker A and B motifs of the second monomer. This neatly solved the problem of why the signature sequence is so highly conserved throughout all NBDs, as well as why the ATP binding site is so open to solvent. Biochemical evidence supporting this hypothesis soon followed with the observation of ATP-dependant NBD dimers harboring a mutation of the highly conserved Glu at the end of the Walker B motif to Gln (Moody et al., 2002; Verdon et al., 2003b). Structural evidence of the proposed head-to-tail dimer came with additional crystal structures of MJ0796 and GlcV NBD dimers, and the structures of the full length ABC transporters BtuCD and MsbA which showed the NBDs in an orientation conducive to forming a head-to-tail dimer (Chang, 2003; Chen et al., 2003; Locher et al., 2002; Reyes and Chang, 2005; Smith et al., 2002; Verdon et al., 2003a) (Figure 3-3).

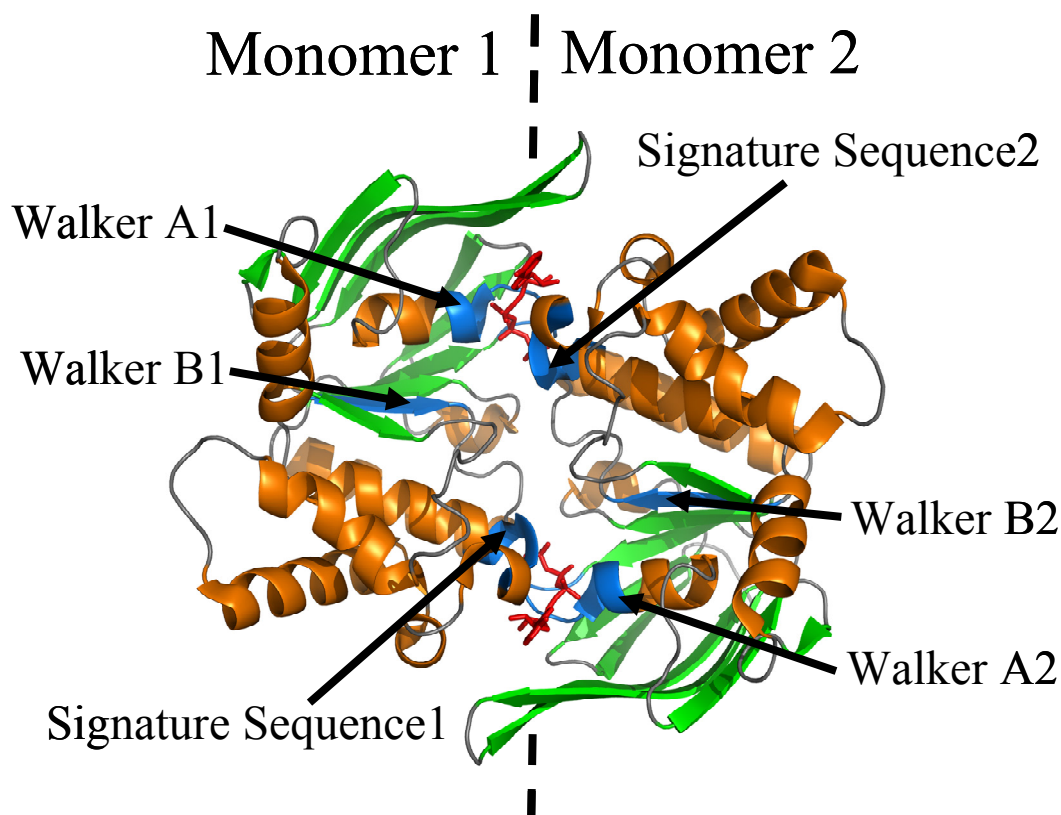


Figure 3-3: **Crystal structure of a “head-to-tail” dimer of MJ0796.** The crystal structure of E171Q MJ0796 (PDB: 1L2T) is shown as a ribbon diagram with α -helices and β -strands colored orange and green respectively, and the conserved Walker A and B motifs along with the signature sequence are colored blue. Notice that the Walker A and B motifs from one monomer along with the signature sequence from the opposing monomer form the ATP binding sites of MJ0796.

In spite of all the data on homologous NBDs, a number of questions still remained regarding the CFTR NBDs. The structure of CFTR NBD1 with and without F508 remained an important question as it had been suggested that the Δ F508 mutation was a folding mutation with a minimal effect on the native state of the domain (Qu et al., 1997; Qu and Thomas, 1996; Strickland et al., 1997). In

addition, a number of questions regarding the ATPase activity and dimerization state of CFTR NBD1 still remained. A number of reports suggested that recombinantly expressed CFTR NBD1 could bind ATP (Ko et al., 1997; Ko et al., 1993; Qu et al., 1997; Qu and Thomas, 1996; Yike et al., 1996), while others indicated that NBD1 had the capacity for ATP hydrolysis (Annereau et al., 2003; Duffieux et al., 2000; Howell et al., 2000; Ko and Pedersen, 1995). The data suggesting that CFTR NBD1 can hydrolyze ATP is interesting because the sequence lacks the highly conserved Glu residue at the end of the Walker B motif which is thought to be the catalytic base for ATP hydrolysis (Hung et al., 1998), as well as the conserved His in the H-loop (Figure 3-1). The reports of ATP hydrolysis suggest that the ATPase cycle of CFTR NBD1 may differ slightly from other NBDs. Complicating the conflicting ATP binding and hydrolysis data is the fact that the domain boundaries of CFTR NBD1 have not yet been well established, and a number of N-terminal and C-terminal boundaries have been reported (Annereau et al., 2003; Chan et al., 2000; Duffieux et al., 2000; Howell et al., 2000; Ko et al., 1997; Ko and Pedersen, 1995; Ko et al., 1993; Qu et al., 1997; Qu and Thomas, 1996; Strickland et al., 1997; Townsend et al., 1996; Wang et al., 2002a; Yike et al., 1996).

In addition to the ATPase data, the dimeric state of the CFTR NBD1 is unclear. Recent biochemical and structural data suggest that the NBDs most likely form symmetrical dimers which are thought to be necessary for ATP

hydrolysis (Chen et al., 2003; Horn et al., 2003; Locher et al., 2002; Moody et al., 2002; Smith et al., 2002; Verdon et al., 2003b). Despite the fact that CFTR is thought to be functional as a monomer (Ramjeesingh et al., 2001), there are reports of CFTR existing in a higher order oligomers (Ramjeesingh et al., 2003a; Ramjeesingh et al., 2003b; Schillers et al., 2001; Schillers et al., 2004). If CFTR exists as a monomer *in vivo* then the functional dimer would have to be a NBD1-NBD2 heterodimer. If CFTR is functional as a dimer or as a higher oligomeric species, then NBD1 and NBD2 homodimers would be possible in addition to NBD1-NBD2 heterodimers. To help establish the domain boundaries of the CFTR NBDs, to resolve the dimeric state of NBD1 and NBD2, and to produce protein constructs suitable for structural studies, we expressed and purified the soluble fraction of recombinantly expressed and purified human CFTR NBD1 (amino acids 419-655) and CFTR NBD2 (amino acids 1206-1456). Size exclusion chromatography experiments were performed to monitor the oligomeric state of the NBDs in the presence and absence of saturating concentrations of ATP. In addition, we tested the ATP binding and ATPase activity of solubly expressed and purified mouse CFTR NBD1 389-673, and monitored the dimeric state of the domain by gel filtration chromatography in the presence and absence of saturating ATP concentrations.

Experimental Procedures:

CFTR NBD1/2 plasmid construction: CFTR amino acids 419-655 (NBD1) or 1206-1456 (NBD2) were cloned into the pET 28 and pET 19 expression vectors respectively, down stream of the N-terminal His-tag using standard techniques. Positive clones were selected and correct sequences were confirmed by DNA sequencing. Plasmids harboring the correct sequences were transformed into *E. coli* CondonPlus (DE3)-RIL cells (Stratagene) for protein expression.

CFTR NBD1/2 expression and purification: *E. coli* CondonPlus (DE3)-RIL cells harboring the NBD1 or NBD2 expression plasmids were grown in 8L of LB to an O.D. of 1.0, and shifted to 14°C and induced with 100 µM IPTG for 24 hr. Cells were collected via centrifugation and were resuspended in 60 ml lysing buffer (15 mM Tris pH 8.0, 600 mM NaCl, 5% glycerol, 1% Triton X 100, 1 mg/ml Lysozyme and Roche Complete Protease Inhibitor Tablets) and lysed via sonication. The lysate was centrifuged at 60,000 rcf, and Ni chromatography was performed with the following buffers: binding buffer (15 mM Tris pH 8.0, 600 mM NaCl, 5% glycerol), wash buffer (15 mM Tris pH 8.0, 600 mM NaCl, 5% glycerol, 40 mM imidazole), and elution buffer (15 mM Tris pH 8.0, 600 mM NaCl, 5% glycerol, 400 mM imidazole).

Fractions containing the NBD were pooled and 10 mM octyl-β-glucoside was added to the protein which was concentrated to 2 ml. The protein was loaded

onto a HiPrep 16/60 Sephacryl S-300 column and run with a 0.3 ml/min flow rate in 15 mM Tris pH 8.0, 600 mM NaCl, 5% glycerol, 10 mM octyl- β -glucoside, 1 mM DTT. NBD1 protein eluting from the gel filtration column in the monomeric region was pooled and concentrated as needed.

Analytical HPLC gel filtration chromatography: The purified NBDs were subjected to analytical gel filtration chromatography on a Tosoh QC-PAK TSK 200GL column, and the absorbance was monitored at 220 nm. All samples were filtered through a 0.2 μ M filter before injection onto the column, and all chromatography was performed at room temperature in 50 mM Tris pH 7.6, 200 mM NaCl.

Mouse CFTR NBD1 purification and ATP binding and hydrolysis: The mouse CFTR NBD1 protein (amino acids 389-673) was expressed and purified as previously described (Lewis et al., 2004). Briefly, the Smt3-NBD1 fusion was expressed in *E. coli* and Ni column chromatography was performed followed by Ulp1 cleavage and gel filtration chromatography. A subsequent Ni chromatography step was performed to ensure complete removal of the Smt3 tag.

The ATP binding and hydrolysis methods for mouse CFTR NBD1 have been described previously (Lewis et al., 2004). Briefly, standard filter binding assays were performed on mouse CFTR NBD1 using α - 32 P ATP. The filters were

washed twice with ice cold buffer and bound α - ^{32}P ATP was measured in a scintillation counter. The hydrolysis reactions were performed using a colorimetric assay where ATP hydrolysis is coupled to the conversion of NADH to NAD^+ (Rosing et al., 1975). This conversion was monitored by the absorbance at 340 nm on a UV spectrophotometer.

Results:

As a first step in characterizing the biochemical and biophysical properties of CFTR NBD1 and NBD2 the amino acid boundaries of the domain had to be identified. To guide this effort a multiple sequence alignment was generated using the program T-coffee to compare the human CFTR sequence to a number of bacterial NBDs. A number of the bacterial NBD sequences used in the multiple sequence alignment had crystal structures available; which aided in predicting the amino acid boundaries of CFTR NBD1 and NBD2 to be amino acids 419-655 and 1206-1456 respectively (Figure 3-1).

CFTR NBD1 expression constructs were produced by cloning CFTR amino acids 419-655 into the pET 28 vector and transforming this plasmid into *E. coli* BL21 CodonPlus (DE3)-RIL for protein expression and purification. Large quantities of CFTR NBD1 were produced, but unfortunately most of the protein was found in the insoluble fraction after cell lysis (Figure 3-4). Nevertheless, some NBD1 was expressed solubly and could be captured via Ni affinity chromatography and further purified by gel filtration chromatography in the presence of octyl- β -glucoside (Figure 3-4).

CFTR amino acids 1206-1456 were cloned into the pET 19 vector which was then transformed into *E. coli* BL21 CodonPlus (DE3)-RIL for protein expression and purification. As with CFTR NBD1, NBD2 was also predominantly expressed as an insoluble aggregate, and only a small fraction of

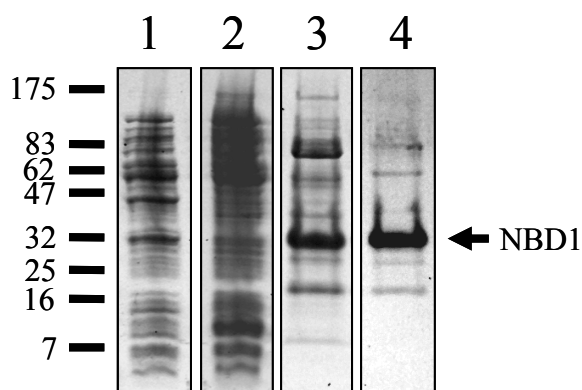


FIGURE 3-4: CFTR NBD1 expression and purification. Coomassie-stained gel lanes of the expression and purification of human CFTR NBD1 (amino acid boundaries 419-655) from *E. coli*. The molecular weight markers are measured in kDa and the protein samples loaded are as follows: 1) Induced cell lysate; 2) supernatant fraction of the cell lysate; 3) Ni column elution; 4) monomeric NBD1 elution off of a Sephacryl S-300 gel filtration column.

NBD2 remained in the supernatant fraction (Figure 3-5). However, a small amount of soluble CFTR NBD2 could be captured via Ni column chromatography, and then further purified by gel filtration chromatography in the presence of 10 mM octyl- β -glucoside (Figure 3-5).

It had been previously shown that some of the bacterial NBDs dimerized in an ATP

dependant fashion (Chen et al., 2003; Horn et al., 2003; Locher et al., 2002; Moody et al., 2002; Smith et al., 2002; Verdon et al., 2003a), so the oligomeric state of NBD1 and NBD2 was monitored by gel filtration chromatography. The purified NBD1 and NBD2 proteins were flowed through a Tosoh QC-PAK TSK 200GL column in the presence and absence of ATP, and the UV absorbance at 220 nm was monitored to track any changes in the oligomeric state of the proteins upon the addition of nucleotide (Figure 3-6). No apparent shift in the retention time of NBD1 or NBD2 was observed upon the addition of 20 mM ATP, nor was

there a change in the retention time of NBD1 and NBD2 upon equimolar mixing of the two proteins in the presence or absence of 20 mM ATP (Figure 3-5).

Approximately the same time these experiments were performed, the company SGX had discovered a way to produce soluble mouse CFTR NBD1, and had approached us to perform some biochemical experiments to help characterize the protein. They cloned mouse CFTR amino acids 389-673 into the His₆-Smt3 expression vector (Mossessova and Lima, 2000) and were able to

express and purify milligram quantities of soluble protein (Figure 3-7A). SGX shipped us purified protein and ATP binding and hydrolysis experiments were performed. Using a coupled ATPase assay it was found that mouse CFTR NBD1 had no measurable ATPase activity but did bind ATP with an affinity of 200 μ M using a standard filter binding assay (Figure 3-7B and C) (Lewis et al., 2004). As a positive control for the coupled ATPase assay a *Methanococcus jannaschii* NBD, MJ0796, was used and a hydrolytic rate of 7.8 ATP min⁻¹ was measured.

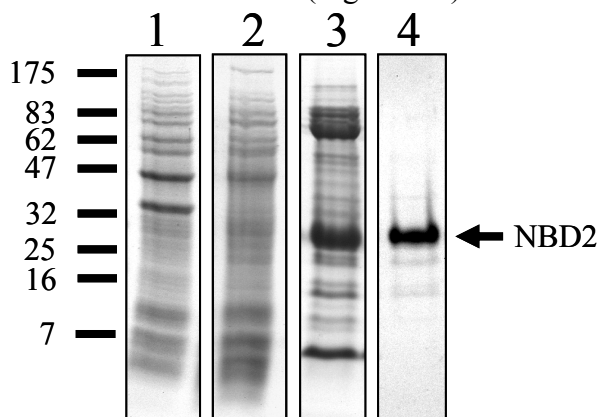


FIGURE 3-5: CFTR NBD2 expression and purification. Coomassie-stained gel lanes of the expression and purification of human CFTR NBD2 (amino acids 1206-1456) from *E. coli*. The molecular weight markers are measured in kDa and the protein samples loaded are as follows: 1) Induced cell lysate; 2) supernatant fraction of the cell lysate; 3) Ni column elution; 4) monomeric NBD1 elution off of a Sephacryl S-300 gel filtration column.

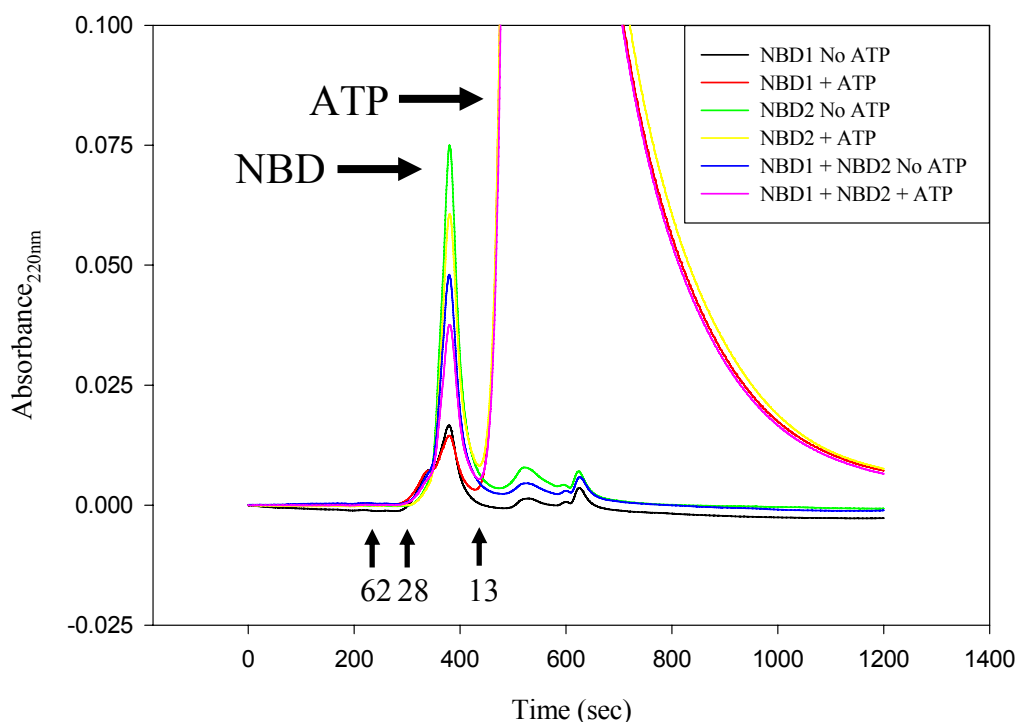


FIGURE 3-6: Oligomeric state of CFTR NBD1/2. The oligomeric state of the purified human CFTR NBD1 and NBD2 was monitored on a Tosoh QC-PAK TSK 200GL HPLC analytical gel filtration column. The retention time of the NBDs, either in isolation, or mixed in equimolar ratios, was measured in the presence or absence of 20 mM ATP. The NBD and ATP peaks are labeled, and the flow rate of the experiment was 0.75 ml/min.

In addition to the ATP binding and hydrolysis experiments, the oligomeric state of the mouse CFTR NBD1 protein was monitored by gel filtration chromatography in the presence and absence of ATP (Figure 3-8). As a positive control for the dimerization of NBDs, MJ0796 E171Q was used. This protein had been purified by Jonathan Moody in our lab, who had previously shown MJ0796 E171Q dimerized in the presence of ATP (Figure 3-8A) (Moody et al., 2002).

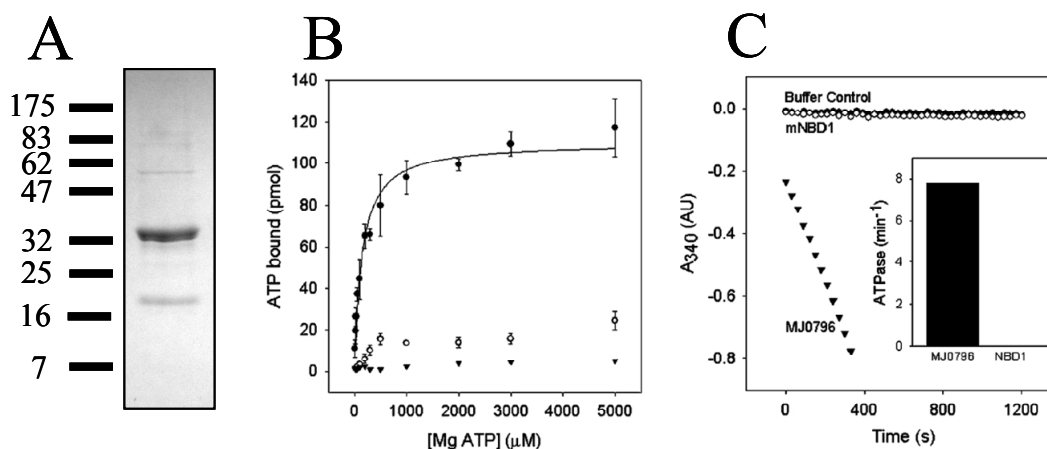


FIGURE 3-7: ATP binding and hydrolysis of mouse NBD1. The ability of mouse CFTR NBD1 to bind and hydrolyse ATP was tested on protein expressed and purified by SGX Pharmaceuticals. (A) A Coomassie-stained gel of the purified mouse CFTR NBD1 protein. (B) ATP binding of mouse CFTR NBD1. (C) ATPase activity of mouse CFTR NBD1. Adapted by permission from Macmillan Publishers Ltd: Lewis *et al.* EMBO (2004) 23(2):282-93.

Performing the same experiment on mouse CFTR NBD1 resulted in no observable ATP dependant dimerization upon the addition of 10 mM ATP (Figure 3-8B).

SGX was able to crystallize the mouse CFTR NBD1 protein and solve the three dimensional structure of the domain at 2.2 Å resolution (Figure 3-9). Several interesting observations were made from this crystal structure. First of all, the overall fold of the protein domain was similar to the other NBD crystal structures. However, the N-terminus of the domain was further upstream in the primary sequence than had originally been predicted. In addition there was a large loop between the first and second β-strands that was not visible in the crystal structure, and the F508 side chain was largely exposed to solvent (Figure

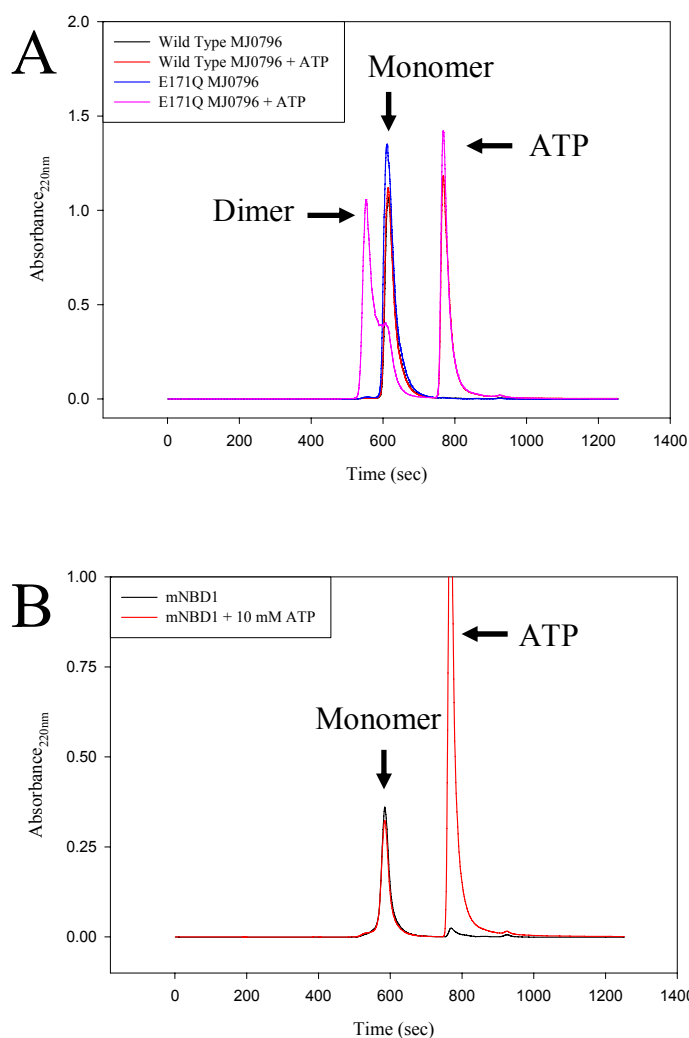


FIGURE 3-8: Oligomeric state of mouse CFTR NBD1. The oligomeric state of the mouse CFTR NBD1 was tested in the presence and absence of 10 mM ATP. (A) The *Methanococcus jannaschii* NBD homolog of LolD, MJ0796, was used as a positive control for NBD dimerization. An absorbance chromatogram of wild type and E171Q MJ0796 protein eluting from a Tosoh QC-PAK TSK 200GL HPLC analytical gel filtration column in the presence or absence of 10 mM ATP. (B) An absorbance chromatogram of mouse wild type CFTR NBD1 eluting from a Tosoh QC-PAK TSK 200GL HPLC analytical gel filtration column in the presence or absence of 10 mM ATP.

3-9). The N-terminal boundary of CFTR NBD1 was predicted to be between amino acids 415 and 419 based upon existing multiple sequence alignments as well as the HisP structure. However, if a careful multiple sequence alignment is constructed using CFTR NBD1 amino acids 380-670 the true N-terminus can be identified using the multiple sequence alignment program T-coffee. (Figure

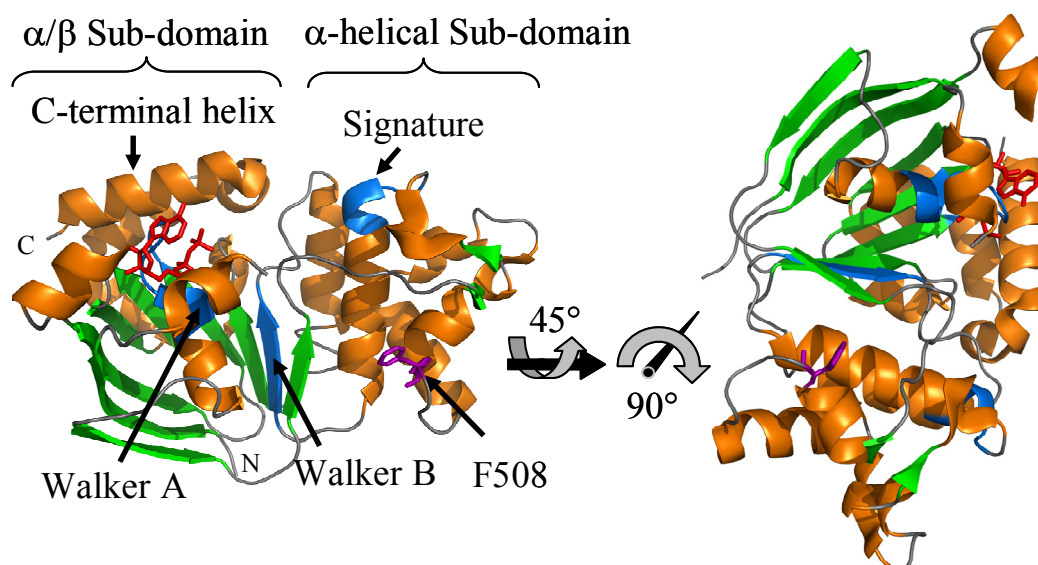


FIGURE 3-9: The crystal structure of mouse CFTR NBD1. The crystal structure of mouse CFTR NBD1 (PDB: 1R0X) is shown as a ribbon diagram with α -helices colored orange and β -strands colored green. The protein was crystallized in the presence of 2 mM ATP which is shown in red. Two views of the NBD are shown; on the left is the conventional view of the NBD while on the right the NBD is rotated to show one half of the putative “head-to-tail” dimer. F508 is colored purple and shown in stick representation, while the highly conserved Walker A, B, and signature motifs are colored blue.

3-10). In addition to this insertion in NBD1, the C-terminus of the domain was found to be further downstream in the primary amino acid sequence than was predicted. The final secondary structural element of NBD1 is a helix which lies close to the Walker A and B motifs and resides in the putative dimer interface. A helix in this position would not be conducive to forming a NBD dimer which is the current model for ABC transporter NBD1’s ATPase cycle, suggesting that the position of this helix is influenced by the crystallization conditions or perhaps this is a novel regulatory mechanism not found in other NBDs.

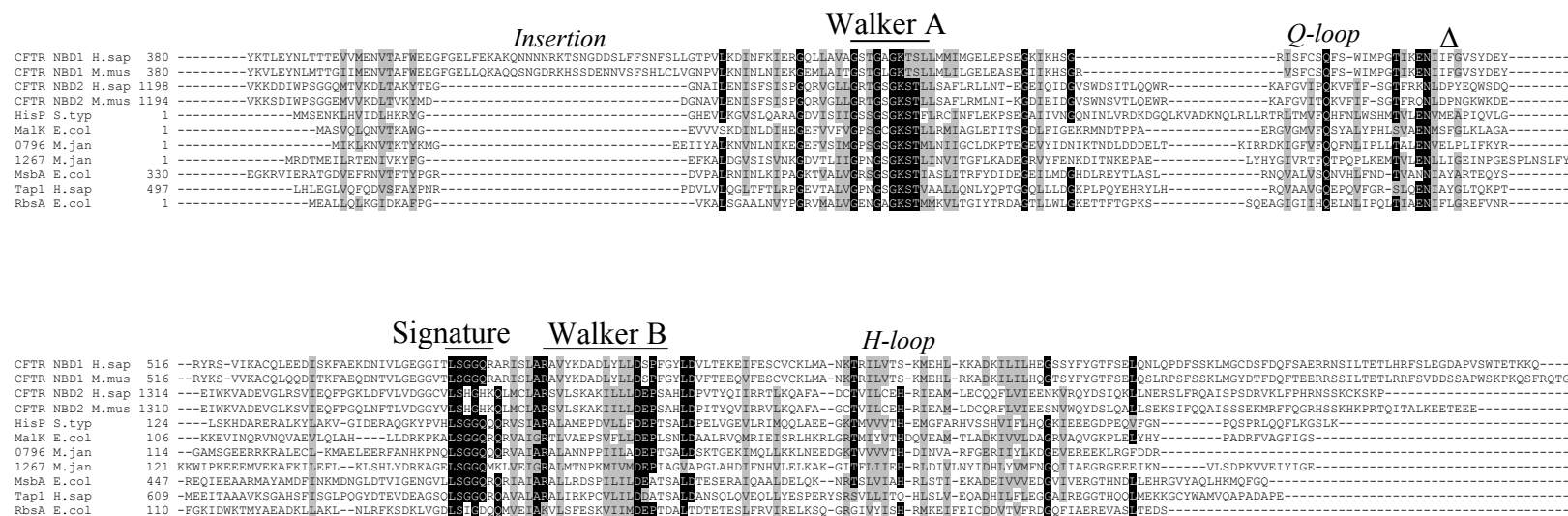


FIGURE 3-10: A revised nucleotide binding domain alignment. A multiple sequence alignment of ABC transporter nucleotide binding domains was constructed using the T-coffee and Boxshade programs, and highlights the N-terminal insertion in CFTR NBD1 which was missed in our previous alignments. The highly conserved Walker A and B motifs along with the signature sequence are denoted with a bar above the sequence and are labeled accordingly. The conserved Gln and His are labeled the Q and H-loops respectively, and the most common CF causing mutation, Δ F508, is denoted with a Δ . The names of the proteins and the numerical position of the first amino acid in the alignment are labeled on the left along with the species the protein sequence is from (H.sap-*Homo sapiens*, M.mus-*Mus musculus*, S.typ-*Salmonella typhimurium*, M.jan-*Methanococcus jannaschii*, E.col-*Escherichia coli*).

Discussion:

I set out to identify the domain boundaries of CFTR NBD1 and NBD2, along with resolving the controversy surrounding the ATP binding and hydrolysis ability of CFTR NBD1. In addition, I hoped to create a method for expressing and purifying CFTR NBD1 which would be suitable for NMR and crystallographic studies. To aid in reaching these goals I produced a multiple sequence alignment with human CFTR NBD1 and NBD2 along with a number of other prokaryotic and eukaryotic ABC transporter NBDs (Figure 3-1). From this alignment the beginning of the CFTR NBD1 was predicted to be amino acid 419, and the C-terminal end of the domain was predicted to be amino acid 655.

The multiple sequence alignment was not significantly different than those previously published (Hung et al., 1998; Jones and George, 1999; Karpowich et al., 2001; Locher et al., 2002; Smith et al., 2002; Yuan et al., 2001). In addition, our predicted domain boundaries were similar to those of Duffieux and colleagues who used CFTR amino acids 422-650 as their NBD1 boundaries. Using this construct Duffieux and coworkers were able to purify soluble NBD1 and obtain an NMR HSQC spectrum of NBD1, suggesting that this construct was suitable for high resolution structural studies (Duffieux et al., 2000). Similarly, I was also able to express and purify soluble NBD1 protein. However, my NBD1 construct suffered from low levels of soluble expression and tended to aggregate unless in the presence of octyl- β -glucoside. Duffieux and colleagues NBD1 construct was

also purified in the presence of 0.1% Triton X-100, and perhaps in the absence of detergent their NBD1 construct may suffer from aggregation and poor solubility similar to my experiences with CFTR NBD1 419-655.

The NBD1 construct most similar to the mouse CFTR NBD1 boundaries previously reported in the literature was that of Yike and colleagues (Yike et al., 1996). They reported testing the expression levels of constructs containing two different N-terminal and four C-terminal NBD1 boundaries, and concluded that CFTR amino acids 383-650 encompassed the NBD1 (Yike et al., 1996). Their domain boundaries were a few amino acids longer on the N-terminus and approximately 20 amino acids shorter on the C-terminus. Interestingly, this construct was expressed as an insoluble protein; however, upon refolding this protein was stable in solution for up to 10 days (Yike et al., 1996). In addition, they mention in the text that Dulhanty and Riordan had defined the C-terminal boundary of CFTR to be between 672 and 679 (Dulhanty and Riordan, 1994). In this sense the domain boundaries of CFTR NBD1 had been identified in the literature eight years earlier. However, without combining this data to produce the 383-672 construct, and identify soluble expression conditions for CFTR NBD1, these domain boundaries were not widely adopted until the mouse CFTR NBD1 crystal structure was solved (Lewis et al., 2004).

A number of studies have now identified ATP dependent dimerization of ABC transporter NBDs (Chen et al., 2003; Howell et al., 2000; Locher et al.,

2002; Lu and Pedersen, 2000; Moody et al., 2002; Smith et al., 2002; Verdon et al., 2003a; Verdon et al., 2003b). The ATP dependent dimers are not typically stable in solution unless catalytic mutations are introduced into the protein to stabilize the dimeric state of the NBDs. When the highly conserved Glu in the Walker B sequence is mutated to Gln (E/Q), putatively removing the catalytic base required for ATP hydrolysis, stable ATP dependent dimers have been observed (Moody et al., 2002; Smith et al., 2002; Verdon et al., 2003b). In addition to the head-to-tail dimers observed in E/Q mutant NBDs, dimers have also been observed in crystal structures of wild type sequences for BtuCD and MalK (Chen et al., 2003; Locher et al., 2002). However, in both the BtuCD and MalK structures additional protein domains were present which held the NBDs in close proximity to one another, allowing the head-to-tail dimers to form in the absence of the E/Q mutation. Our experimental results on the short (419-655 and 1206-1456) and long (389-673) CFTR NBD constructs suggest that neither NBD1 nor NBD2 form stable ATP dependent dimers in solution (Figure 3-6 and 3-8). One possible explanation for why ATP dependant dimerization was not observed in our experiments is that we did not make the E/Q mutation that allows efficient dimerization (Moody et al., 2002; Verdon et al., 2003b). However, the conserved Walker B Glu is not present in the CFTR NBD1 sequence (Figure 3-1 and 3-10). Instead a Ser residue takes the place of the highly conserved Glu, which we hypothesized might inhibit the ATPase activity of the domain and allow stable

dimers to form. In addition, the highly conserved His of the H-loop is also a Ser residue in CFTR NBD1, which provides further evidence that NBD1 probably cannot hydrolyze ATP, and suggested that we may be able to observe ATP dependent dimerization of the wild type CFTR NBD1 sequence. However, this was not the case. Perhaps if the E/Q mutations were made in CFTR NBD1 and NBD2, ATP dependant dimerization could be observed for the two NBDs either alone (homodimers) or when mixed together (heterodimers).

ATP binding experiments with mouse CFTR NBD1 indicated that this protein construct was capable of binding ATP with an affinity of $149 \pm 48 \mu\text{M}$ (Figure 3-7B). Our results are consistent with previous reports that human CFTR NBD1 has ATP binding properties (Ko et al., 1997; Qu et al., 1997; Qu and Thomas, 1996; Yike et al., 1996). However, the measured ATP binding affinity for the various NBD1 constructs reported in the literature range from the low micromolar to several millimolar (Ko et al., 1997; Qu et al., 1997; Qu and Thomas, 1996; Yike et al., 1996). The differences observed between our ATP binding constant of $149 \pm 48 \mu\text{M}$ with the complete domain and those previously reported most likely stem from differences in the domain boundaries of CFTR NBD1. Nonetheless, sequence variation between mouse and human NBD1 may also contribute to the differences in measured ATP binding affinity.

In addition to ATP binding experiments, ATPase activity assays were also performed on the mouse NBD1 protein. No ATP hydrolysis activity was

measured for mouse CFTR NBD1 using a standard coupled assay (Rosing et al., 1975) (Figure 3-7C). MJ0796 was used as a positive control, and an ATPase activity of 7.8 min^{-1} was measured, which is similar to the previously reported value (Moody et al., 2002). The lack of observed ATPase activity for the mouse CFTR NBD1 contrasts with previous reports of ATPase activity for human CFTR NBD1 (Annereau et al., 2003; Duffieux et al., 2000; Howell et al., 2000; Ko and Pedersen, 1995). Differences in the domain boundaries of the protein constructs used as well as the sequence differences between species may account for the observed differences in the ATPase activity. In addition, the lack of the critical Glu and His residues at the end of the Walker B motif and H-loop respectively, may contribute significantly to the lack of ATPase activity. The fact that CFTR NBD1 lacks these critical residues known to be important for ATP hydrolysis suggests that it is unlikely that CFTR NBD1 supports ATP hydrolysis on its own. Indeed, studies of full length CFTR have suggested that NBD1 and NBD2 form a head-to-tail dimer in which the NBD1 site has ATP binding properties with minimal to no ATPase activity while the NBD2 site can bind and hydrolyze ATP (Aleksandrov et al., 2002; Basso et al., 2003; Berger et al., 2005; Vergani et al., 2005; Vergani et al., 2003). A recent report on the isolated NBDs demonstrated that the ATPase activity of CFTR NBD1 and NBD2 increases approximately three fold when the domains are mixed together compared to their individual activities alone (Kidd et al., 2004). These data taken together suggest that NBD1

and NBD2 may form a functional heterodimer in CFTR which regulates channel opening and closing.

The crystal structure of the mouse CFTR NBD1 revealed that the domain is highly similar to other NBDs whose crystal structures had been solved (Chang, 2003; Chang and Roth, 2001; Chen et al., 2003; Diederichs et al., 2000; Gaudet and Wiley, 2001; Horn et al., 2003; Hung et al., 1998; Karpowich et al., 2001; Locher et al., 2002; Reyes and Chang, 2005; Schmitt et al., 2003; Smith et al., 2002; Verdon et al., 2003a; Yuan et al., 2001). The mouse NBD1 structure has a mixed α/β domain that contains the highly conserved Walker A and B motifs, and an α -helical sub-domain which contains F508, the deletion of which is the most prevalent CF causing mutation (Figure 3-9). The structure also revealed that there was an insertion between the first and second β -strands of the domain which had previously not been identified (Figure 3-9 and 3-10). A large portion of this insertion was not visible in the electron density map which suggests that this region of the domain is highly flexible in the crystal. As a result of this previously undetected insertion, the N-terminus of NBD1 is earlier in sequence than most had predicted. This implies that most of the previously reported NBD1 constructs were truncated domains, which may help explain some of the poor biochemical properties previously observed. In addition to the insertion and the location of the N-terminus, the C-terminus was further in sequence than most had predicted.

Interestingly, the final secondary structural element in NBD1 is an α -helix that is not found in other NBDs, and lies across the putative head-to-tail dimer interface (Figure 3-11A and B). If the models for ABC transporter NBD ATPase activity are correct then ATP binding to two NBDs allows for a dimer to form which places the signature sequence of one NBD opposite to the Walker A and B motifs from the opposing dimer. The three highly conserved sequences come together to complete the ATP binding site and allow for ATP hydrolysis to occur; this results in NBD dimer dissociation and product release (Figure 3-11A). The gating models for CFTR suggest that the formation of NBD1-NBD2 heterodimers control channel gating (Aleksandrov et al., 2002; Basso et al., 2003; Berger et al., 2005; Vergani et al., 2005; Vergani et al., 2003). The position of this α -helix would sterically inhibit the formation of a head-to-tail dimer and prevent the ATPase cycle from occurring unless there are substantial changes to the NBD2 dimer interface which can accommodate this C-terminal helix (Figure 3-11B). Based upon sequence similarity it is probably a safe assumption that the dimer interface between CFTR NBD1 and NBD2 is similar to the previously reported head-to-tail dimers. This suggests that this C-terminal helix might play a role in regulating the formation of an NBD1-NBD2 heterodimer. The sequence that forms the C-terminal helix has traditionally been assigned to the R-domain of CFTR and it contains a PKA phosphorylatable Ser at position 660 (Cheng et al., 1991). It is possible that PKA phosphorylation of the R-domain

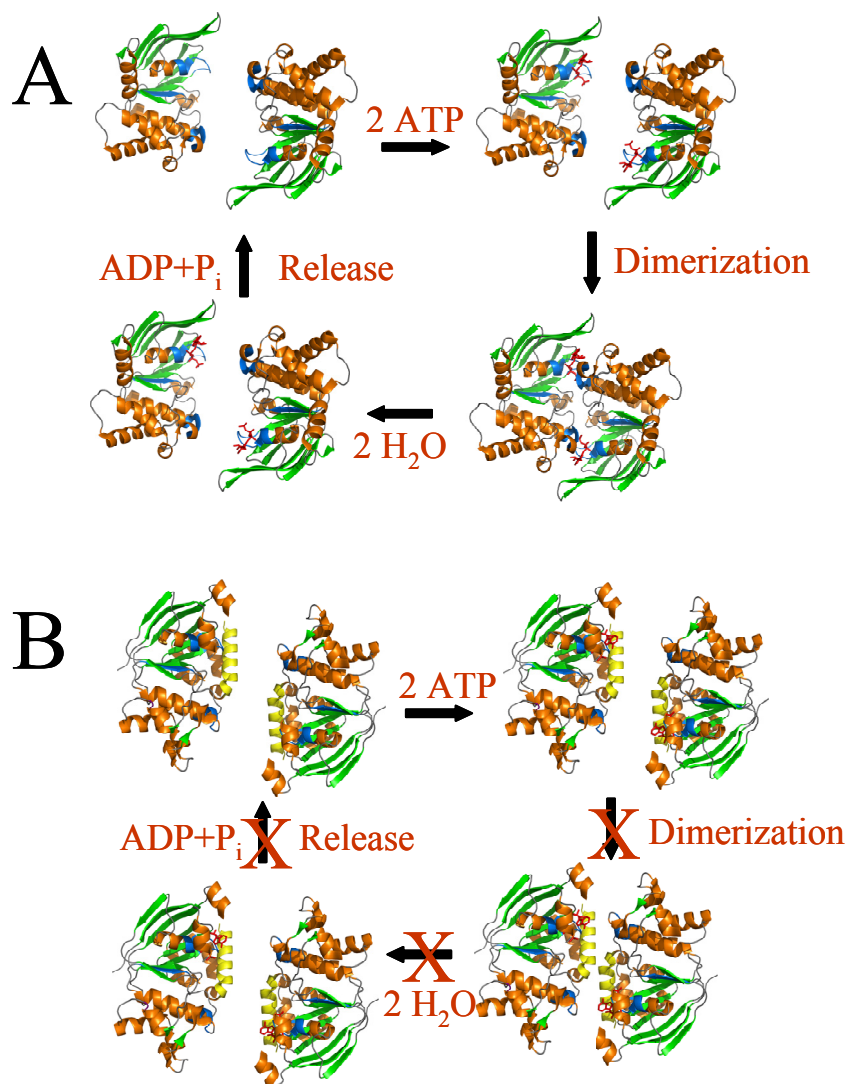


FIGURE 3-11: Proposed ATPase cycle for ABC transporter NBDs. Crystal structures of MJ0796 (PDB: 1L2T) and mouse CFTR NBD1 (PDB: 1R0X) are shown with α -helices colored orange and β -strands colored green. The highly conserved Walker A and B motifs along with the signature sequence are colored blue while the nucleotides are colored red. (A) The proposed ATPase cycle of MJ0796 begins with two MJ0796 monomers binding two ATP molecules which allow the formation of the "head-to-tail" dimer (note: the Walker A and B sequences are in close proximity of the signature sequence of the opposing monomer). Once a dimer is formed, the NBDs can hydrolyze ATP to ADP and P_i , and upon product release the ATPase cycle is completed, and the NBDs are ready for another cycle of ATP binding and hydrolysis. (B) In the absence of a NBD2 structure, a mouse CFTR NBD1 homodimer is modeled to demonstrate that the C-terminal helix (colored yellow) is sterically preventing the typical "head-to-tail" dimer from forming, which in turn would prevent the proposed NBD ATPase cycle from occurring.

influences the position of this C-terminal helix, providing a molecular mechanism for the PKA dependant regulation of CFTR's Cl^- channel activity. However, phosphorylation of Ser660 alone does not affect the location of this helix which can be seen in the phosphorylated (PDB: 1R0Z) and unphosphorylated (PDB: 1R0X) mouse CFTR NBD1 structures which have an RMSD of 0.558 Å. It is also possible that the position of the C-terminal helix is influenced by the crystallization conditions and the contacts formed in the crystal lattice, and under physiological conditions it does not reside at the dimer interface. Additional crystallographic studies which have found that this C-terminal helix can adopt at least two different conformations, suggesting this helix may be loosely associated with the dimer interface (Figure 3-12).

Subsequent to the mouse CFTR NBD1 crystal structure, a number of other CFTR NBD1 structures with different mutations have been solved in various space groups. This gives us the opportunity to look at different crystal packing arrangements to determine if the crystal contacts are possibly influencing the position of the C-terminal helix. Figure 3-12A shows the structure of human CFTR NBD1 containing the ΔF508 mutation which crystallized in the space group P4_32_12 , while Figure 3-12B shows human CFTR NBD1 F508A which crystallized in the space group C222_1 . Both structures contain bound ATP yet they show very different positions of the NBD1 C-terminal helix, suggesting that

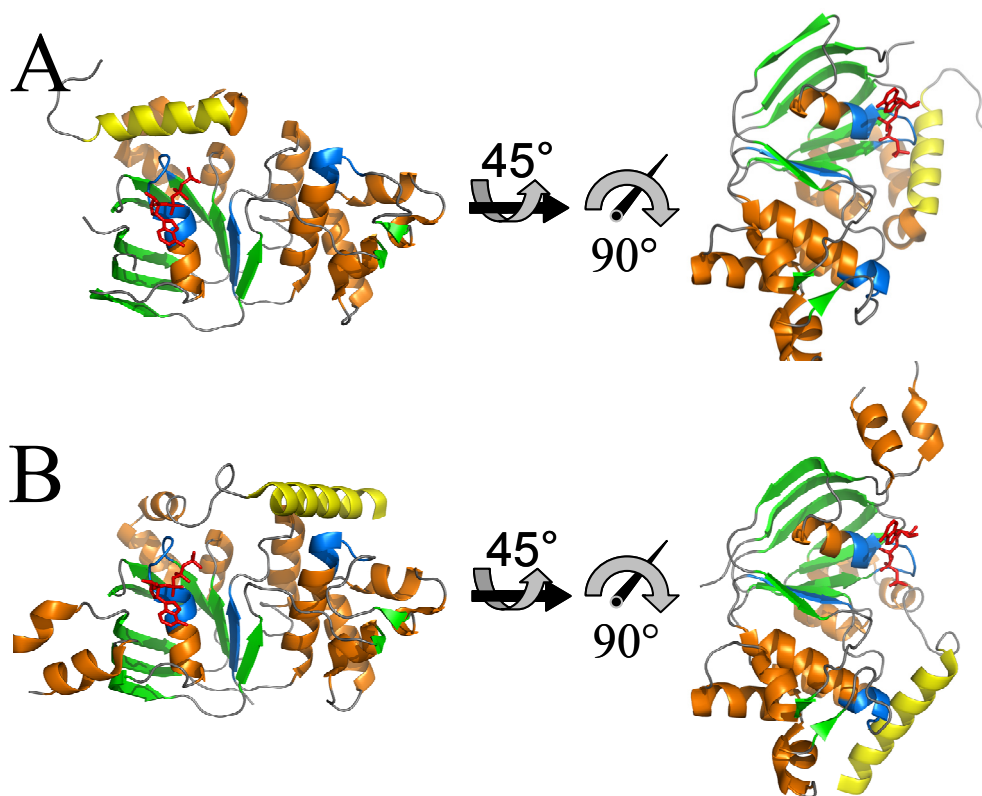


FIGURE 3-12: The C-terminal helix is found in multiple conformations. Human CFTR NBD1 crystal structures of $\Delta F508$ (PDB: 1XMJ) and F508A (PDB: 1XMI) are shown with α -helices colored orange and β -strands colored green. The highly conserved Walker A and B motifs along with the signature sequence are colored blue while the C-terminal helix is colored yellow and the nucleotides are colored red. The C-terminal helix is shown to occupy two different conformations, both of which reside in the putative dimer interface. The two respective positions of the C-terminal helix have different crystal contacts which suggest that the position of this helix is dependent upon the crystal packing of the protein.

the crystal contacts may be influencing the location of this helix. Upon closer inspection of the two structures, the F508A structure reveals that the C-terminal helix is involved in very different crystal contacts than is the C-terminal helix of the $\Delta F508$ structure, suggesting that these crystal contacts may be influencing the position of this helix. However, even in this altered crystal form the C-terminal

helix still resides in the dimer interface and would prevent a dimer from forming. Since all of the high resolution structural data on CFTR NBD1 are from crystallographic studies, a NMR structure of CFTR NBD1 would help to clarify where this C-terminal helix is in solution. In addition, crystal structures of NBD1-R as well as a full length CFTR may shed additional light on the location of this helix relative to the putative NBD1 dimer interface, which may in turn give us a better understanding of the biological role of C-terminal helix.

The crystal structure of mouse CFTR NBD1 revealed a number of important findings regarding the domain boundaries of the domain as well as providing the CF community with a structure in which the structural implications of various CF causing mutations could be better understood. In addition, the mouse NBD1 structure provided a key stepping stone which led to additional crystal structures of human wild type and $\Delta F508$ CFTR NBD1 corrected with additional mutations (Lewis et al., 2005). The corrected $\Delta F508$ crystal structure is consistent with previous reports that the loss of F508 did not have a large effect on the native state of the protein; rather, the defect manifested itself along the folding pathway (Qu et al., 1997; Qu and Thomas, 1996). Hopefully the recent advances in the structural understanding of CFTR NBD1 will lead to a true $\Delta F508$ structure and expand into other domains of CFTR, ultimately leading to a structure of the complete CFTR molecule.

Chapter Four:

Characterization of the human SLC26A3 STAS domain

Introduction:

One of the hallmarks of the SLC26A family of proteins is that they all have a C-terminally conserved domain which is homologous to the SpoIIAA anti-sigma factor antagonists (Aravind and Koonin, 2000) (see Figure 2-18). As a result, the C-terminal domain of the SLC26A transporters has been named STAS for sulfate transporters and anti-sigma factor antagonists. The function of the SpoIIAA family of antisigma-factor antagonists is to regulate sporulation of bacteria through a protein-protein interaction with the antisigma-factors (Duncan et al., 1996; Duncan and Losick, 1993; Garsin et al., 1998). The anti-sigma factor antagonist binds to the anti-sigma factor protein, and in the process displaces the sequestered sigma factor which is then free to bind the RNA polymerase holoenzyme and alter gene transcription.

Since the SLC26A STAS domains are homologous to the SpoIIAA proteins, they have been used as a structural model for the SLC26A STAS domains. However, there are some important differences between the SLC26A3 STAS domains and the SpoIIAA proteins. The biggest difference is that the SLC26A3 STAS domains have an insertion of sequence between the first α -helix

and the third β -strand of the SpoIIAA fold (Aravind and Koonin, 2000) (see Figure 2-18). We have named this insertion the intervening sequence (IVS), and it varies in length between the different SLC26A transporters from no IVS to 140 residues for SLC26A11 and SLC26A8 respectively. In addition, the highly conserved STAS domain N-terminal sequence $G\Phi XI\Phi XXXSPIYFAN$ and C-terminal sequence $F\Phi S/TVXD/EA\Phi$ (where X is any amino acid and Φ is a hydrophobic amino acid) are not as highly conserved in the SpoIIAA proteins, yet the high degree of conservation of these sequences suggests that they are critical for the function of the SLC26A STAS domains. As a result of the low sequence identity between the STAS domains and the SpoIIAA proteins it is difficult to predict the exact boundaries of the domain. Thus, the boundaries of the STAS domain must be determined experimentally.

The biological function of the STAS domain is not known. However, CFTR has been demonstrated to stimulate the Cl^-/HCO_3^- exchange activity of SLC26A transporter's, and this activation is inhibited by a CLD-causing mutation in the SLC26A3 STAS domain (Ko et al., 2002). This result suggests that the STAS domain may be important for this regulatory interaction. Therefore we hypothesized that the STAS domain may be required for this regulatory interaction. To test this hypothesis we decided to biochemically and biophysically characterize the STAS domain and identify the domain(s) of CFTR that are required for the activation of the SLC26A3 Cl^-/HCO_3^- exchange activity.

Experimental Procedures:

Plasmid Construction: The GST and GST-R domain constructs were constructed by first inserting a longer linking region between the GST and target protein in the pGEX-2T vector (Amersham). The new linker region was put in shortly after the Thrombin cleavage site, and has the sequence LVPRGSPGISGGGGGILDH. The CFTR R-domain constructs (654-855, 654-835, 670-855, 670-835) were cloned into this vector so that the first residue of the R-domain followed the last His of the elongated linker.

The wild type CFTR pCMV plasmid was a gift from Dr. J. Rommens (Hospital for Sick Children, Toronto). The mouse SLC26A3 STAS domain myc-pCMV vector has been previously described (Ko et al., 2004). Briefly, amino acids 518-713 were cloned into the myc-pCMV vector and confirmed by DNA sequencing.

Human SLC26A3 STAS domain was produced either as an MBP or Smt3 fusion. The MBP fusion protein was constructed by cloning human SLC26A3 amino acids 503-764 into the pMAL C2X vector. Nine human SLC26A3 STAS domain constructs were produced by cloning them into the Smt3 pET28 vector (Mossessova and Lima, 2000) using three N-terminal boundaries (503, 510, 525) and three C-terminal boundaries (720, 741, 764). All constructs were verified to be correct by DNA sequencing.

GST and GST-R Expression and Purification: GST and GST-R protein was expressed in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene) in 1L and 4L of LB respectively. The cells were grown at 37°C, to an O.D. of 0.65 and then induced with 100 μ M IPTG and shifted to 20°C for overnight expression. The following day the cells were collected by centrifugation and resuspended in 40 ml of lysing buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.2% Triton X 100 + Roche Protease Inhibitors) per liter of cells and lysed via sonication on ice. The lysates were cleared via centrifugation at 40,000 rcf for 30 min, and the supernatant was removed. The GST and GST-R supernatant fraction had 225 μ l of resuspended glutathione beads (Amersham Biosciences) added, and the lysates were rocked at 4°C for 2 hr. The beads were then washed 5 times with 1.4 ml of wash buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.2% Triton X 100) and after the final wash the beads were resuspended in 1 ml of wash buffer.

Phosphorylation of GST and GST-R: GST and GST-R beads were washed 3 times in 1 ml of PKA buffer (50 mM Tris pH 7.5, 10 mM MgCl₂ and 0.1 mg/ml BSA), then resuspended in 550 μ l of PKA buffer. 1 μ l of bovine heart PKA catalytic subunit (a generous gift from Dr. Melanie Cobb) was used per 50 μ l reaction volume, and incubated with 1 mM ATP at 30°C for 1hr. After the incubation the

beads were washed 3 times with 1.4 ml wash buffer, and then resuspended in 600 μ l of wash buffer.

STAS Domain Expression and Purification: The MBP and MBP-STAS 503-764 constructs were transformed into *E. coli* BL21 CodonPlus (DE3)-RIL (Stratagene) and positive clones selected. Cells were grown in LB to an O.D. of 0.5 at 37°C and induced with 100 μ M IPTG and allowed to express protein for 4 hr. Cells were collected via centrifugation and resuspended in 40 ml of lysing buffer (20 mM Tris pH 7.5, 200 mM NaCl, Roche protease inhibitors) per L of bacterial culture. Cells were lysed via sonication and the resulting lysates were cleared via centrifugation at 40,000 rcf for 30 min at 4°C. The supernatant fraction was run over an amylose column, and protein was eluted from the column in lysing buffer containing 10 mM maltose.

The Smt3-STAS constructs were expressed in *E.coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene) and grown at 37°C in LB containing 50 μ g/L Kanamycin and 34 μ g/L Chloramphenicol to an O.D.₆₀₀ of 0.8. The cells were then induced with 750 μ M IPTG (Research Products International) and shifted to 15°C for overnight expression. The following day the cells were pelleted at 3000 rcf and then resuspended in buffer A (20 mM Tris pH 7.9, 200 mM NaCl, 1 mM BME). The cells were then lysed via sonication and the resulting lysates were cleared by centrifugation at 40,000 rcf. Standard Ni chromatography was then

performed on the resulting supernatants using 10 ml bed volume of resin (Novagen) using buffer A as the equilibration buffer, buffer A containing 40 mM imidazole as the wash buffer and buffer A containing 400 mM imidazole as the elution buffer. Fractions containing the Smt3-STAS fusion protein were pooled together and digested overnight with a 1:1000 dilution of the Ulp1 protease at 4°C.

The following day 750 mM Ammonium Sulfate was added to the cleaved Smt3 STAS protein and then subjected to butyl hydrophobic interaction chromatography (Amersham Biosciences). The equilibration/binding buffer contained 20 mM Na₂PO₄ pH 5.9, 750 mM Ammonium Sulfate, 1 mM DTT and the elution buffer contained 20 mM Na₂PO₄ pH 6.2, 1 mM DTT. Fractions containing the STAS protein were pooled together and 350 mM NaCl was added to the solution. The protein was then concentrated to 2 ml and run over a HiPrep 16/60 Sephacryl S-200 column (Amersham Biosciences) in 20 mM Na₂PO₄ pH 6.2, 350 mM NaCl, 1 mM DTT.

GST and GST-R Pull-Downs: HEK 293 cells were transiently transfected with mouse SLC26A3 myc-STAS and allowed to express the protein for 48 hr. The cells were lysed in 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X 100 and protease inhibitors on ice for 30 min and the lysates were cleared via centrifugation at 12,000 rcf. 130 µl of the STAS lysates were added to 50 µl of

GST or GST-R beads and incubated overnight at 4°C on a rocking platform. The following day the beads were allowed to settle by gravity, and the supernatant aspirated off. The beads were washed 4 times with 500 µl of wash buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.2% Triton X 100), and the bound proteins were eluted using sample buffer and subjected to western blot analysis.

A similar protocol was used for the Smt3-STAS pull-down experiments except 200 µl of Ni column purified Smt3-STAS protein was used in place of mouse SLC26A3 myc-STAS protein lysate from HEK 293 cells.

CFTR Cl⁻ Current Recordings: Whole cell recordings of CFTR Cl⁻ currents in HEK 293 cells have been described elsewhere (Ko et al., 2004; Zeng et al., 1997). Briefly, the bath and pipette solutions were such that the only permeable ion was Cl⁻ allowing for the direct measurement of Cl⁻ currents. The membrane potential was held at -60 mV throughout the experiment, and the resistances of the pipet, access and seals were 3-7 MΩ, 10 MΩ and >10 GΩ respectively.

Analytical HPLC Gel Filtration Chromatography: A Tosoh QC-PAK TSK 200GL column was used to monitor the oligomeric state of the purified human STAS 510-741 protein. 150 µg of STAS protein in 20 mM Na₂PO₄ pH 6.5, 1 mM DTT and either 0, 250 or 500 mM NaCl, was injected onto the column which had a flow rate of 1 ml/min and a mobile phase of 20 mM Na₂PO₄ pH 6.5.

Analytical Ultracentrifugation: Analytical ultracentrifugation was performed on the purified STAS domain at 1, 5 and 10 mg/ml in 20 mM Na_2PO_4 pH 5.9, 350 mM $(\text{NH}_4)_2\text{SO}_4$ and 1 mM DTT. The protein was centrifuged in a Beckman XLI centrifuge in an AN60Ti rotor at 17 and 24 Krpm. Interference optics data was collected at 675 nm at 4°C using the Beckman Optima software. Partial specific volume and the solvent densities were calculated using Sednterp.

Trypsin Digests: Overnight trypsin digests were performed on the purified STAS protein in 20 mM Na_2PO_4 pH 5.9, 200 mM NaCl, 1 mM DTT at 4°C with various STAS to trypsin ratios (1:10,000, 1:1,000 and 1:100). The digest reactions were stopped by the addition of sample buffer and the protein fragments visualized by SDS-PAGE.

Mass Spectrometry and N-terminal Sequencing: The purified human STAS domain 510-741 was given to the UT Southwestern Biopolymer Laboratory for electrospray mass spectrometry analysis and by Edman Degradation N-terminal sequencing.

STAS Urea Denaturation: Tryptophan fluorescence spectra were taken of the purified STAS domain 510-741 protein with increasing concentrations of urea

with an excitation wavelength of 290 nm. The folded STAS domain has a tryptophan fluorescence maximum at 327 nm, and the denatured STAS domain (in 6 M urea) has a tryptophan fluorescence maximum at 345 nm. A ratio of the folded to unfolded fluorescence maximums was calculated by dividing the fluorescence intensity at 327 nm by the fluorescence intensity at 345 nm for each urea concentration. This fluorescence ratio was then plotted as a function of urea concentration and pre and post-transition baselines were calculated. The fraction native was then calculated using the following equation: $F_n(C) = (Y_x(C) - Y_u(C)) / (Y_n(C) - Y_u(C))$. Where $F_n(C)$ is the fraction native, $Y_x(C)$ is the fluorescence ratio at urea concentration x , $Y_u(C)$ is the value of the post-transition baseline at urea concentration x and $Y_n(C)$ is the value of the pre-transition baseline at urea concentration x . The average and standard deviation of three independent experiments is shown.

NMR of the STAS Domain and CFTR R-domain: ^{15}N labeled CFTR R-domain (CFTR amino acids 654-838) was expressed and purified by Jennifer Baker in Dr. Julie Forman-Kay's laboratory following our protocol. A $^{15}\text{N}/^1\text{H}$ TROSY spectrum was collected of 50 μM CFTR R-domain on an 800 MHz spectrometer in 20 mM Na_2PO_4 pH 6.8, 350 mM NaCl, 2 mM DTT for 1½ hr in the presence or absence of 150 μM unlabeled purified human SLC26A3 STAS.

Results:

To biochemically and biophysically characterize the SLC26A3 STAS domain I needed protein constructs with which I could produce milligram quantities of soluble, well folded protein. I also wanted to produce protein that was biologically active, and the only known function of the STAS domain to date is its role in SLC26A3 activation by CFTR. It was hypothesized that the STAS domain may be interacting with the R-domain of CFTR since PKA was required for the observed activation. To test this hypothesis I began by producing GST-R domain fusion proteins with different N and C-terminal boundaries for use in pull-down experiments to test for a physical interaction between the STAS and R-domains.

Four different GST-R-domain fusion proteins (CFTR amino acids 670-835, 670-855, 645-835, 654-855) were constructed and then tested for expression and purification in *E. coli* (Figure 4-1). All four of the fusion proteins were expressed however; only a very small fraction of the total protein was soluble. Differing amounts of degradation were also observed for the four different GST-R constructs. The two constructs whose C-terminal boundaries are CFTR amino acid 855, showed high levels of protein degradation. This contrasts with the GST-R constructs whose C-terminal boundary is CFTR amino acid 835 which showed very little to no protein degradation. The GST-R construct with domain boundaries 654-835 was selected for pull-down experiments because it had the

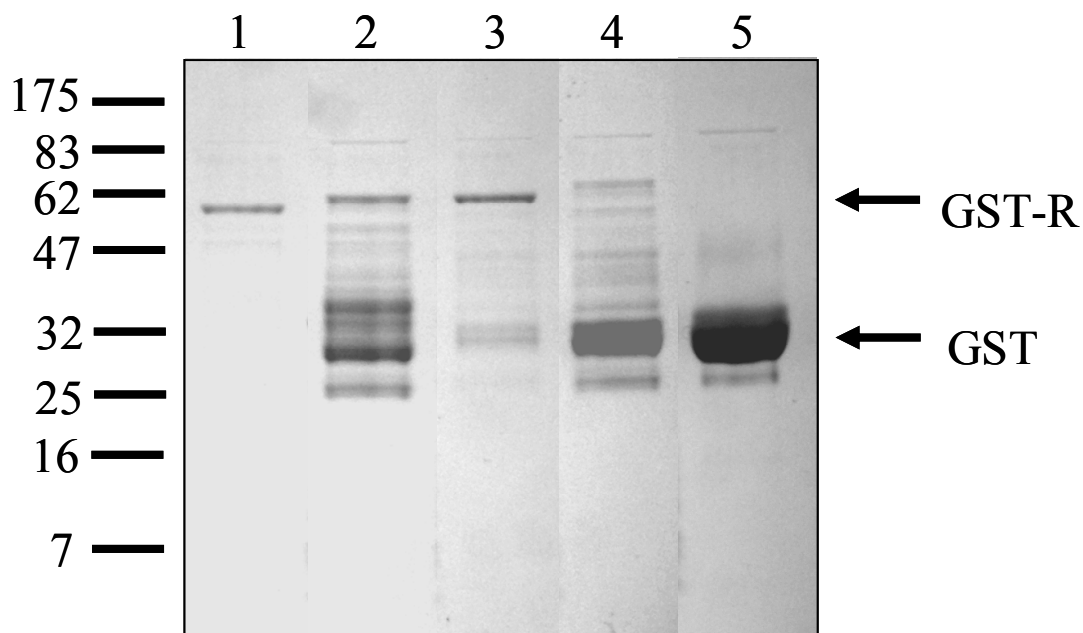


FIGURE 4-1: GST-R production. A Coomassie-stained gel showing the purity of the GST and the four GST-R proteins. The proteins were expressed and purified from *E. coli* using glutathione beads as described in experimental procedures. The molecular weight markers are measured in kDa, and the protein samples in each lane are as follows: Lane 1) GST-R 670-835, 2) GST-R 670-855, 3) GST-R 654-835, 4) GST-R 654-855, 5) GST.

highest level of soluble expression with relatively low levels of degradation, and without precise knowledge of what residues were important for the interaction between the STAS and R-domains, we wanted to include as many of the R-domain residues as possible.

Since the functional interaction between CFTR and SLC26A3 only occurs when the R-domain of CFTR is phosphorylated by PKA (Ko et al., 2004), we decided to perform our pull-down experiments with both unphosphorylated and phosphorylated GST-R. Phosphorylation reactions were set up of the GST and

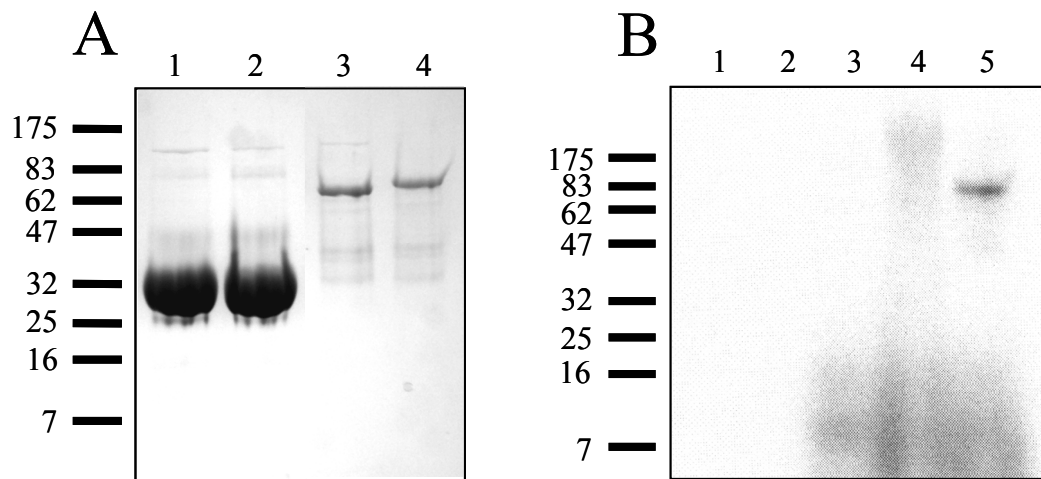


FIGURE 4-2: Phosphorylation of GST and GST-R with PKA. The purified GST and GST-R 654-835 were incubated with PKA and ATP for *in vitro* phosphorylation. Panel (A) shows a Coomassie-stained gel of purified GST and GST-R 654-835 before and after *in vitro* phosphorylation. The molecular weight markers are measured in kDa and the protein samples in each lane are as follows: 1) GST, 2) GST + PKA and ATP, 3) GST-R 654-835, 4) GST-R 654-835 + PKA and ATP. (B) A phosphoimage of GST and GST-R 654-835 before and after an *in vitro* phosphorylation reaction using γ - ^{32}P labeled ATP is shown. The molecular weight markers are measured in kDa and the protein samples in each lane are as follows: 1) GST, 2) GST-R 654-835, 3) PKA and ATP, 4) GST + PKA and ATP, 5) GST-R 654-835 + PKA and ATP.

GST-R proteins in the presence of PKA and ATP. A Coomassie-stained gel is shown of GST and GST-R phosphorylation reactions in the presence or absence of ATP (Figure 4-2A), along with a phosphoimage of the reactions in the presence of γ - ^{32}P -ATP (Figure 4-2B). A shift in the electrophoretic mobility of the GST-R construct can be seen in the Coomassie-stained gel indicating that all the GST-R protein has been phosphorylated. This is in contrast to the GST protein where no shift in the electrophoretic mobility is observed. In addition, the phosphoimage of

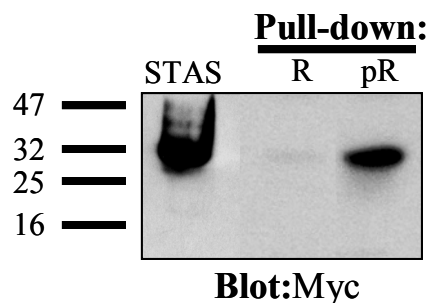


FIGURE 4-3: Phosphorylated GST-R pulls down the STAS domain more readily than unphosphorylated GST-R. HEK 293 cells transiently transfected with the mouse STAS domain were used with GST-R 654-835 for pull down experiments plus and minus phosphorylation. A western blot is shown of the expressed STAS domain (first lane), the unphosphorylated GST-R pull down of the STAS domain (second lane) and the phosphorylated GST-R pull down of the STAS domain (lane 3). The molecular weight markers are measured in kDa. Adapted by permission from Macmillan Publishers Ltd: Ko *et al.* Nat Cell Biol. (2004) 6(4):343-50.

STAS domain more readily than was the unphosphorylated GST-R protein, suggesting that the phosphorylation of the R-domain strengthens this interaction (Figure 4-3).

These results suggest that CFTR amino acids 654-835 and mouse SLC26A3 518-713 are sufficient for the physical association of these domains. To characterize the interaction between the human SLC26A3 STAS domain and

the gel shows only the GST-R construct being labeled with ^{32}P . Using the specific activity of the ^{32}P and the protein concentration a stoichiometry of 4 phosphates for every GST-R molecule was calculated.

Now that I was able to produce phosphorylated or unphosphorylated GST-R protein, GST pull-down experiments were performed with HEK 293 cell lysates which had been transiently transfected with mouse SLC26A3 STAS domain (amino acids 518-713). The phosphorylated GST-R protein was able to pull-down the mouse

the CFTR R-domain, the human SLC26A3 STAS domain was cloned into a suitable expression vector. To aid in identifying the appropriate human SLC26A3 STAS domain boundaries, a multiple sequence alignment of the SLC26A transporters and four bacterial anti-sigma factor antagonists which have been identified as structural homologs of the SLC26A STAS domains was constructed (see Figure 2-18). From this alignment human SLC26A3 amino acids 525-720 were cloned into the pMAL C2X vector to produce an MBP-STAS fusion construct. When expressed in *E. coli*, the MBP-STAS domain 525-720 protein was produced but was not well behaved biochemically. The fusion protein was soluble to roughly 3 mg/ml and Factor Xa was able to cleave the MBP tag from the STAS domain. However, when the STAS domain was run over a gel filtration column the protein was eluted in the void volume. As a result, this construct was deemed to be unfit for further biochemical and biophysical studies and new improved constructs had to be produced.

I thought that perhaps our human STAS domain boundaries were too short on the N and C-termini, so I produced another MBP-STAS fusion with SLC26A3 amino acids 503-764. This construct could be concentrated approximately 4.5 mg/ml before precipitation was observed. However, the STAS domain itself still migrated in the void volume of gel filtration column after cleavage from MBP, while MBP eluted as expected. Interestingly, the longer MBP-STAS fusion protein was functional in terms of its ability to activate CFTR channel activity.

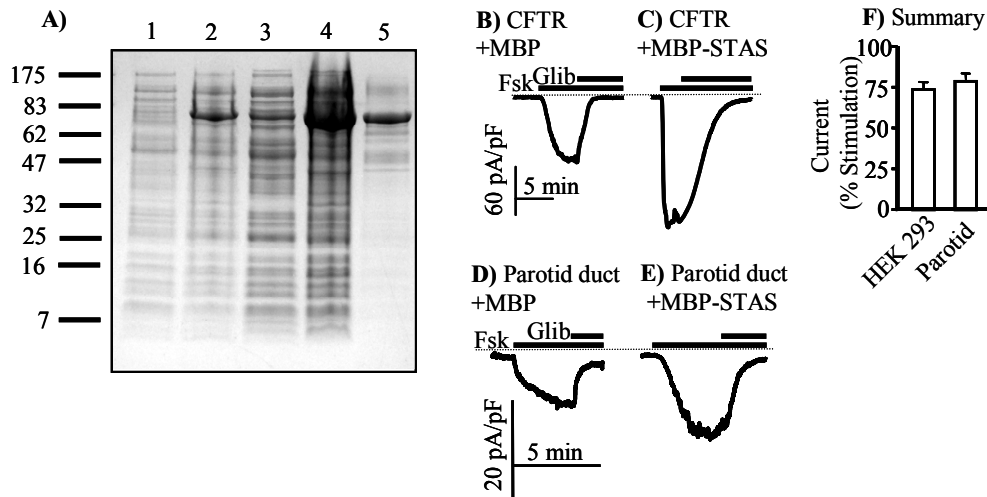


FIGURE 4-4: MBP-STAS activates CFTR in HEK 293 cells and in parotid duct cells. (A) A Coomassie-stained gel of the human STAS domain expressed and purified from *E. coli* as an MBP fusion with domain boundaries 503-764 is shown. The molecular weight markers are measured in kDa and the protein samples loaded on the gel are as follows: lane 1) uninduced cell lysate, 2) induced cell lysate, 3) supernatant fraction of cell lysate, 4) pellet fraction of cell lysate, 5) MBP-STAS elution off of an amylose column. (B) and (C) show Cl⁻ current recordings from HEK 293 cells transiently transfected with CFTR and infused with either MBP or MBP-STAS and treated with forskolin (Fsk) or glibenclamide (Glib). (D) and (E) show Cl⁻ current recordings from mouse parotid duct cells infused with MBP (panel D) or MBP-STAS (panel E) and treated with forskolin (Fsk) and glibenclamide (Glib). (F) A summary of the Cl⁻ current stimulation of the parotid duct cells and the transiently transfected HEK 293 cells. Adapted by permission from Macmillan Publishers Ltd: Ko *et al.* Nat Cell Biol. (2004) 6(4):343-50.

MBP and MBP-STAS 503-764 fusion proteins were purified using amylose affinity chromatography (Figure 4-4A), and the purified protein was given to Dr. Weizhong Zeng who infused the protein into HEK 293 cells transiently transfected with CFTR, or freshly dissected mouse Parotid duct cells (Figure 4-4B-E) (Ko *et al.*, 2004). It was found that the MBP-STAS protein was capable of activating CFTR's Cl⁻ channel activity in both HEK 293 cells and mouse parotid duct cells.

From this data I knew I could produce functional human STAS domain protein, but I still needed a way to express and purify biochemical amounts of soluble protein. I decided to try cloning the STAS domain into the Smt3 pET 28 expression vector to test if a different fusion partner might aid the expression and purification of soluble human SLC26A3 STAS domain. I chose to produce nine different STAS domain constructs to test various N and C-terminal boundaries for the domain. Human SLC26A3 amino acids 503, 510, and 525 were chosen as the N-terminal boundaries while 720, 741, and 764 were selected as the C-terminal boundaries. Using these N and C-termini, I produced the nine possible constructs as Smt3-STAS fusion proteins and tested their soluble expression, purification and ability to be cleaved from the Smt3 tag (Figure 4-5). All three of the constructs produced with amino acid 720 as their C-terminal boundary were expressed exclusively as insoluble proteins. However, the remaining six constructs were all expressed with some improved degree of solubility. In addition, all of the solubly produced protein was efficiently cleaved by the Ulp1 protease and the free STAS domain remained soluble, allowing for further purification of the domain (Figure 4-5).

This left six soluble SLC26A3 STAS constructs which all had similar expression and purification characteristics. In order to identify which construct to more extensively characterize biochemically and biophysically, I performed GST pull-down experiments to test the ability of the Smt3-STAS

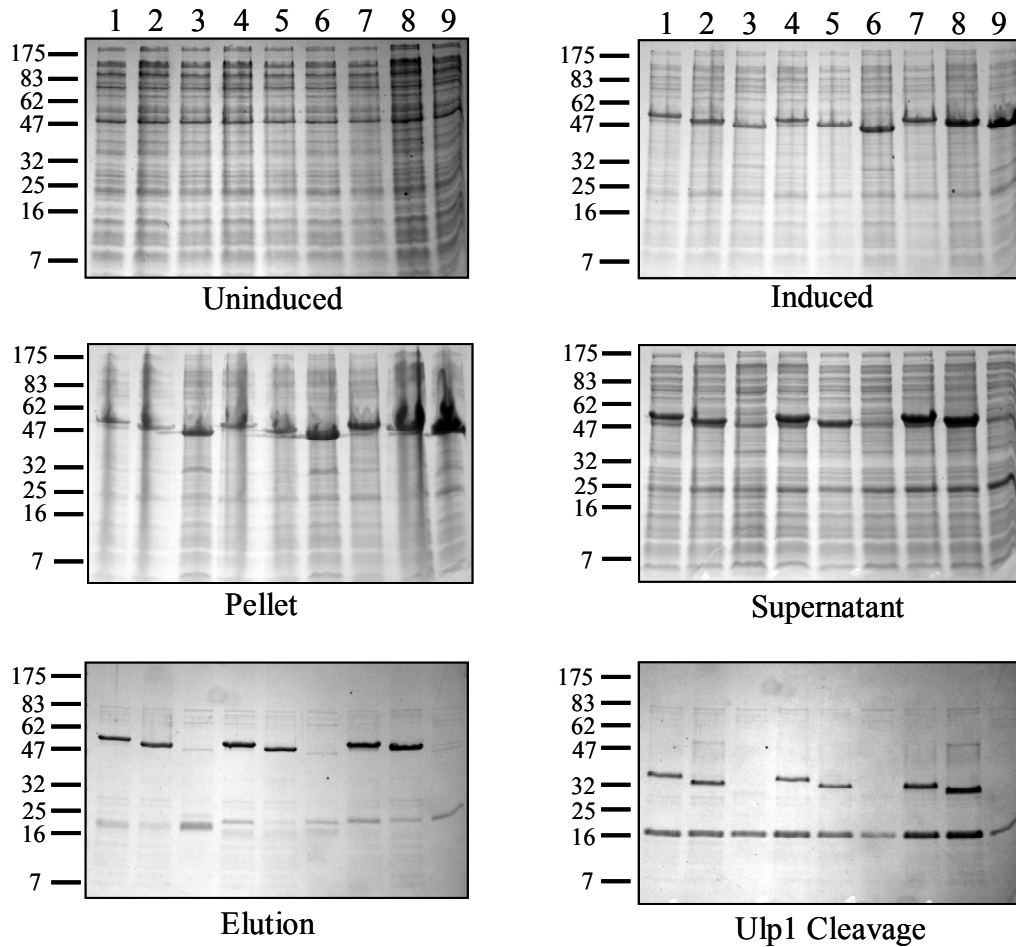


FIGURE 4-5: Defining the STAS domain boundaries. Nine different STAS domain constructs were produced as Smt3 fusions with three different N-termini (amino acids 503, 510 and 525) and three C-termini (amino acids 720, 741 and 764). Six different gels are shown of uninduced, induced, pellet, supernatant, Ni Bead Elution and Ulp1 cleaved samples for the various constructs. The molecular weight markers are measured in kDa and the protein constructs in each lane are as follows: 1) Smt3-503-764 2) Smt3-503-741, 3) Smt3-503-720, 4) Smt3-510-764, 5) Smt3-510-741, 6) Smt3-510-720, 7) Smt3-525-764, 8) Smt3-525-741, 9) Smt3-525-720.

proteins to interact with the R-domain of CFTR (Figure 4-6A). Five of the six Smt3-STAS constructs showed non-specific binding to GST, while the STAS domain with amino acid domain boundaries 510-741 showed specific binding to

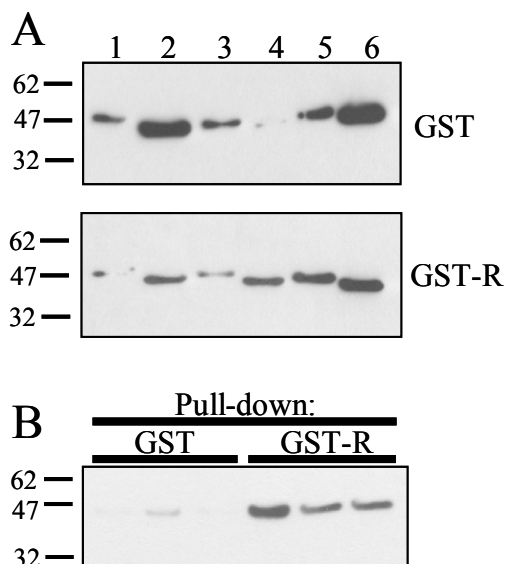


FIGURE 4-6: GST-R pull-downs of the Smt3-STAS fusion proteins. (A) An anti-His₆ western blot is shown of the six soluble Smt3-STAS fusion proteins pulled down by GST (top panel) or by GST-R (bottom panel). Lane 1) Smt3-STAS 503-764, lane 2) Smt3-STAS 503-741, lane 3) Smt3-STAS 510-764, lane 4) Smt3-STAS 510-741, lane 5) Smt3-STAS 525-764, lane 6) Smt3-STAS 525-741. (B) An anti-His₆ western blot of GST and GST-R pull-down experiments performed in triplicate with the Smt3-STAS 510-741 construct. The molecular weight markers shown to the left of the gels are measured in kDa.

GST-R. For this reason the STAS domain with amino acid boundaries 510-741 was selected as the construct for further biochemical and biophysical characterization (Figure 4-6B).

In order to biochemically and biophysically characterize the SLC26A3 STAS domain 510-741, I needed to purify it to homogeneity. This was accomplished using a three step purification process (Figure 4-7). Standard Ni affinity chromatography was performed as the initial purification step followed by fusion protein cleavage with Ulp1. The resulting protein mixture was eluted

from a Butyl column and the fractions containing the STAS domain were pooled and run over a Sephacryl S-200 gel filtration column.

The gel filtration column allowed me to estimate the monomeric state of the STAS domain. The elution profile from the Sephacryl S-200

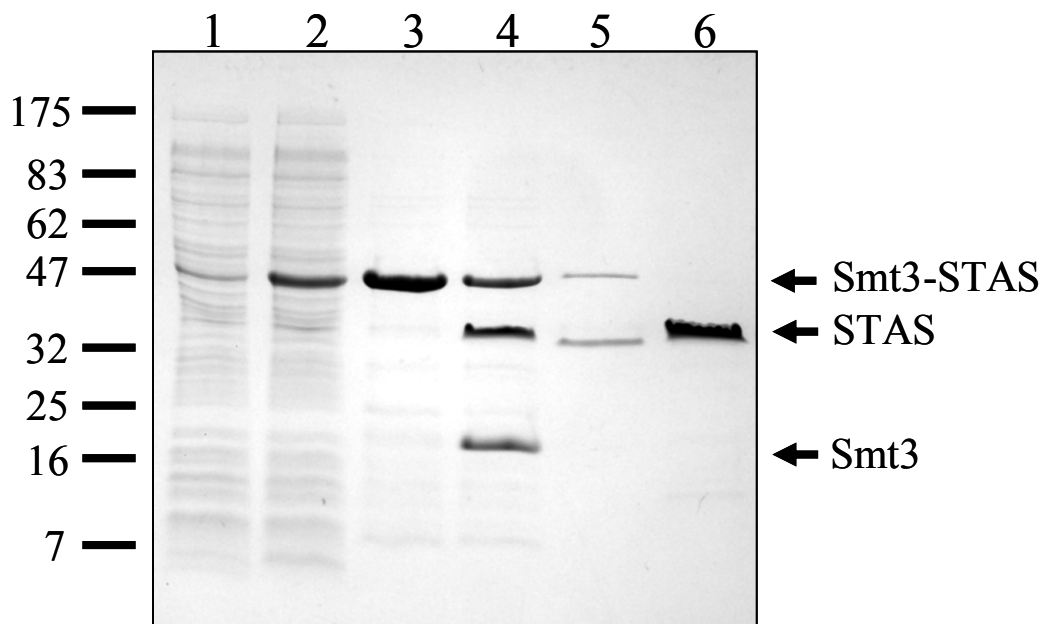


FIGURE 4-7: Purification of the Smt3-STAS 510-741 construct. A representative Coomassie-stained gel showing the purity of the STAS 510-741 protein after each purification step. The samples loaded in each lane are as follows: 1) uninduced cell lysate, 2) induced cell lysate, 3) Ni column elution, 4) Ulp1 digest of Ni column elution, 5) Butyl column elution, 6) Sephacryl S-200 gel filtration elution. The molecular weight markers are shown to the left of the gel and are measured in kDa.

column was consistent with the STAS domain being a monomer (Figure 4-8A). The STAS domain was also run over an analytical HPLC Tosoh QC-PAK TSK 200GL gel filtration column to more carefully monitor the oligomeric state of the protein. The STAS domain eluted from the analytical Tosoh HPLC gel filtration column in two peaks, one with a 5.7 min retention time which is consistent with it being a monomer, and another peak with a retention time of 6.3 min which is consistent with a smaller protein species (Figure 4-8B). Both of these peaks were collected and run on an SDS-PAGE gel to determine if the second peak was

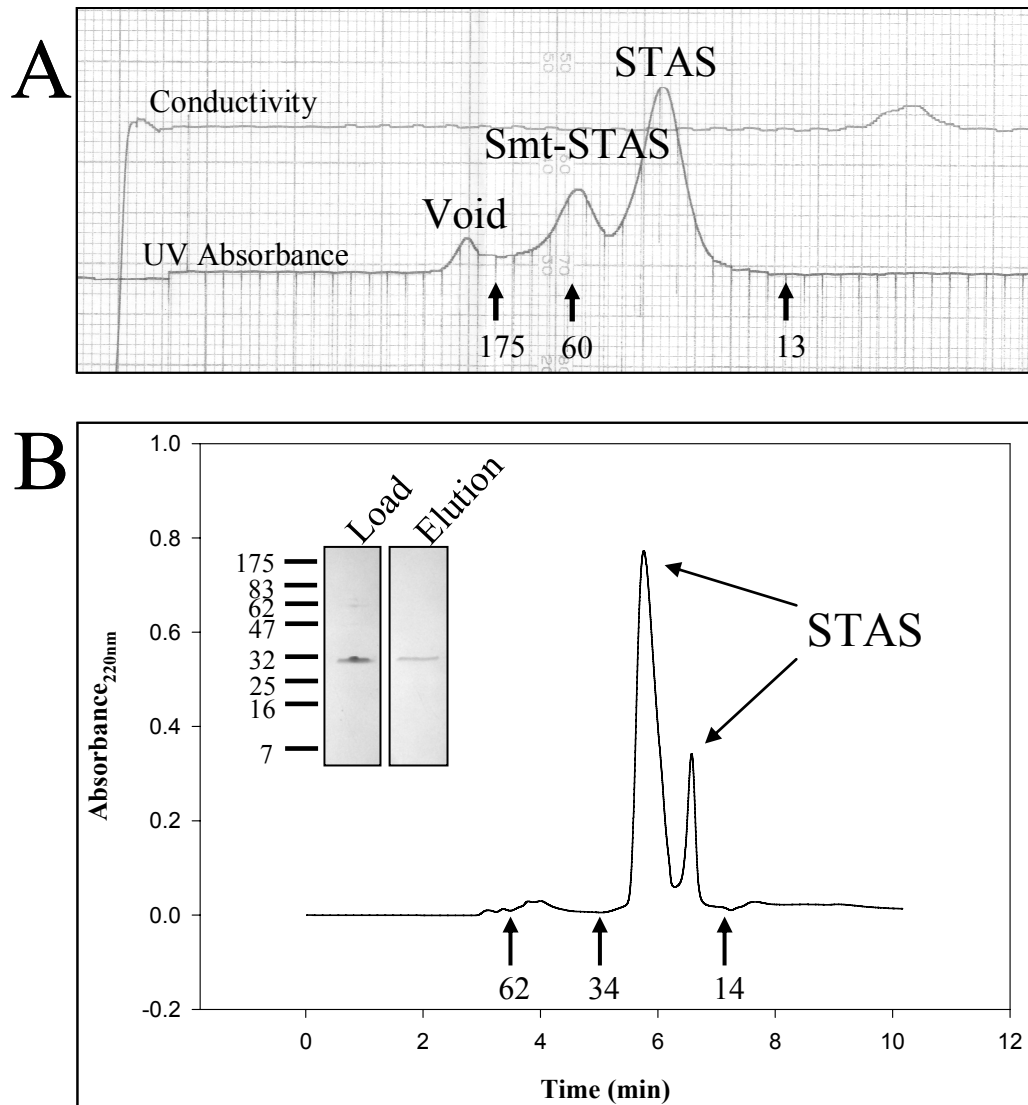


FIGURE 4-8: STAS 510-741 gel filtration chromatography. (A) A representative HiPrep Sephacryl S-200 Hi Resolution gel filtration chromatogram of the STAS 510-741 protein. Conductivity and UV absorbance (280 nm) traces are labeled along with the void, Smt3-STAS and STAS UV absorbance peaks. The retention times of Catalyase (175 kDa), BSA (62 kDa), and Lysozyme (13 kDa) are indicated below the UV absorbance trace. (B) The UV absorbance (220 nm) profile of purified STAS 510-741 protein eluting from an analytical Tosoh QC-PAK TSK 200GL gel filtration column. Retention times of the molecular weight standards BSA (62 kDa), Carbonic Anhydrase (28 kDa) and Lysozyme (13 kDa) are shown below the trace. The inset Coomassie-stained gel shows the purity of the protein loaded onto and eluted from the column with molecular weight standards measured in kDa shown to the left of the gel.

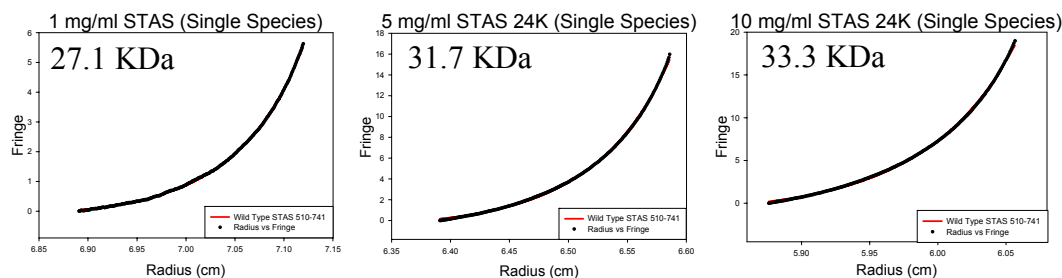


FIGURE 4-9: STAS 510-741 analytical ultracentrifugation. Three different concentrations of STAS 510-741 were subjected to analytical ultracentrifugation at 17,000 rpm for 48 hr. The resulting fringe data was best fit by a single species model, estimating the molecular weight of the protein to be 27.1, 31.7 and 33.3 kDa at the 1, 5 and 10 mg/ml concentrations respectively. A monomer-dimer model did not significantly improve the fitting of the experimental data.

a degradation product of the STAS domain, however, both samples appeared to be full length protein (Figure 4-8B inset). In addition the peak heights appeared to be modulated by the NaCl concentration. As the NaCl concentration increased so did the absorbance of the 6.3 min peak.

There are two possible explanations for the two STAS elution peaks from the Tosoh gel filtration column. One possible explanation is that the STAS domain is adopting two different conformations, one of which is much more compact than the other. The second possibility is that the STAS domain is indeed dimerizing, and both the monomer and dimer species have greater retention times than would be predicted for a protein of their size. To distinguish between these two possibilities, analytical ultracentrifugation was performed as an additional method to monitor the oligomeric state of the protein (Figure 4-9). The analytical ultracentrifugation was performed at three different protein concentrations; the

experimental data was fit to a single species which predicted the STAS molecular weight to be 27.7, 31.7 and 33.3 kDa at 1, 5 and 10 mg/ml respectively. Fitting the experimental data to a monomer-dimer equilibrium did not significantly improve the fit of the data, consistent with the STAS domain being a monomer.

To look at the stability of the STAS domain we performed trypsin digests with varying molar ratios of STAS to trypsin, and allowed the digest to proceed overnight at 4°C. The trypsin digest revealed two stable bands which were present at approximately 18 and 10 kDa (Figure 4-10). N-terminal sequencing was

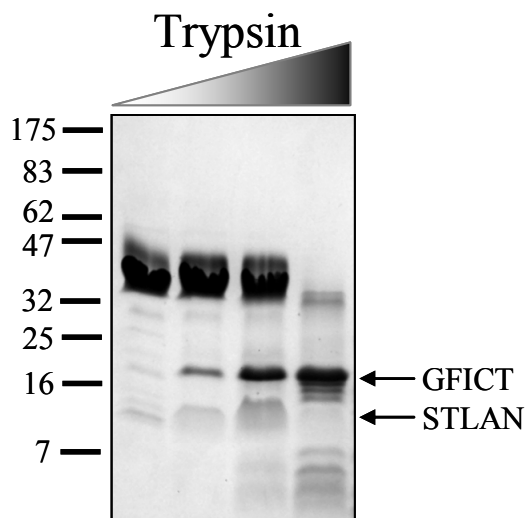


FIGURE 4-10: Trypsin digest of the STAS 510-741 protein. A Coomassie-stained gel is shown of a trypsin digest experiment which was performed on purified STAS 510-741 protein at 4°C overnight with increasing concentrations of trypsin. Two stable bands were observed at the intermediate trypsin concentrations and were subjected to N-terminal sequencing which yielded the amino acid sequences GFICT (amino acids 593-597) and STLAN (amino acids Ser-510-513). Note: the Ulp1 cleavage of the STAS 510-741 protein results in a single N-terminal Ser residue left on the STAS domain construct, which happens to be the 509th residue of SLC26A3. The molecular weight markers left of the gel are measured in kDa.

performed on these two bands to determine the trypsin cleavage site. N-terminal sequencing of the smaller of the two bands yielded the sequence STLAN which is the beginning of the STAS domain construct, while the larger band yielded the

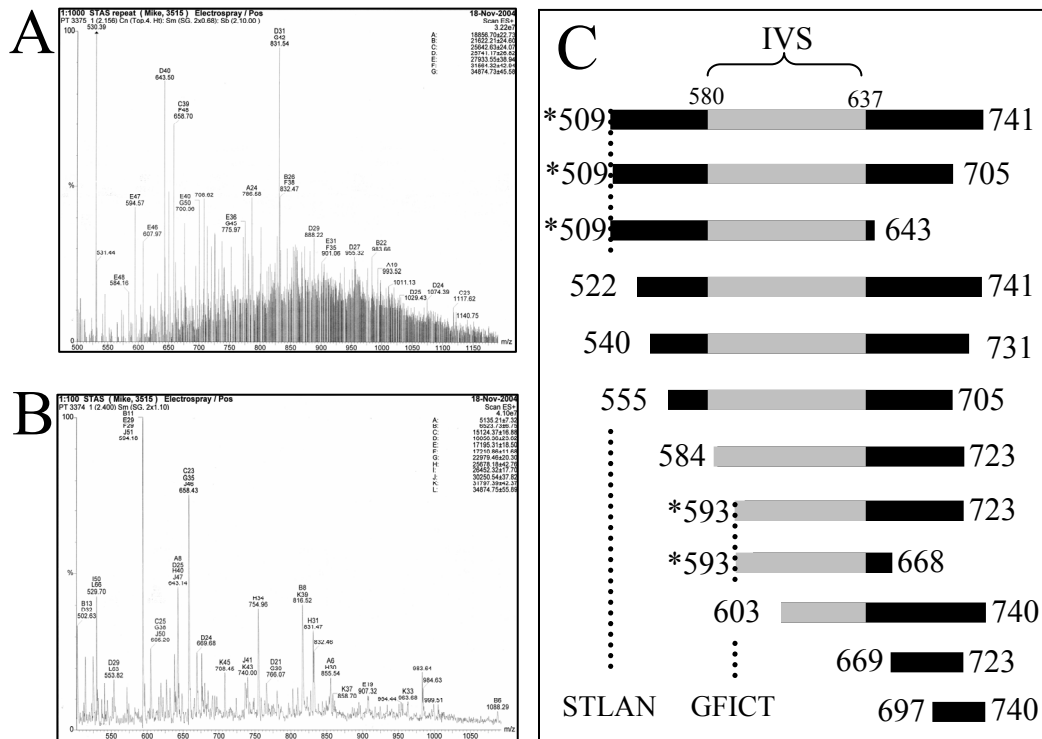


FIGURE 4-11: Electrospray mass spectrometry of the Trypsin digested STAS 510-741 protein. (A) The trypsin digested STAS 510-741 protein shown in Figure 4-12 (lane 2) was subjected to electrospray mass spectrometry. Multiple masses were observed and are listed A-G in the top right hand corner of the figure. (B) The trypsin digested STAS 510-741 protein shown in Figure 4-12 (lane 3) was subjected to electrospray mass spectrometry. Multiple masses were observed and are listed A-L in the top right hand corner of the figure. (C) The mass spectrometry tools on the Protein Prospector website were used to identify the various STAS 510-741 protein fragments which are shown schematically with the intervening sequence (IVS) shown in gray and the rest of the STAS sequence shown in black. The N and C-terminal residues of each fragment are listed on the left and right side of the schematic respectively, and the asterisks denote the N-termini (STLAN and GFICT) which were observed in the N-terminal sequencing reactions performed on the two most predominant bands in the trypsin digest shown in Figure 5-10.

amino acid sequence GFICT which corresponds to SLC26A3 amino acids 593-597 in the IVS.

Samples from the trypsin digest were also given to the UT Southwestern biopolymer laboratory for mass spectrometry analysis (Figure 4-11). The electrospray spectrum of the 1:1000 trypsin to STAS ratio shows seven observed peptides and their respective molecular weights (Figure 4-11A), while the 1:100 molar ratio digest shows twelve peptide fragments and their molecular weights (Figure 4-11B). The mass spectrometry tools on the Protein Prospector website were used to determine the various cleavage sites in the STAS domain that were cleaved. The various cleaved protein peptides identified from the two mass spectrometry experiments are shown schematically in Figure 4-13C.

The stability of the STAS domain was also assessed by performing urea denaturation experiments. Tryptophan fluorescence emission scans were collected by exciting the protein at 290 nm in various concentrations of urea (Figure 4-12). The fluorescence ratio of 327 nm (folded STAS fluorescence maximum) to 345 nm (denatured STAS fluorescence maximum) was calculated and plotted as a fraction folded after baseline corrections were made. From this fraction folded plot the midpoint of the urea denaturation curve is calculated to be 3.1 M urea, and hysteresis is observed for the folding reaction of the STAS domain (open circles in Figure 4-14). The inset panel of Figure 4-14 shows fluorescence spectra of the folded and unfolded STAS domain.

After characterization of the purified SLC26A3 STAS domain 510-741, we sent our protein constructs to the laboratory of Dr. Julie Forman-Kay to look

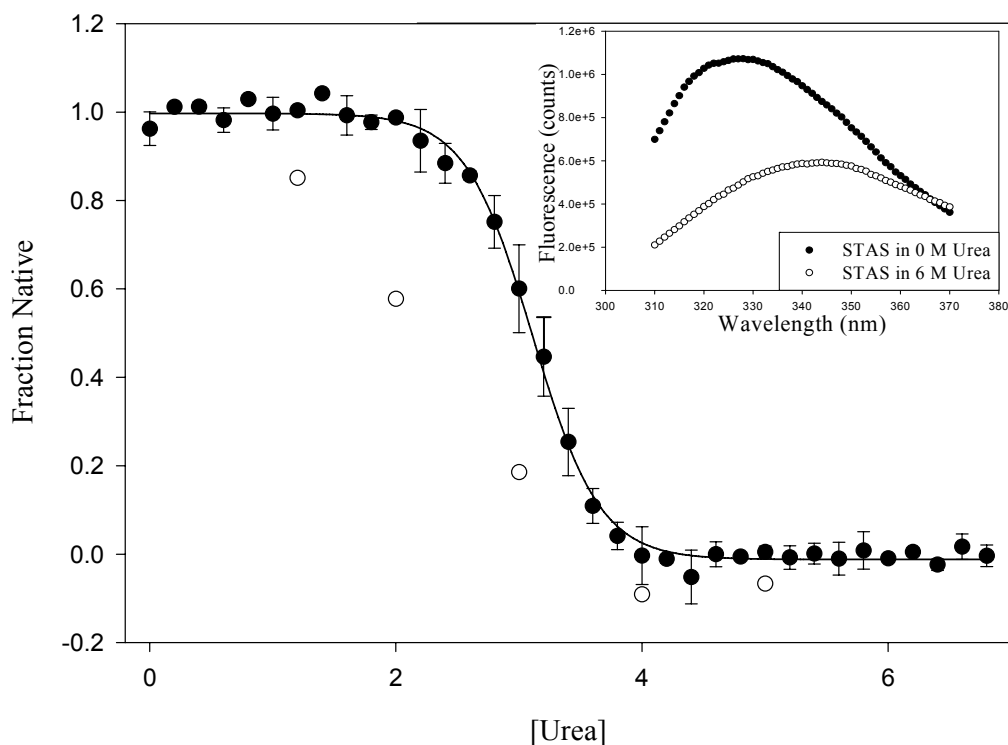


FIGURE 4-12: STAS 510-741 folding and unfolding transitions. Purified STAS 510-741 protein was unfolded using various concentrations of urea (filled circles) and tryptophan fluorescence spectra were collected. The folded STAS 510-741 protein fluorescence peak maximum was at 327 nm, while the unfolded protein had a fluorescence peak maximum at 345 nm. A ratio of the 327 nm/345 nm fluorescence intensity was calculated and converted into a fraction native and plotted as a function of urea concentration. The midpoint of the unfolding transition for the STAS 510-741 protein is 3.1 M urea. Hysteresis is observed upon refolding (open circles). The inset shows the folded STAS domain tryptophan fluorescence spectra in 0 M urea or the unfolded spectra in 6 M urea.

at the purified protein's ability to interact with the R-domain of CFTR. TROSY-HSQC experiments using 50 μ M purified 15 N labeled CFTR R-domain (amino acids 654-838) were conducted in the presence or absence of 150 μ M unlabeled

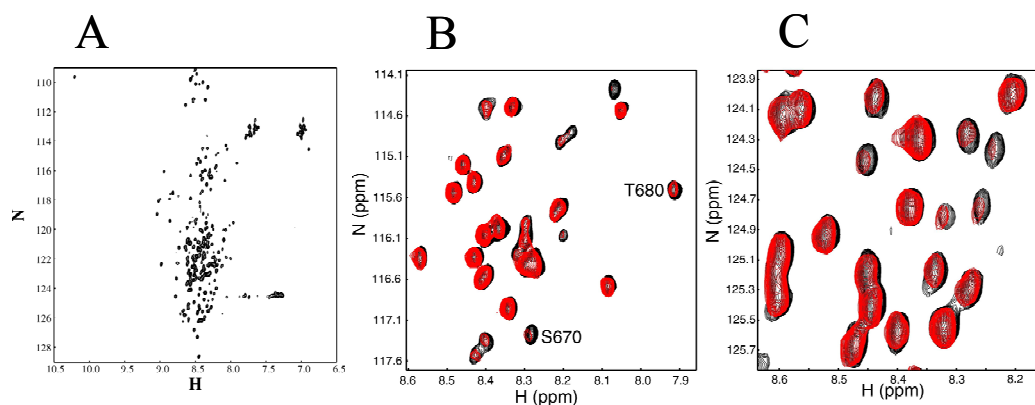


FIGURE 4-13: HSQC spectrum of CFTR R-domain in the presence or absence of the STAS domain. (A) A TROSY-HSQC spectra of purified ^{15}N labeled CFTR R-domain. (B) TROSY-HSQC spectrum of the ^{15}N labeled phosphorylated R-domain (black peaks) or ^{15}N labeled phosphorylated R-domain mixed with unlabeled STAS domain (red peaks). Ser670 is experiencing a significantly different chemical environment in the presence of the STAS domain than in its absence. (C) A different portion of the TROSY-HSQC spectrum shown in B highlighting the overlap of most of the proton-nitrogen coupled cross peaks.

STAS domain. Several specific residues in the R-domain, including Ser670, show significant line broadening in the presence of the STAS domain, suggesting that these residues are involved in the binding interaction with the STAS domain (Figure 4-13). This result suggests that the STAS domain 510-741 is not only biochemically well behaved but also functionally competent to bind to the R-domain after purification.

Discussion:

I set out to biochemically and biophysically characterize the SLC26A3 STAS domain and to determine if it interacted with the R-domain of CFTR. Thus, I needed to create STAS and R-domain constructs which would be useful for binding experiments. The GST-R 654-835 construct was chosen for the pull-down experiments because it was the longest construct which had the least amount of degradation (Figure 4-1). The R-domain of CFTR has been previously demonstrated to be phosphorylated by PKA *in vivo* (Cheng et al., 1991; Picciotto et al., 1992), so to test my recombinantly produced GST-R protein's ability to be phosphorylated, *in vitro* phosphorylation reactions were performed using PKA and ATP. Most if not all of the GST-R protein was phosphorylated by PKA, and the phosphorylated GST-R had a stoichiometry of four moles of phosphate per mole of protein which is similar to previously reported values (Cheng et al., 1991; Picciotto et al., 1992; Townsend et al., 1996).

GST pull-down experiments were performed using both unphosphorylated and phosphorylated GST-R to monitor the potential physical interaction between the STAS and R-domains. I found that the mouse SLC26A3 STAS domain interacted with the phosphorylated R-domain of CFTR more readily than it did with the unphosphorylated R-domain (Ko et al., 2004) (Figure 4-3). This is consistent with the STAS domain of SLC26A3 binding to the phosphorylated R-

domain of CFTR *in vivo* and increasing CFTR's open probability (Ko et al., 2004).

The SLC26A3 STAS domain encompassing amino acids 503-764 was expressed and purified as an MBP fusion protein. The purified MBP-STAS 503-764 protein was competent to activate CFTR Cl⁻ current when it was infused into CFTR expressing HEK 293 cells or freshly dissected mouse Parotid duct cells (Figure 4-4). This is consistent with previous observations that the STAS domain is required for the activation of CFTR's Cl⁻ channel activity (Ko et al., 2002). In addition, these results also suggest that my recombinantly expressed STAS domain is biologically active even though the biochemical properties of the protein are not ideal.

In an attempt to produce milligram quantities of monomeric purifiable STAS domain protein, nine different human SLC26A3 STAS domain constructs were cloned into the Smt3 pET 28 expression vector (Figure 4-5) (Mossessova and Lima, 2000). The three protein constructs which contained SLC26A3 amino acid 720 as their C-terminal boundary were expressed almost exclusively as insoluble protein. Interestingly, amino acid 720 was also the same domain boundary used in the short MBP-STAS 525-720 construct which showed elevated levels of aggregation and poor solubility compared to the longer MBP-STAS 503-764 construct. This is consistent with the hypothesis that the human SLC26A3 STAS domain probably includes residues C-terminal to amino acid 720. In

addition, residue 720 in human SLC26A3 is analogous to mouse SLC26A3 amino acid 713, which was the C-terminal residue of the mouse SLC26A3 STAS domain construct used in the initial GST pull-down experiments (Figure 4-3). This suggests that there may be domain boundary differences between the mouse and human SLC26A3 STAS domains, or that mammalian cells can fold and maintain the shorter STAS domain in solution with greater efficiency than can bacterial cells. Additional GST pull-down experiments were performed on the six soluble Smt3-STAS fusion proteins with unphosphorylated GST-R, and the Smt3-STAS 510-741 construct was found to specifically bind with GST-R protein in three out of five experiments (Figure 4-6). Since these pull-down reactions were performed with purified GST-R and purified Smt3-STAS protein, these results also indicate that the interaction between the STAS domain and the R-domain is direct and no additional adaptor or bridging proteins are necessary for their interaction. From these results, the STAS 510-741 construct was chosen for further biochemical and biophysical characterization.

After I had established that I would use amino acid 510 as the N-terminal boundary of the SLC26A3 STAS domain construct, a report was published that used SLC26A6 amino acid 497 as their N-terminal STAS domain boundary (Alvarez et al., 2005). Amino acid 497 in SLC26A6 is analogous to amino acid 513 in SLC26A3 which indicates that two independent groups have identified very similar N-terminal boundaries for the STAS domain (Alvarez et al., 2005).

However, Alvarez and colleagues defined the C-terminal boundary of the SLC26A6 STAS domain to be amino acid 633, which from our multiple sequence alignment is predicted to be near the end of the IVS, suggesting that their GST-STAS domain construct is likely incomplete, and truncated on the C-terminus (Alvarez et al., 2005).

The purified SLC26A3 STAS domain 510-741 yielded soluble and highly pure protein which appeared monomeric from the elution profile of a HiPrep Sephacryl S-200 gel filtration column (Figure 4-8A). However, when the purified STAS domain was run over the Tosoh size exclusion column a large absorbance peak was observed at a retention time of 5.7 min, consistent with a 27 kDa protein, and a second absorbance peak was observed with a retention time of 6.3 min which is consistent with a 22 kDa protein. SDS-PAGE results suggested that both elution peaks contained full-length STAS domain protein eliminating the possibility that the 22 kDa peak was a degradation product (Figure 4-8B inset). This suggested that the STAS protein may be dimerizing, and the two observed absorbance peaks could correspond to a monomer and dimer which elute from the column with a greater retention time than predicted for typical globular proteins. Another possible explanation for this data is that the STAS domain protein may adopt at least two distinct conformations which can be separated by gel filtration chromatography, or the STAS domain may be interacting with the matrix of the column. In addition, the ionic strength of the protein buffer seems

to modulate the relative intensities of these two peaks, with increasing ionic strengths giving rise to a larger absorption peak at 6.3 min. This suggests that the salt concentration is somehow affecting the oligomeric state of the STAS domain or its conformation.

The potential dimerization of the STAS domain is of interest because it is a homolog of the SpoIIAA proteins which have been shown to dimerize with the SpoIIAB protein (Aravind and Koonin, 2000; Duncan et al., 1996; Garsin et al., 1998). As a result, it may be possible that the STAS domain has retained the protein binding function of the SpoIIAA protein, and can either form dimers on its own, or form complexes with additional proteins such as the R-domain of CFTR (Aravind and Koonin, 2000; Duncan et al., 1996; Garsin et al., 1998; Ko et al., 2004).

To further characterize the oligomeric state of the STAS domain, equilibrium analytical ultracentrifugation (AUC) experiments were performed on the purified protein (Figure 4-9). At a STAS domain concentration of 1 mg/ml, the single species fit to the AUC data was 27.1 kDa, which is very close to the 27.0 kDa molecular weight predicted for the STAS domain from the amino acid sequence. However, when the STAS domain concentration was increased to 5 mg/ml or to 10 mg/ml the single species fit to the AUC data increased from 27.1 kDa to 31.7 and 33.3 kDa respectively, suggesting that the STAS domain or some other contaminant in our sample was beginning to oligomerize. Since the

equilibrium AUC was performed under different buffer conditions than were the Tosoh gel filtration experiments, it is difficult to directly compare these two sets of data. Nonetheless, since AUC is an equilibrium method which is insensitive to protein shape (Lebowitz et al., 2002), it seems likely that the STAS domain protein is monomeric at least at protein concentrations of 1 mg/ml and below.

Limited proteolysis experiments performed on the STAS domain yielded two stable fragments with the N-terminal sequence STLAN, which corresponds to the N-terminus the STAS protein construct (amino acids 509-513), and GFICT which corresponds to amino acids 593-597. This data indicated that our STAS domain construct was not being proteolytically cleaved on the N-terminus during the expression and purification procedure. It also indicated that our domain was relatively resistant to proteolysis, suggesting that our STAS domain is well folded.

In addition to N-terminal sequencing of the two main proteolytic bands from the limited proteolysis experiment, the 1:100 and 1:1000 trypsin to STAS ratio digests were analyzed by mass spectrometry. Using the mass spectrometry tools from the Protein Prospector website (<http://prospector.ucsf.edu/>) the identification of some of the minor proteolytic fragments observed in our digest were determined (Figure 4-11). Twelve proteolytic cuts were observed throughout the STAS domain out of a predicted 34 possible cleavage sites. The trypsin cleavage sites are scattered throughout the domain without any significant

clustering. One interesting set of predicted cleavage sites which were not identified as being cleaved in our mass spectrometry experiments were the nine predicted trypsin cleavage sites found from amino acids 566 to 580. It is possible that these sites are readily cut by trypsin and simply not detected in our mass spectrometry experiments. However, it is also possible that there is another secondary structural element in this sequence that is absent from the SpoIIAA structures, which is preventing trypsin cleavage.

The sequence from 565-645 is defined as the IVS which is not found in the SpoIIAA structural homologs and currently has no known function. Using the secondary structure prediction program Jpred (<http://www.compbio.dundee.ac.uk/~www-jpred/>), the IVS sequence is predicted to be largely unstructured except for residues 565-583, which are predicted to be α -helical in nature. This is consistent with the idea that trypsin sites in this sequence may not be readily cleaved by trypsin, which would explain why these peptides were not observed in our mass spectrometry experiments. It is also worth noting that the next trypsin cleavage site that we observed in our mass spectrometry experiments is 583, which is predicted by Jpred to be the end of an α -helix. In addition, there is significant homology between the various SLC26A transporters (with the exception of SLC26A11) up to human SLC26A3 amino acid 577 where the sequence similarity drops off until the end of the IVS, suggesting that perhaps most of the SLC26A STAS domains contain an extra

secondary structural element which the SpoIIAA domains lack. Finally, the proteolytically cleaved peptides observed by mass spectrometry include some which contain the N and C-termini, confirming that our STAS domain is not being proteolytically cleaved on the N or C-termini during protein expression and purification.

To further characterize the stability of the STAS domain, urea denaturation experiments were performed and tryptophan fluorescence spectra of the protein samples were collected. The tryptophan fluorescence spectra show a fluorescence maximum at 327 nm, consistent with the single tryptophan being in a relatively hydrophobic environment. This is noteworthy because W631 is located towards the end of the IVS which is not predicted to contain any regular secondary structural elements. This suggests that the secondary structure prediction of the STAS domain IVS is not completely accurate, or that the W631 packs up against the STAS domain in a relatively apolar environment. When the STAS domain is unfolded by 6.0 M urea, the fluorescence maximum is red-shifted to 345 nm, consistent with the tryptophan being exposed to a relatively polar environment (Figure 4-12 inset). A urea denaturation curve was constructed by collecting fluorescence spectra of the STAS domain in the presence of varying concentrations of urea as described in the experimental procedures. The unfolding and refolding curves of the STAS domain did not overlay one another, indicating that there was hysteresis under these experimental conditions and thus,

the folding and unfolding reactions of the STAS domain were not at equilibrium. As a result, no thermodynamic parameters for STAS domain folding could be calculated.

It had previously been shown that the Smt3-STAS 510-741 construct was competent to bind to the GST-R construct but it had not yet been shown that the purified STAS domain without the Smt3 tag could bind to the R-domain of CFTR. NMR experiments conducted by Jennifer Baker in Dr. Julie Forman-Kay's laboratory suggest that indeed purified STAS domain does interact with the R-domain of CFTR (Figure 4-13). Specifically, Ser670 experienced significant line broadening upon the addition of the unlabeled STAS domain. Ser670 is an interesting residue in the sense that it can be phosphorylated by PKA (Wilkinson et al., 1997) and it resides in the C-terminal helix of NBD1 (see Figure 3-11 and chapter 3 for more details). This is of great interest because the CFTR NBD1 structures have revealed that this C-terminal helix which lies across the dimer interface which would prevent the typical ATPase cycle for NBDs from occurring (see chapter three for details). One possibility is that this C-terminal helix does in fact lie across the dimer interface under physiological conditions, and upon binding of the STAS and R-domains the C-terminal helix undergoes a conformational change which moves it away from the dimer interface. This reorientation of the C-terminal helix would allow the typical "head-to-tail" NBD dimer to form and allow the proposed NBD ATPase cycle to occur, thereby

opening the Cl⁻ channel of CFTR. Alternatively, the position of the C-terminal helix may be influenced by the crystallization conditions, and this helix may rarely adopt this conformation *in vivo*. The C-terminal helix is not part of the core NBD fold as it is absent in all other NBD structures solved to date (Chang, 2003; Chang and Roth, 2001; Chen et al., 2003; Diederichs et al., 2000; Gaudet and Wiley, 2001; Horn et al., 2003; Hung et al., 1998; Karpowich et al., 2001; Locher et al., 2002; Reyes and Chang, 2005; Schmitt et al., 2003; Smith et al., 2002; Verdon et al., 2003a; Yuan et al., 2001). This suggests that the C-terminal helix may be better ascribed to the R-domain of CFTR than the NBD1. It also suggests that in solution the C-terminal helix of the CFTR NBD1 may not reside in the NBD1 dimer interface, and the position of this helix may be influenced by the crystallization conditions and crystal contacts. Indeed, this helix can adopt two distinct conformations with respect to the core fold of NBD1 depending upon the crystal contacts, and perhaps in the context of additional domains of CFTR this helix would reside elsewhere, and not be found in the putative dimer interface (see chapter three discussion for more details). Additional studies will be required to fully understand the position of this helix relative to the dimer interface of NBD1, as well as what residues in the C-terminal helix and the rest of the R-domain are required for its interaction with the STAS domain.

Chapter Five:

The Effect of CLD Causing Mutations on SLC26A3 Structure and Function

Introduction:

SLC26A3 was first identified as a gene that was down regulated in human colon adenocarcinomas and subsequently named DRA (Rouached et al., 2005). Thus far SLC26A3 has not been conclusively demonstrated to be a tumor suppressor gene (Chapman et al., 2002; Hemminki et al., 1998), but it has been unequivocally demonstrated to be a $\text{Cl}^-/\text{HCO}_3^-$ exchanger with a 2:1 transport stoichiometry (Chapman et al., 2002; Hemminki et al., 1998; Ko et al., 2002; Ko et al., 2004; Shcheynikov et al., 2006). The transport function of SLC26A3 is thought to play an important role in Cl^- absorption and HCO_3^- secretion in the colon and pancreas respectively (Chapman et al., 2002; Hemminki et al., 1998; Hoglund et al., 1996b; Ko et al., 2002; Ko et al., 2004; Moseley et al., 1999).

The highly conserved C-terminal STAS domain of the SLC26A proteins has been demonstrated to be important for proper ion transport activity (Chernova et al., 2003; Ko et al., 2002; Ko et al., 2004; Rouached et al., 2005; Shibagaki and Grossman, 2004; Shibagaki and Grossman, 2006). This C-terminal domain has been shown to be homologous to the SpoIIAA family of antisigma-factor

antagonists (Aravind and Koonin, 2000) (see Figure 2-18). The SLC26A3 STAS domain has been demonstrated to undergo a protein-protein interaction with the R-domain of CFTR, which is regulated by phosphorylation (Ko et al., 2004). The result of the STAS and R-domain interaction is the reciprocal activation of CFTR's Cl^- channel activity and SLC26A3's $\text{Cl}^-/\text{HCO}_3^-$ exchange activity (Ko et al., 2004), which is thought to be important for proper pancreatic function (Ko et al., 2002; Ko et al., 2004).

Mutations found in the SLC26A3 gene (also known as DRA or CLD) have been linked to congenital chloride diarrhea (CLD) (Hoglund et al., 1996b), a disease in which patients suffer from watery diarrhea containing elevated Cl^- concentrations which can prove fatal if left untreated (Holmberg, 1986). Clinical management of the disease includes increased dietary uptake of water, KCl and NaCl (Hihnala et al., 2006a; Holmberg, 1986). However, a recent report also suggests butyrate is effective in controlling the patient's diarrhea (Canani et al., 2004). There are currently over 30 mutations identified in SLC26A3 which have been linked to CLD (Dawson and Markovich, 2005; Holmberg, 1986; Makela et al., 2002). Four of these mutations are missense and/or insertion mutations which reside in the STAS domain of SLC26A3 ($\Delta\text{Y526/7}$, I544N, I675/6ins and G702T followed by the nine amino acid insertion TGFEVKIQNF (G702Tins)). The I544N and I675/6ins mutations have been demonstrated to cause a loss of measurable transport activity of the human and mouse SLC26A3 protein

respectively (Chernova et al., 2003; Ko et al., 2002; Ko et al., 2004). However, no information regarding how these mutations may result in the loss of function has been reported. In addition, no information regarding the effect that the $\Delta Y526/7$ or G702Tins mutations have on the SLC26A3 protein has been published to date. In an attempt to elucidate the molecular mechanism(s) by which the CLD causing mutations residing in the SLC26A3 STAS domain give rise to disease, we functionally, biochemically and cell biologically characterized the wild type and mutant transporters. In addition, we monitored the effect of the CLD causing mutations on the isolated STAS domain. These data has led us to a better understanding of the molecular mechanisms by which the CLD causing mutations in the STAS domain give rise to disease.

Experimental Procedures:

Plasmid construction: Mammalian expression constructs for full-length human SLC26A3 protein were constructed using standard molecular biology techniques and cloned into the BglII and NotI restriction sites of the pCMV-Myc vector (Clontech). Site directed mutagenesis was performed using Pfu Turbo DNA polymerase (Stratagene) to generate the $\Delta Y526/7$, I544N, I675/6ins and G702Tins mutations in the SLC26A3 protein. Human SLC26A3 STAS domain constructs were generated with domain boundaries encompassing residues 509-741, and were cloned into the BglII and NotI sites of the pCMV-Myc vector, while amino acid residues 510-741 were cloned into the BamHI site of the Smt3-pET28 vector (Mossesso and Lima). All constructs were verified by DNA sequencing.

Cl/HCO₃⁻ Exchange: HeLa cells grown on glass cover slips were transfected with 0.5 μ g of the appropriate SLC26A3 plasmid along with 0.5 μ g of a GFP expression plasmid. 3 μ l of Fugene 6 (Roche) was used per transfection, and the cells were used for Cl⁻/HCO₃⁻ exchange experiments 48 hr post transfection. GFP positive cells were selected on a fluorescence microscope (Nikon) and then incubated with 10 μ M 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probes) and 0.2% Pluronic F-127 (Molecular Probes) for 10 min in Cl⁻ Buffer (10 mM Hepes pH 7.4, 140 mM

sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 10 mM Glucose, osmolarity (Osm) 310). Cells were perfused with Cl^- buffer until a steady baseline was achieved, then cells were perfused with HCO_3^- buffer for 5 min (2.5 mM Hepes pH 7.4, 120 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 10 mM Glucose, Osm 260, then 50 mM of sodium bicarbonate was added). Subsequently the cells were perfused with Cl^- free HCO_3^- buffer for 5 min (2.5 mM Hepes pH 7.4, 120 mM sodium gluconate, 5 mM potassium gluconate, 1 mM magnesium sulfate, 1 mM cyclamic acid, 10 mM Glucose, Osm 260 then 50 mM sodium bicarbonate was added), followed by a 5 min perfusion with HCO_3^- buffer and finally the cells were perfused with Cl^- buffer for 5 min. Carbon dioxide gas was bubbled through buffers containing bicarbonate throughout the experiment. The change in the fluorescence excitation ratio of BCECF-AM was monitored at 490 nm/440 nm with the emission at 510 nm. pH_i was calibrated using three standard buffers containing 10 mM Hepes, 140 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 10 mM glucose and 5 μM nigericin at pH 6.65, 7.4 and 8.15.

Immunofluorescence: HeLa and COS7 cells were grown on glass cover slips (Fisher) in 35 mm dishes using DMEM (Gibco) containing 10% calf serum (Gemini) and supplemented with penicillin and streptomycin at 37°C in a

humidified incubator with 5% CO₂. HeLa cells were transfected with 1 µg of wild type or mutant SLC26A3 pDNA using Fugene 6 following the manufacture's protocol. 48 hr post transfection cells were washed twice with PBS and then fixed in 3.7% formaldehyde for 20 min and subsequently washed twice for 5 min in PBS. Cells were permeablized with 0.1% Triton X 100 for 10 min at room temperature followed by two washes with PBS. The permeablized cells were incubated for 20 min in blocking solution (1.3% fish skin gelatin and 1% BSA in PBS) followed by a 1 hr incubation with a 1:100 dilution of rabbit anti-Grp78 (Bip) (Stressgen), 1:500 anti-c-Myc (Santa Cruz Biotechnology) or 1:500 anti-STAS, and a 1:500 Phalloidin conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Sigma) in blocking solution. The cells were subsequently washed twice with PBS for 5 min and then incubated with a 1:100 dilution of anti-rabbit antibody conjugated to cyanine-5.18 (cy5) (Jackson Immuno Research) and a 1:500 dilution of anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson Immuno Research) in blocking solution. Cells were washed and mounted using gel mount (Biomedica). Stained cells were visualized and images collected using a Ziess confocal microscope and Zeiss LSM 510 software version 2.8.

Glycosylation Assay: COS7 cells were grown in DMEM (Gibco) containing 10% calf serum (Gemini) supplemented with penicillin and streptomycin at 37°C or

30°C in a humidified incubator with 5% CO₂. 35 mm dishes of COS7 cells were transfected with 1 µg of wild type or mutant SLC26A3 pDNA using Fugene 6 (Roche) following the manufacture's guidelines. 48 hr post transfection the cells were washed twice with 0.5 ml TBS and then lysed in 0.5 ml of RIPA buffer (20 mM Tris pH 8.0, 140 mM sodium chloride, 0.1% SDS, 0.5% DOC, 1% IGEPAL and protease inhibitors (Roche)) on ice for 30 min. The lysates were centrifuged at 12,000 rcf for 5 min and then 100 µl of lysate was incubated at 37°C for 1 hr with 500 units of EndoH (NEB), 500 units of PNGaseF (NEB) or no enzyme. The proteins were separated by SDS-PAGE and transferred to PVDF membrane for western blot analysis.

STAS Solubility assay: *E. coli* BL21 Codon Plus RIL cells (Stratagene) containing the human wild type and mutant Smt3-SLC26A3 STAS domain plasmids were grown at 37°C in LB (Qbiogene) containing 50 µg/L Kanamycin and 34 µg/L Chloramphenicol to an O.D.₆₀₀ of 1.0. The cells were induced with 750 µM IPTG (Research Products International) and shifted to 15°C for overnight expression. The following day O.D.₆₀₀ measurements were taken and used to ensure that the same number of cells were pelleted at 4°C at 3000 rcf. The cell pellets were then resuspended in 20 ml of lysing buffer (20 mM Tris pH 7.9, 200 mM sodium chloride, 1 mM 2-mercapto ethanol) and lysed via sonication with a Branford 450 Sonicator. The resulting lysates were cleared via a 40,000

ref centrifugation step at 4°C for 30 min. Equal volumes of cell lysates were separated by SDS-PAGE and stained with coomassie blue or transferred for western blot analysis.

HEK 293 cells were transiently transfected with wild type and mutant pCMV-Myc SLC26A3 STAS domains, and incubated at 37°C for 48 hr and subsequently scraped off the plate in 0.5 ml of lysing buffer. The cells were lysed by sonication and centrifuged at 60,000 rcf for 30 min at 4°C. Equivalent amounts of protein were loaded on the gel as determined by a Bradford assay.

Western Blotting: SDS-PAGE gels for western blots were transferred to PVDF membrane (Millipore) using the wet transfer method in Tris/Glycine transfer buffer containing 10% methanol. The membranes were blocked using 5% milk (BioRad) in TBS containing 0.05% Tween 20 (Sigma), and primary and secondary antibody incubations were performed in blocking buffer. Primary antibody dilutions were as follows; a 1:1,000 dilution of the anti-His₆ (Novagen), a 1:3,000 dilution of the anti-c-Myc (Santa Cruz Biotechnology) and a 1:5,000 dilution of the anti-STAS antibody. Secondary antibody dilutions were as follows; 1:10,000 of the anti-mouseHRP (Jackson Immuno Research) and 1:10,000 of the anti-rabbitHRP (Jackson Immuno Research). ECL Plus (Amersham Biosciences) or SuperSignal (Pierce) detection reagent was used following the manufacture's guidelines and visualized using the STORM 840

(Amersham Biosciences) or Hyblot CL autoradiography film (Denville Scientific).

STAS expression and purification: Five liters of *E.coli* BL21 Codon Plus RIL cells (Stratagene) containing the wild type or mutant Smt3-STAS domain plasmids were grown at 37°C in LB containing 50 µg/L Kanamycin and 34 µg/L Chloramphenicol to an O.D.₆₀₀ of 0.8. The cells were induced with 750 µM IPTG (Research Products International) and shifted to 15°C for overnight expression. The following day the cells were pelleted at 3,000 rcf and resuspended in 70 ml of lysing buffer. The cells were then lysed via sonication and the resulting lysates cleared by centrifugation at 40,000 rcf. Standard Ni chromatography was performed on the resulting supernatants using 10 ml bed volume of resin (Novagen). The equilibration/binding buffer (buffer A) contained 20 mM Tris pH 7.9, 200 mM sodium chloride, 1 mM 2-mercapto ethanol, the column was washed using buffer A containing 40 mM imidazole and the protein was eluted from the column using buffer A containing 400 mM imidazole. Fractions containing the Smt3-STAS fusion protein were pooled together and digested overnight with a 1:1,000 dilution of the Ulp1 protease at 4°C.

750 mM Ammonium Sulfate was added to the cleaved protein solution and subjected to butyl hydrophobic interaction chromatography (Amersham Biosciences). The equilibration/binding buffer contained 20 mM sodium

phosphate pH 5.9, 750 mM ammonium sulfate, 1 mM DTT and the elution buffer contained 20 mM sodium phosphate pH 6.2, 1 mM DTT. Fractions containing the STAS protein were pooled together and 350 mM sodium chloride was added to the solution. The protein was concentrated to 2 ml and run over a HiPrep 16/60 Sephacryl S-200 column (Amersham Biosciences) in 20 mM sodium phosphate pH 6.2, 350 mM sodium chloride, 1mM DTT.

Circular Dichroism: Circular dichroism spectra were measured using an AVIV 62DS spectrophotometer in a 0.1 cm quartz cuvette. The STAS CD spectra and thermal denaturation curves were collected at 10 μ M in 20 mM sodium phosphate pH 6.2, 350 mM sodium chloride, 1 mM DTT.

NMR Spectroscopy: 15 N labeled wild type and Δ Y526/7 SLC26A3 STAS domain were expressed in M9 defined media with 15 N ammonia chloride as the only nitrogen source. The proteins were purified as described above with the only change being that the protein was eluted off of the gel filtration column in 50 mM potassium phosphate pH 6.8, 400 mM potassium chloride, 100 mM arginine, 2% glucose, 2 mM DTT. A TROSY-HSQC spectrum of 90 μ M purified STAS domain was collected on a 600 MHz spectrometer at 30°C for 12 hr.

Tryptophan Fluorescence: Fluorescence emission spectra (300-370 nm) of the wild type, Δ Y526/7 and I544N STAS domains were collected using an excitation wavelength of 290 nm and a protein concentration of 10 μ M. The fluorescence spectra of the folded STAS were taken in 20 mM Na_2PO_4 pH 6.2, 350 mM NaCl, 1 mM DTT and the denatured samples were in the same buffer with the addition of 6 M GdnHCl.

Δ STAS and STAS Co-transfection: HEK 293 cells were transfected with 1 μ g of the Δ STAS, STAS, or co-transfected with both the Δ STAS and STAS constructs. The HEK 293 cells were allowed to express the proteins for 48 hr and then were lysed in RIPA buffer. The resulting lysates were used for a glycosylation assay as described above.

STAS Domain Antibody Production: Recombinantly expressed and purified SLC26A3 STAS domain protein (described above) was used as the antigen for rabbit polyclonal antibody production. Briefly, five milligrams of purified protein was shipped to Proteintech Group Inc. for injection of two rabbits following their standard 102 day protocol. Two production bleeds and a final bleed from each animal were tested for their reactivity to the STAS domain protein, and all six antiserum samples were found to detect the STAS domain protein in a similar fashion.

SLC26A Recognition by the STAS Domain Antibody: COS7 cells were transfected with various SLC26A expression constructs and were allowed to express protein for 48 hr. The cells were lysed in RIPA buffer and the resulting lysates were subjected to western blot analysis using a 1:5,000 dilution of the STAS domain antibody. In addition to expressed SLC26A proteins, freshly dissected mouse Kidney and Submandibular gland were dounce homogenized in 5 ml of RIPA buffer and then briefly sonicated to ensure cell lysis.

SLC26A3 Immuno-precipitation: HEK 293 cells were transiently transfected with the wild type and mutant SLC26A3 transporters and allowed to express protein for 48 hr. The cells were then lysed in 0.5 ml RIPA buffer and 180 μ l of the lysate was incubated in the presence or absence of 2 μ l of the STAS domain antibody for 90 min at 4°C on a rocking platform. 20 μ l (bed volume) of protein G agarose was added to the immuno-precipitation reactions and incubated overnight at 4°C on a rocking platform. The protein G agarose beads were washed three times for 3 min with 200 μ l of RIPA buffer and the bound protein was eluted from the beads with SDS sample buffer.

Trypsin Digest: Transiently transfected HEK 293 cells were allowed to express the wild type and mutant SLC26A3 proteins for 48 hr at which time they were

washed twice with TBS and then scraped off the plate in 500 μ l of 10 mM HEPES pH 7.4 and 200 mM sucrose. The cells were then sonicated twice for 20 seconds on the lowest power setting and placed on ice. The resulting lysates were incubated in the presence of increasing concentrations of trypsin (0.3, 1, 3, 10...300 μ g/ml) at room temperature for 12 min. The digests were stopped using SDS sample buffer and the digested protein was separated by SDS-PAGE and the proteins transferred to PVDF membrane. Western blots were performed as described above using the anti-STAS antibody, and densitometry was performed on representative western blots using the ImageJ program.

Biotinylation Assay: HEK 293 cells were transiently transfected with wild type STAS domain, wild type SLC26A3 and the mutant SLC26A3 transporters and allowed to express the proteins for 48 hr. Cells were harvested and washed twice in ice cold PBS, and then left in 0.5 ml of ice cold PBS for 20 min on ice. Proteins were biotinylated in the presence of 0.74 mM EZ-Link Sulfo-NHS-SS-Biotin (Pierce) for 30 min at 4°C. The cells were then washed three times in ice cold wash buffer (25 mM Tris pH 8.0, 200 mM Glycine) and then lysed in 0.5 RIPA buffer. 350 μ l of cell lysate was then incubated with 25 μ l (bed volume) of ImmunoPure Immobilized Streptavidin agarose beads at 4°C over night on a rocking platform. The streptavidin beads were washed the following day three

times with 350 μ l of RIPA buffer, and proteins were eluted from the beads using SDS sample buffer and subjected to SDS-PAGE and western blot analysis.

Results:

To better understand how the identified CLD causing mutations in the SLC26A3 STAS domain give rise to CLD, I began by measuring the $\text{Cl}^-/\text{HCO}_3^-$ exchange activity of the wild type and mutant transporters in HeLa cells using the pH sensitive fluorescent dye BCECF-AM as previously described (Park et al., 2002). $\text{Cl}^-/\text{HCO}_3^-$ exchange activity was observed for the wild type and $\Delta\text{Y526/7}$ SLC26A3 proteins (Figure 5-1B), but no significant $\text{Cl}^-/\text{HCO}_3^-$ exchange activity was observed for the I544N, I675/6ins and G702Tins mutant transporters when compared to HeLa cells transfected with vector alone (Figure 5-1C). By calculating the rate of intracellular pH (pH_i) alkylization upon removal of extracellular Cl^- , I was able to compare the activities of the wild type and mutant transporters. A $58 \pm 21\%$ reduction in $\text{Cl}^-/\text{HCO}_3^-$ exchange activity was measured for the $\Delta\text{Y526/7}$ mutant when compared to wild type SLC26A3 activity, and no significant activity for the I544N, I675/6ins and G702Tins mutant transporters was measured over background $\text{Cl}^-/\text{HCO}_3^-$ exchange activity (Figure 5-1D).

Two different models can account for the functional effects of the CLD causing mutations on the transporters; 1) the mutations affect the mechanochemistry of the transporter and therefore disrupt the $\text{Cl}^-/\text{HCO}_3^-$ exchange activity of the protein or 2) the CLD causing mutations result in protein misfolding and or mistrafficking, and therefore prevent the transporters from

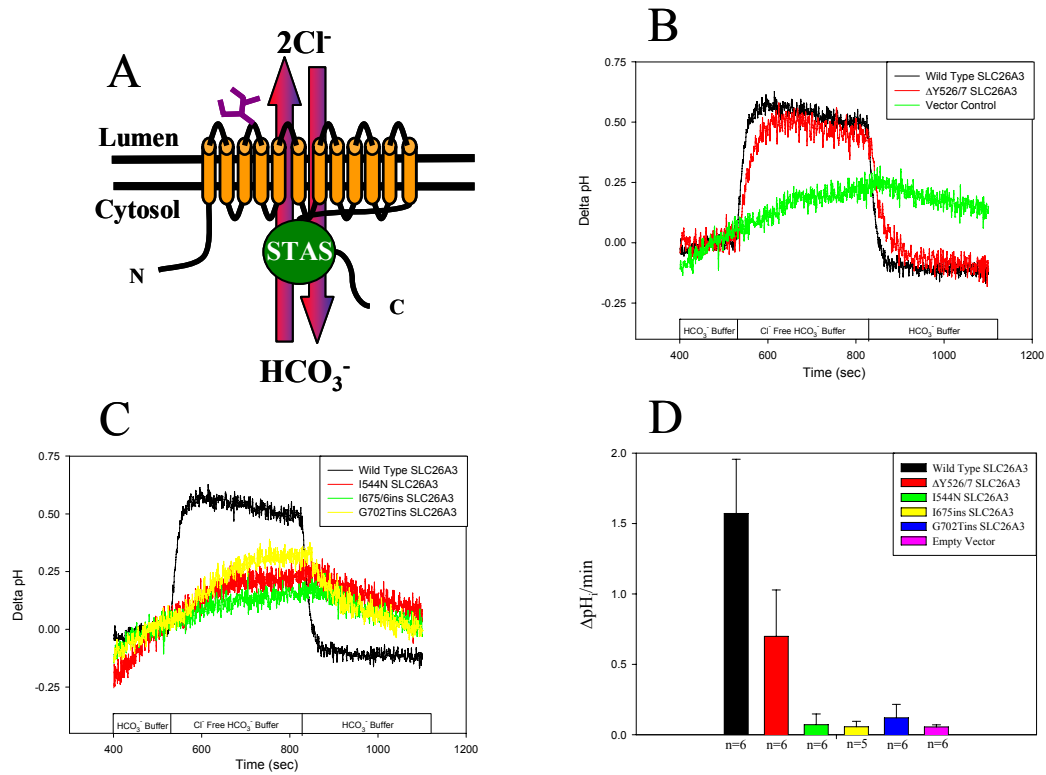


FIGURE 5-1: SLC26A3 $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. (A) A cartoon representation of the SLC26A3 protein depicting the predicted twelve transmembrane spanning segments, the C-terminal STAS domain and the predicted N-linked glycosylation site. The $\text{Cl}^-/\text{HCO}_3^-$ exchange function of SLC26A3 has been demonstrated to have a stoichiometry of $2\text{Cl}^-/1\text{HCO}_3^-$, allowing the indirect assessment of HCO_3^- transport by measuring pH_i . (B) and (C) Averaged $\text{Cl}^-/\text{HCO}_3^-$ exchange activity measurements recorded from HeLa cells transiently transfected with wild type SLC26A3, $\Delta\text{Y526/7}$ SLC26A3, I544N SLC26A3, I675/6ins SLC26A3, G702Tins SLC26A3 or empty vector. The cells were loaded with BCECF-AM and ratiometric fluorescence excitation measurements were recorded and converted to pH_i as described in experimental procedures. (D) Quantified $\text{Cl}^-/\text{HCO}_3^-$ exchange activity between the HCO_3^- buffer and Cl^- free buffer transition for the indicated SLC26A3 construct. The number of experiments used in the quantification is indicated below the bar graph.

reaching the plasma membrane where we can monitor the $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. To distinguish between these two possibilities, biochemical experiments were performed on transiently-transfected COS7 cells to monitor the

glycosylation state of the protein. Transfected COS7 cells were allowed to express the wild type or mutant transporters for 48 hr at 37°C, lysed in RIPA buffer, and incubated with either EndoH or PNGaseF enzyme, or left untreated (Figure 5-2A). The digestion reactions were visualized via western blot analysis using the anti-c-Myc antibody. The western blot revealed two bands for the untreated wild type transporter with the higher molecular weight species being the predominant band. Upon treatment of the wild type lysates with EndoH the lower molecular weight species migrated more quickly on a SDS-PAGE gel, consistent with this species being core glycosylated. Upon PNGaseF treatment of the wild type lysates, both bands migrated as this lowest molecular weight species on a SDS-PAGE gel, consistent with the upper molecular weight species being complexly glycosylated SLC26A3 protein. The $\Delta Y526/7$ SLC26A3 protein was mostly core glycosylated and only a small amount was complexly glycosylated when compared to the wild type protein. The I544N, I675/6ins and G702Tins mutants were all found to be core glycosylated but contained no observable complexly glycosylated protein, suggesting that these CLD causing mutations give rise to the disease by preventing proper trafficking to the plasma membrane. In addition, it is worth noting that at steady state the expression levels of the I675/6ins and G702Tins mutant SLC26A3 proteins are consistently reduced compared to the wild type, $\Delta Y526/7$ and I544N mutant transporters.

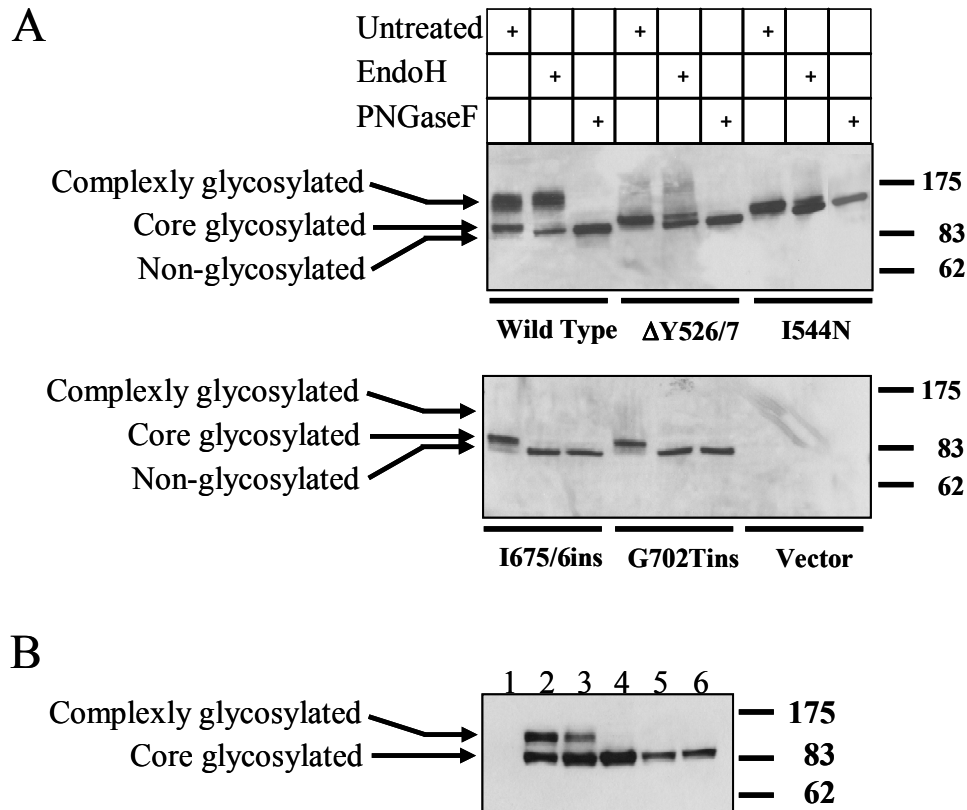


FIGURE 5-2: The glycosylation state of SLC26A3. (A) COS7 Cells transiently transfected with wild type or mutant SLC26A3 vectors were allowed to express protein for 48 hr at 37°C. Cells were lysed with RIPA buffer and lysates were incubated in the presence or absence of the endoglycosidase enzymes EndoH or PNGaseF for 1 hr at 37°C. The reactions were quenched by the addition of sample buffer, and western blot analysis was performed using the anti-c-Myc antibody for SLC26A3 detection. (B) COS7 cells were transiently transfected with the wild type and mutant SLC26A3 proteins and grown for 24 hrs at 37°C, and then grown for an additional 24 hr at 30°C. The cells were lysed in RIPA buffer and western blot analysis was performed using the anti-c-Myc antibody for detection of the SLC26A3 proteins. SLC26A3 protein lysate loaded in each lane are as follows: lane 1) vector control, 2) wild type, 3) ΔY526/7, 4) I544N, 5) I675/6ins, 6) G702Tins SLC26A3 protein. The molecular weight markers shown to the right of the gels are measured in kDa.

The CLD causing mutations affect the glycosylation state of the SLC26A3 transporter, suggesting that these mutations prevent proper trafficking out of the

ER. In an attempt to rescue this phenotype, we reduced the expression temperature from 37°C to 30°C and monitored the amount of the complexly glycosylated protein produced. Transiently transfected COS7 cells were incubated at 37°C for 24 hr, and then either allowed to express the proteins for an additional 24 hr at 37°C, or the cells were shifted to 30°C for an additional 24 hr of expression. We observed a substantial increase in the amount of the complexly glycosylated species for the $\Delta Y526/7$ mutant transporter when expressed at 30°C when compared to 37°C expression (Figure 5-2B). This is in contrast to the I544N, I675/6ins or G702Tins transporters where we did not observe any complex glycosylated protein upon low temperature expression (Figure 5-2B).

In addition to monitoring the glycosylation state of the protein, we also directly monitored the cellular location of the wild type and mutant SLC26A3 transporters by performing immunofluorescence experiments. HeLa cells stained with the anti-c-Myc antibody revealed the wild type transporter is found in the plasma membrane and in the ER, consistent with membrane protein synthesis and maturation (Figure 5-3B). Cells transfected with the $\Delta Y526/7$ transporter showed two distinct populations of cells. One population of cells showed the $\Delta Y526/7$ transporter being trafficked to the plasma membrane (Figure 5-3C1), and another which it is completely retained in the ER (Figure 5-3C2). The I544N, I675/6ins and G702Tins mutant transporters all show ER localization (Figure 5-3D, E and F). Immunofluorescence experiments were also performed with transiently

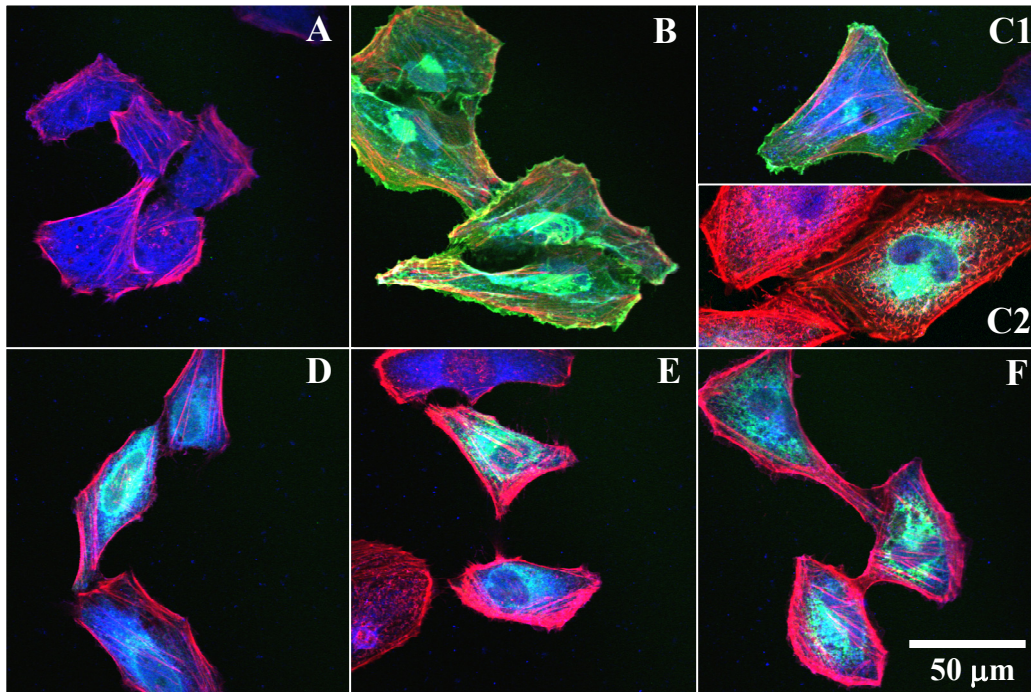


FIGURE 5-3: SLC26A3 immunofluorescence. HeLa cells transiently transfected with the wild type or mutant SLC26A3 proteins were used for immunofluorescence imaging as described in the experimental procedures. Briefly, filamentous actin staining is colored Red, Bip (an ER marker) staining is colored Blue, and Myc staining is colored green. (A) Empty Vector, (B) wild type SLC26A3, (C1 and C2) Δ Y526/7 SLC26A3, (D) I544N SLC26A3, (E) I675/6ins SLC26A3, (F) G702Tins SLC26A3.

transfected COS7 cells to test for any differences in protein trafficking between the two cell lines. The same subcellular localization results were observed in the COS7 cells when compared with the HeLa cells (data not shown).

To further confirm the presence or absence of the wild type and mutant SLC26A3 proteins at the plasma membrane, cell surface biotinylation experiments were performed (Figure 5-4). Cell surface proteins on intact cells were biotinylated using 0.74 mM EZ-link biotin on ice for 20 min, and then

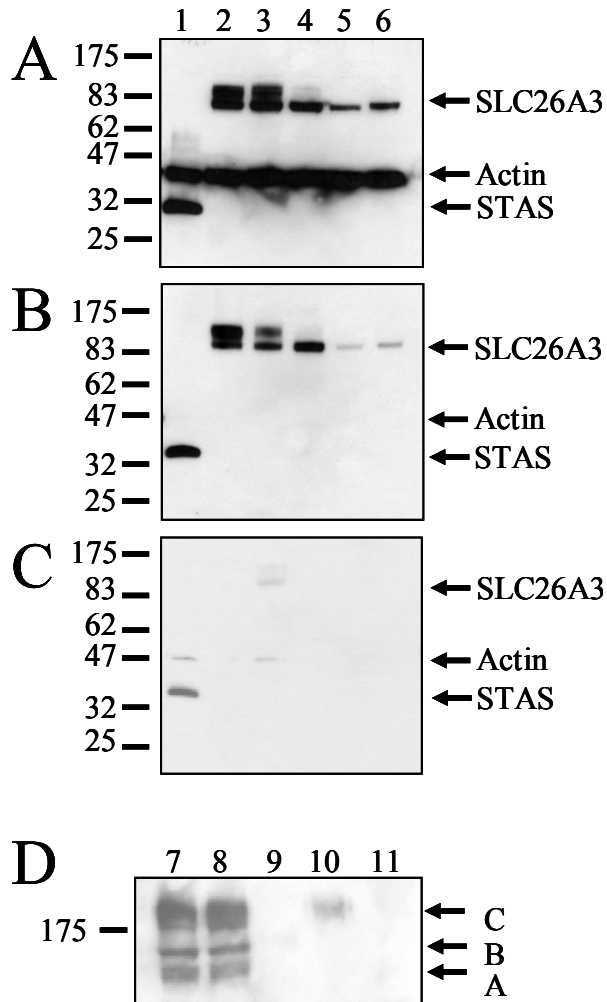


FIGURE 5-4: Cell surface biotinylation of SLC26A3. Cell surface biotinylation reactions were performed as described in experimental procedures on transiently transfected HEK 293 cells expressing the wild type and mutant SLC26A3 proteins, as well as the wild type STAS domain. Briefly, cells were incubated in the presence of 740 μ M biotin for 30 min on ice and then washed three times in 25 mM Tris pH 8.0, 200 mM glycine. Cells were then lysed via sonication and the resulting lysates were incubated in the presence of streptavidin beads and a pull-down reaction was performed. The precipitated protein was then subjected to western blot analysis using a mixture of the anti-c-Myc and anti-actin antibody for detection of actin and the STAS domain (panels A-C) or the M3A7 antibody to detect CFTR (panel D). (A) HEK 293 cell whole cell lysate of cells expressing the

SLC26A3 transporters or the STAS domain. (B) A representative western blot of a cell surface biotinylation reaction. (C) A western blot of a cell surface biotinylation reaction in the absence of biotin (negative control). (D) A representative western blot of a cell surface biotinylation reaction of HEK 293 cells transfected with CFTR. The protein lysate loaded in each lane is as follows: 1) wild type STAS domain, 2) wild type SLC26A3, 3) Δ Y526/7 SLC26A3, 4) I544N SLC26A3, 5) I675/6ins SLC26A3, 6) G702Tins SLC26A3, 7) CFTR whole cell lysate after biotinylation, 8) CFTR whole cell lysate no biotinylation, 9) untransfected HEK 293 whole cell lysate after biotinylation, 10) streptavidin pull-down of biotinylated HEK 293 cells transfected with CFTR, 11) streptavidin pull-down of non-biotinylated HEK 293 cells transfected with CFTR (negative control). The molecular weight markers are measured in kDa.

washed and lysed the cells in RIPA buffer. Streptavidin-linked agarose beads were used to pull-down the cell surface biotinylated proteins which were separated by SDS-PAGE. The proteins were transferred to PVDF membrane for western blot analysis, and the SLC26A3 proteins were detected using the anti-c-myc antibody. The wild type and the mutant SLC26A3 transporters were consistently observed being pulled down by the streptavidin beads after cell surface biotinylation, but not when biotin was omitted from the pull-down reactions (Figure 5-4B and C). As a control for labeling of intracellular proteins, the western blots were probed with an anti-actin antibody along with the anti-c-myc antibody to determine if actin was being labeled. No actin was detected in the biotin-streptavidin pull-down experiments, suggesting that the SLC26A3 proteins were being labeled at the cell surface. To further control for our experimental procedure cell surface biotinylation reactions were performed on HEK 293 cells transiently transfected with CFTR (Figure 5-4D). The complexly glycosylated form of CFTR, band C, was pulled down by streptavidin while the core glycosylated form of CFTR, band B, was not. This further suggests that our biotinylation assay procedure was working as expected.

The reduced ability of the $\Delta Y526/7$ mutant SLC26A3 transporter to traffic to the plasma membrane, and the lack of plasma membrane trafficking of the I544N, I675/6ins and G702Tins mutants suggested that these mutations may be causing the transporters to misfold. To investigate the possibility that these

mutations were specifically disrupting the ability of the STAS domain to fold, I expressed the wild type and mutant STAS domains recombinantly in order to study their biochemical and biophysical properties. A multiple sequence alignment was produced to help guide us in choosing our initial domain boundaries (see Figure 2-18). Initially I choose three N-terminal boundaries (amino acids 503, 510 and 525) and three C-terminal boundaries (amino acids 720, 741 and 764) and produced the nine possible protein constructs for the wild type SLC26A3 STAS domain. The nine constructs all resulted in protein expression. However, the three constructs ending in amino acid 720 were found to be insoluble after cell lysis and a 40,000 rcf centrifugation step (see chapter 4 for details). The remaining six constructs were all solubly produced in *E. coli*, and the domain boundaries for our STAS domain construct were chosen based upon the biochemical properties of the proteins as well as their ability to bind to the R-domain of CFTR (see chapter 4 for details). From this data, the STAS domain construct with domain boundaries of 510-741 was chosen for further study. The mutant STAS domains were cloned with the construct boundaries of amino acids 510-741, and the soluble expression pattern in *E. coli* of the mutants was compared to that of the wild type protein.

The wild type, $\Delta Y526/7$ and the I544N SLC26A3 STAS domains were all found to be solubly expressed in *E. coli* however, the I675/6ins and G702Tins

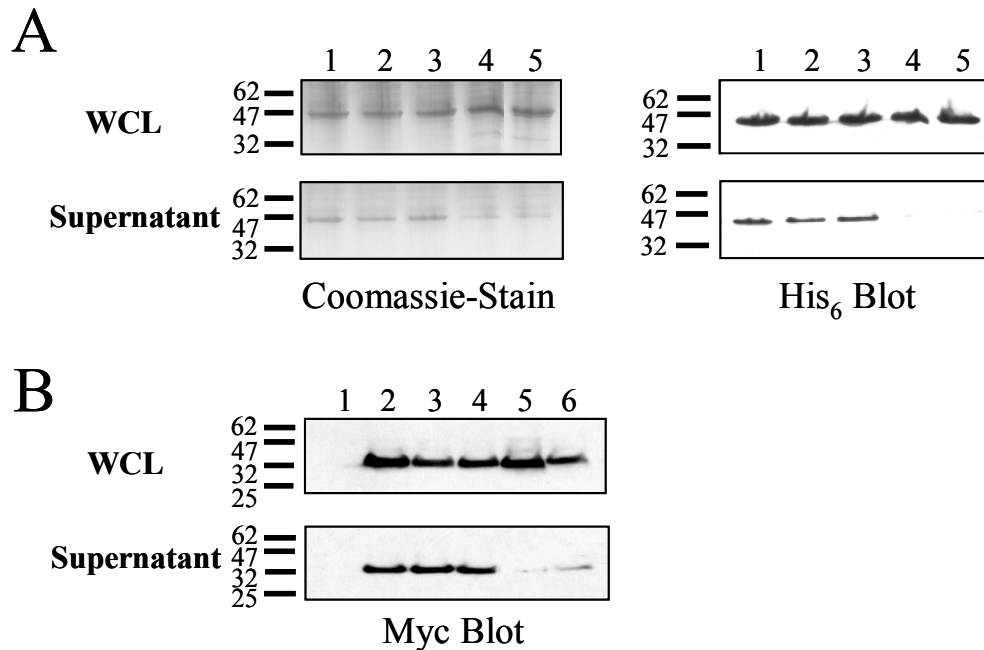


FIGURE 5-5: STAS solubility assay. (A) The wild type and mutant SLC26A3 STAS 510-741 domains were expressed as Smt3 fusions in *E. coli*, and the amount of solubly expressed STAS domain protein, as defined by a 40,000 rcf centrifugation step, was assessed by Coomassie-staining or western blot analysis. Equivalent amounts of STAS protein lysates were loaded on the gels as follows: 1) wild type, 2) $\Delta Y526/7$, 3) I544N, 4) I675/6ins, 5) G702Tins. (B) The wild type and mutant SLC26A3 STAS domains were expressed as myc-tagged fusions in HEK 293 cells, and the amount of solubly expressed STAS domain protein, as defined by a 60,000 rcf centrifugation step, was assessed by western blot analysis. Equivalent amounts of total protein were used in this assay, and the anti-c-Myc antibody was used for protein detection. The STAS protein lysates were loaded on the gel as follows: 1) untransfected cells, 2) wild type, 3) $\Delta Y526/7$, 4) I544N, 5) I675/6ins, 6) G702Tins. The molecular weight standards are shown to the left of the gel and are measured in kDa.

SLC26A3 STAS domains were found to be produced in an insoluble form (Figure 5-5A). This was confirmed by western blot analysis using the His₆ tag on the Smt3 fusion partner. Interestingly, the STAS domains showed a similar pattern of solubility when produced in COS7 or HEK 293 cells with an N-terminal myc tag, suggesting that this solubility phenomenon is due to an intrinsic property of the

STAS domain itself, and not likely an artifact due to fusion protein expression (Figure 5-5B).

To better characterize the wild type and mutant STAS domains I expressed and purified the protein from *E. coli* using Ni affinity chromatography (Figure 5-6). As the STAS solubility assay predicted, significant quantities of the wild type, $\Delta Y526/7$ and I544N STAS domains were readily purified, however, significant quantities of the I675/6ins or G702Tins mutant STAS domains were not. The purified wild type, $\Delta Y526/7$ and I544N Smt3-STAS proteins were all readily cleaved by Ulp1 protease, and were purified to homogeneity using Butyl hydrophobic interaction chromatography and gel filtration chromatography (Figure 5-7A). NMR and CD spectra were taken of the purified proteins to monitor any significant differences in secondary structure (Figure 5-7B, C and D). The wild type and $\Delta Y526/7$ STAS domain TROSY-HSQC spectra are very similar to one another, with only a few differences in the position of proton-nitrogen coupled cross peaks consistent with one of the proteins containing a deletion of a single amino acid (Figure 5-7B). In addition, the high degree of proton-nitrogen coupled cross peak dispersion in TROSY-HSQC spectra indicates that these two domains are well folded (Figure 5-7B). The CD spectra for the wild type and two mutant STAS domains are indistinguishable from one another, and indicate that the STAS domain is a mixed α/β protein as evident by local minima at 220 nm and 207 nm, consistent with the predicted structure of the

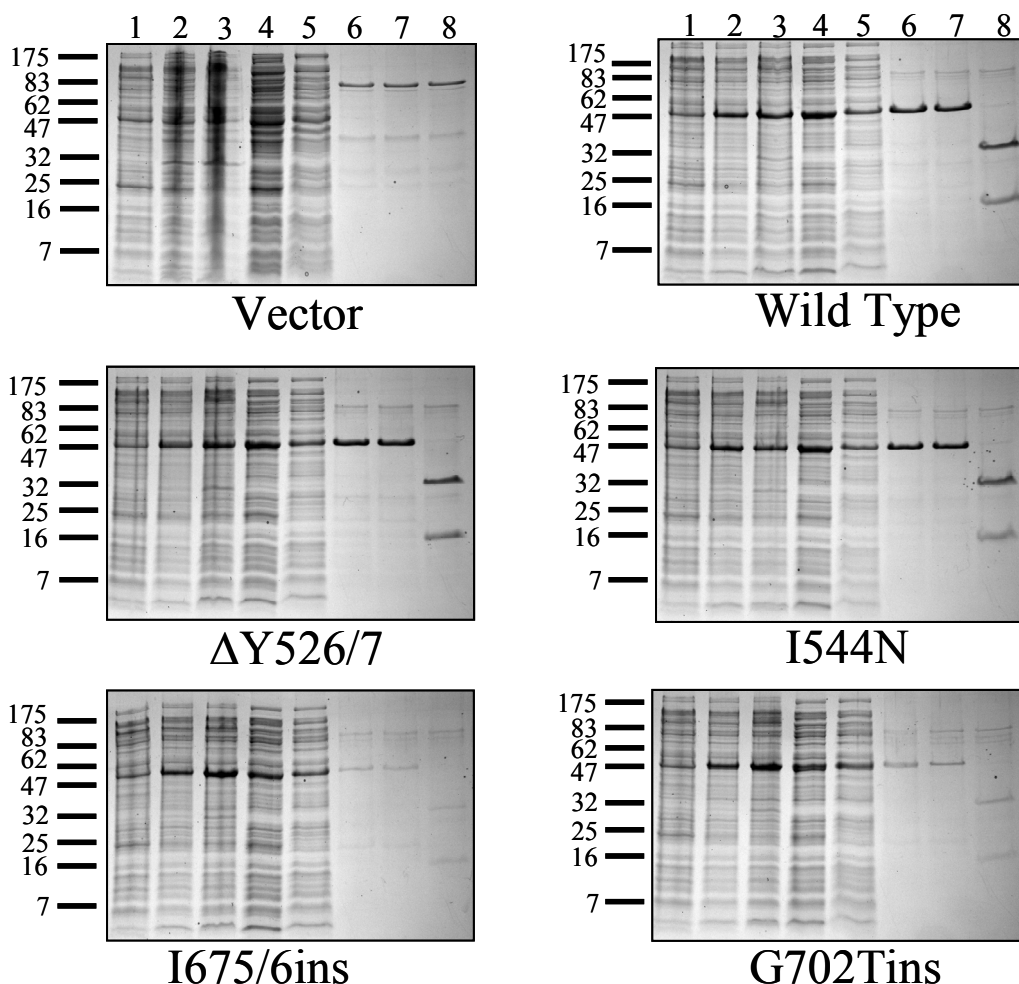


FIGURE 5-6: Wild type and mutant STAS domain purification. Six Coomassie-stained gels are shown of the wild type and mutant STAS domains at different stages of the Ni column purification. The molecular weight markers are shown to the left of the gel and are measured in kDa. The protein samples loaded in each lane are as follows: 1) uninduced cell lysate, 2) induced cell lysate, 3) pellet fraction of induced cell lysate, 4) supernatant fraction of induced cell lysate, 5) Ni column wash, 6) Ni column elution, 7) filtered Ni column elution, 8) filtered Ni column elution cleaved with Ulp1.

STAS domain (Figure 5-7C). Thermal stability measurements were also made of the wild type, $\Delta Y526/7$ and I544N STAS domains by monitoring the loss of molar ellipticity at 222 nm as the temperature was increased from 20°C to 80°C.

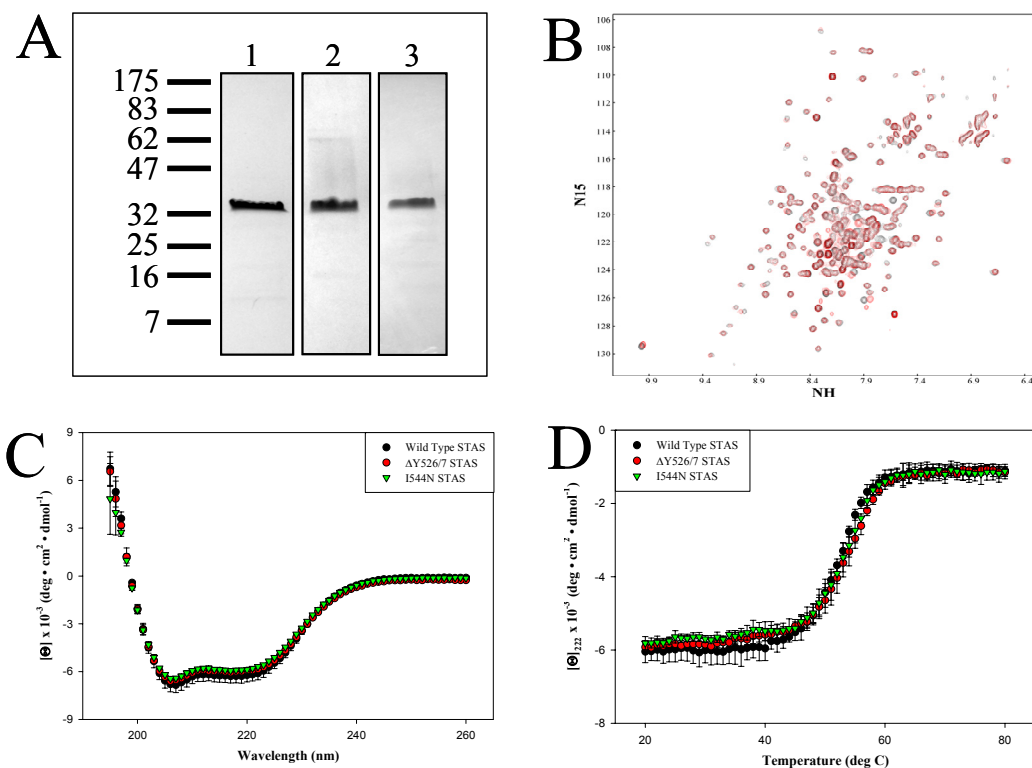


FIGURE 5-7: SLC26A3 STAS structural analysis. (A) The wild type (lane 1), $\Delta Y526/7$ (lane 2) and I544N (lane 3) SLC26A3 STAS domains were produced as Smt3 fusions and purified as described in experimental procedures. Representative Coomassie-stained gels of purified STAS 510-741 domain protein is shown with molecular weight markers measured in kDa on the left. (B) An NMR TROSY-HSQC spectra of purified ^{15}N wild type (black peaks) and $\Delta Y526/7$ (red peaks) SLC26A3 STAS 510-741 domain. (C) Circular dichroism spectra of the wild type, $\Delta Y526/7$ and I544N SLC26A3 STAS domains. The spectra are averages of at least 3 experiments from at least two separate protein preparations. (D) Circular dichroism measurements were recorded at 222 nm of the wild type, $\Delta Y526/7$ and I544N SLC26A3 STAS domains while increasing the temperature of the STAS domain protein from 20°C to 80°C. The data presented are averaged data from at least three experiments from at least two independent protein preparations.

No significant differences were observed between the wild type, $\Delta Y526/7$ and I544N STAS domains denaturation curves with all three proteins having a half melting point temperature of 53°C (Figure 5-7D).

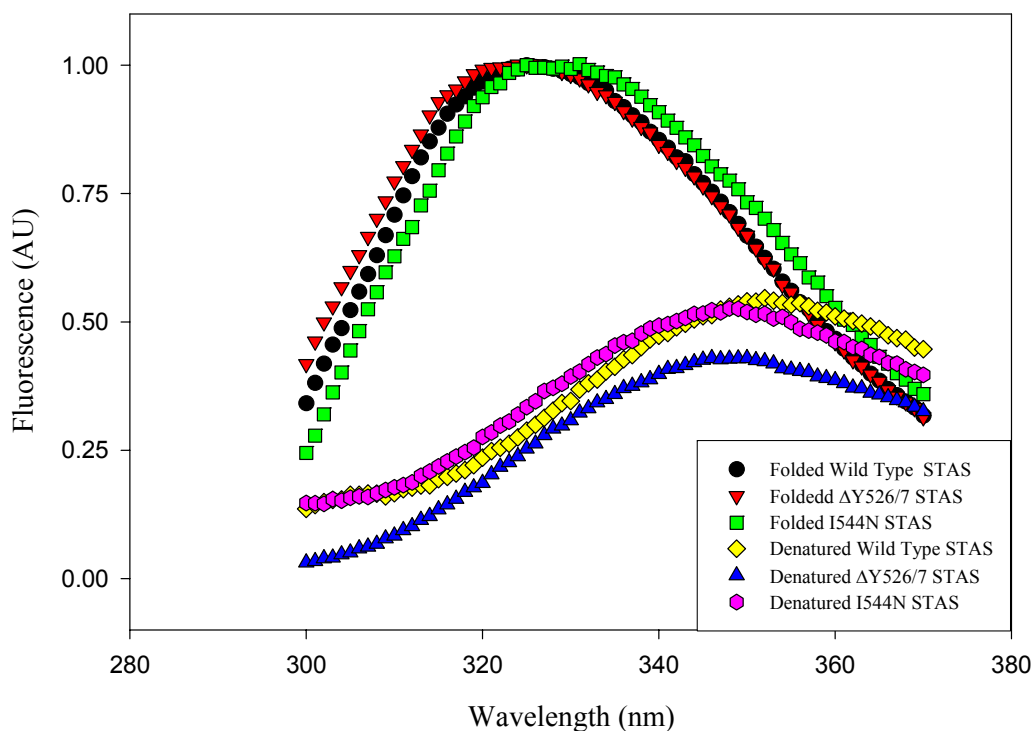


FIGURE 5-8: Normalized tryptophan fluorescence of wild type and mutant STAS domain 510-741. Tryptophan fluorescence spectra were collected from the purified wild type, $\Delta Y526/7$ and I544N STAS domains using an excitation wavelength of 290 nm. Unfolded STAS fluorescence spectra were collected after protein unfolding by the addition of 6 M guanidinium.

In addition to the NMR and CD spectra collected on the wild type, $\Delta Y526/7$ and I544N STAS domains, tryptophan fluorescence spectra were also collected on these proteins (Figure 5-8). The tryptophan fluorescence spectra of the purified STAS domains did not reveal any significant differences in the folded state or unfolded states.

Since no differences were readily observable between the purified wild type, $\Delta Y526/7$ and I544N STAS domains, I reasoned that the folding defect may be due to the inability of the mutant STAS domains to properly associate with the transmembrane domain of the SLC26A3 protein. In an attempt to monitor this interaction the predicted transmembrane spans (Δ STAS) of the SLC26A3 protein (amino acids 1-509) were cloned into the myc-pCMV vector as was the SLC26A3 STAS domain (amino acids 510-741). These two plasmids were either transfected alone or co-transfected into HEK 293 cells, and western blots performed to assess the Δ STAS trafficking (Figure 5-9). The Δ STAS construct produced some complexly glycosylated protein which made interpreting the experimental results difficult. However, it appeared that co-transfection of the wild type STAS domain with the Δ STAS construct does not significantly increase the amount of complexly glycosylated protein, suggesting that the hypothesized association of the STAS domain and the transmembrane spans cannot be monitored with this experimental system.

In another attempt to monitor the interaction between the STAS domain and the transmembrane domain, trypsin digest experiments were performed to test if the mutant SLC26A3 proteins might have a different proteolytic pattern compared to the wild type transporter. HEK 293 cells were transfected with the wild type SLC26A3 protein, and limited proteolysis experiments were performed followed by western blot analysis. Unfortunately, when blotting with the anti-

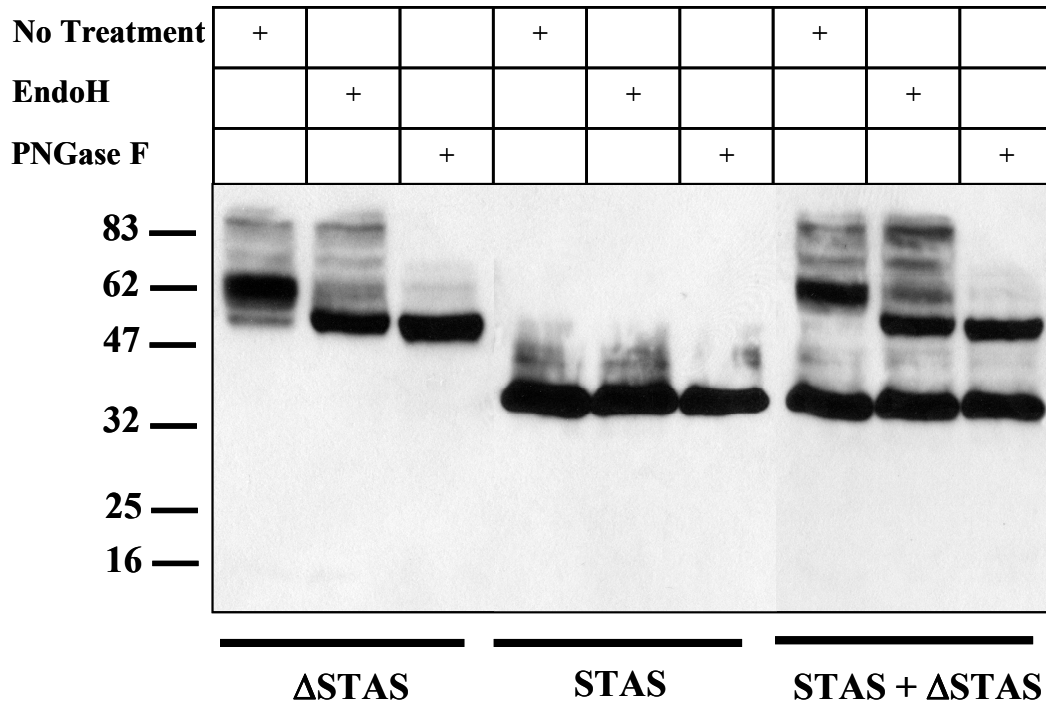


FIGURE 5-9: Co-transfection of wild type STAS with Δ STAS. HEK 293 cells were transiently-transfected with a C-terminally truncated SLC26A3 construct which has the STAS domain deleted (Δ STAS), the STAS domain, or co-transfected with both the Δ STAS and STAS domain constructs. Cells were lysed in RIPA buffer and a glycosylation assay was performed by incubating the cell lysates with EndoH, PNGaseF or no enzyme. The resulting protein samples were separated by SDS-PAGE and an anti-c-Myc western blot was performed to visualize the various protein species. The molecular weight markers to the left of the gel are measured in kDa.

myc antibody no proteolytic fragments were observable, consistent with the myc tag being cleaved off by trypsin. To monitor the trypsin digest of SLC26A3 STAS domain containing fragments directly, a polyclonal STAS domain antibody was produced in rabbits using purified wild type SLC26A3 STAS domain.

Characterization of the STAS domain antibody revealed that it recognized multiple SLC26A proteins from both human and mouse species in western blots (Figure 5-10), and is proficient at immunoprecipitating the wild type and mutant

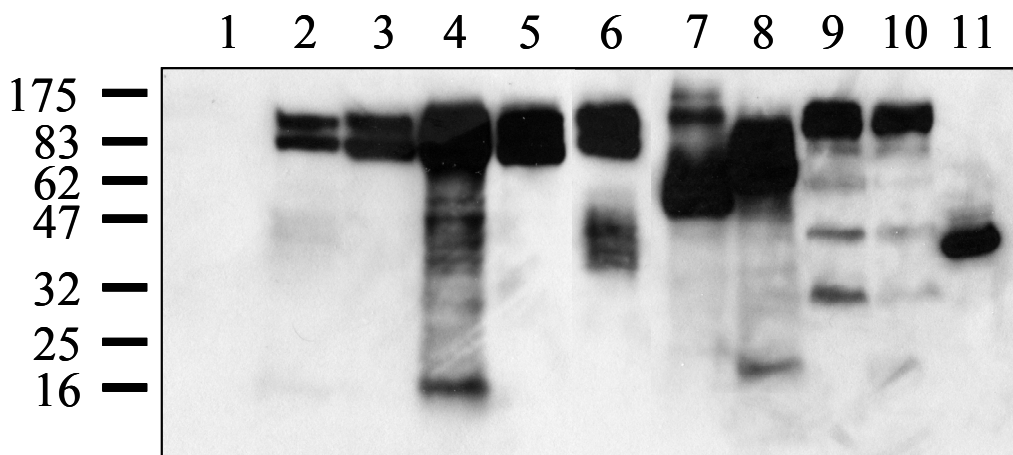


FIGURE 5-10: The STAS domain antibody recognizes multiple SLC26A proteins. A western blot using the rabbit polyclonal STAS domain antibody to recognize various SLC26A transporters expressed in HEK 293 cells (lanes 1-8, 11) or from native tissues (lanes 9 & 10). The molecular weight markers are measured in kDa and the protein samples loaded in each lane are as follows: 1) untransfected, 2) human SLC26A3, 3) mouse SLC26A3, 4) human SLC26A6, 5) mouse SLC26A6, 6) mouse SLC26A7, 7) mouse SLC26A9, 8) human SLC26A11, 9) mouse kidney, 10) mouse sub-mandibular gland, 11) human SLC26A3 STAS domain.

SLC26A3 transporters as well as the wild type STAS domain from HEK 293 cell lysates (Figure 5-11). The STAS domain antibody is also useful in immunofluorescence experiments, and it showed staining patterns for the wild type and mutant SLC26A3 transporters similar to the anti-myc antibody (Figure 5-12).

With the creation of the STAS domain antibody, there were now epitopes on both the N and C-termini of the SLC26A3 constructs that could be probed with either the myc or STAS antibodies respectively. Immunofluorescence experiments were performed with and without permeabilizing the cell membrane

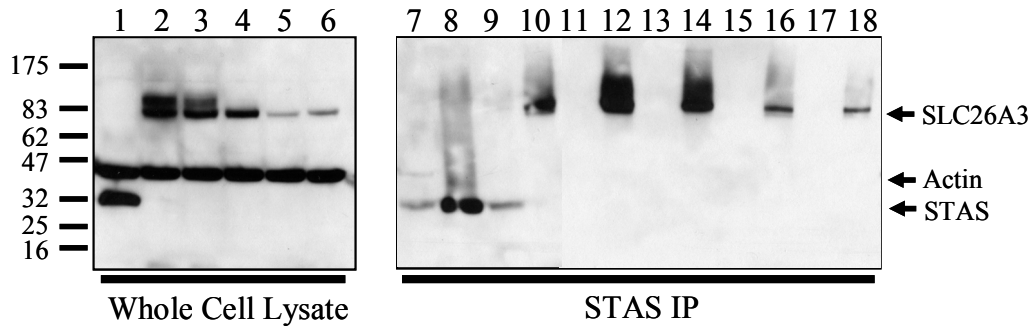


FIGURE 5-11: The STAS domain antibody can immunoprecipitate various SLC26A3 constructs. The left panel shows a western blot developed with a mixture of the anti-c-Myc and anti-actin antibodies showing the HEK 293 cell expression of the STAS domain (lane 1), wild type SLC26A3 (lane 2), $\Delta Y526/7$ SLC26A3 (lane 3), I544N SLC26A3 (lane 4), I675/6ins SLC26A3 (lane 5), G702Tins SLC26A3 (lane 6). The right panel shows a western blot developed with a mixture of the anti-c-Myc and anti-actin antibodies of the immunoprecipitation reactions performed with or without the STAS domain antibody. Lane 7) STAS domain – Ab, 8) STAS domain + Ab, 9) wild type SLC26A3 – Ab, 10) wild type SLC26A3 + Ab, 11) $\Delta Y526/7$ SLC26A3 – Ab, 12) $\Delta Y526/7$ SLC26A3 + Ab, 13) I544N SLC26A3 – Ab, 14) I544N SLC26A3 + Ab, 15) I675/6ins SLC26A3 – Ab, 16) I675/6ins SLC26A3 + Ab, 17) G702Tins SLC26A3 – Ab, 18) G702Tins SLC26A3 + Ab. The molecular weight markers to the left of the gel are measured in kDa.

with detergent to test the hypothesis that the N and C-termini are located intracellularly. The anti-myc and STAS domain antibodies both recognized the STAS domain and the SLC26A3 transporter in transiently transfected HEK 293 cells only when the plasma membrane was permeabilized with detergent, consistent with the N and C-termini of the SLC26A3 protein being located intracellularly (Figure 5-13A and B).

Using the new STAS domain antibody, trypsin digest experiments were performed on the wild type and mutant SLC26A3 transporters to test the hypothesis that the $\Delta Y526/7$ and I544N mutations may be affecting the ability of

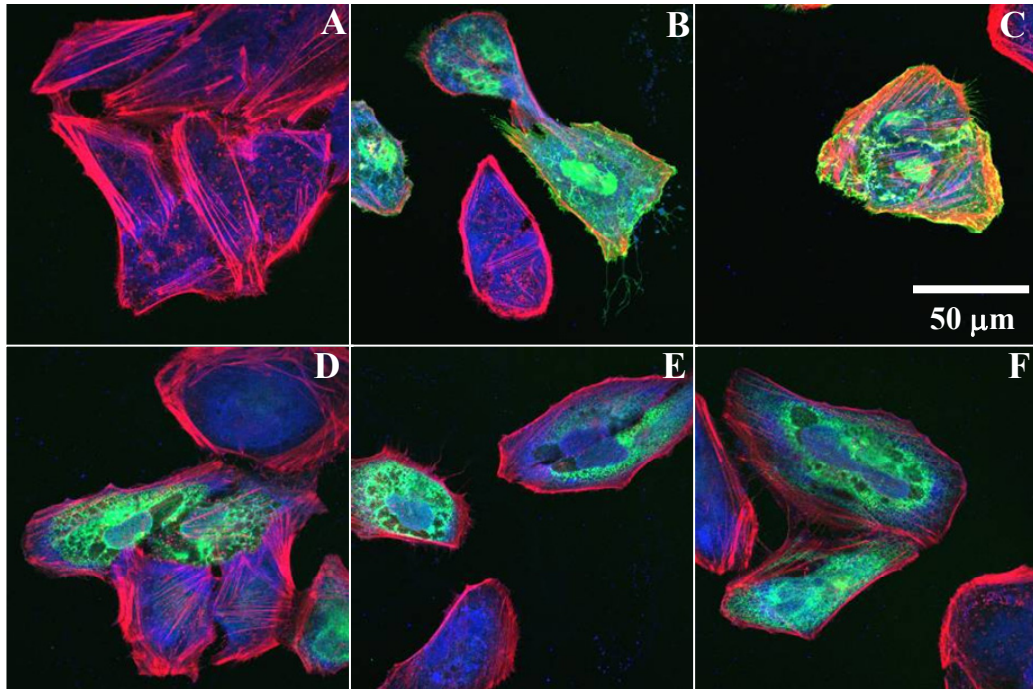


FIGURE 5-12: The STAS domain antibody allows detection of SLC26A3 by immunofluorescence. HeLa cells transiently transfected with the wild type or mutant SLC26A3 proteins were used for immunofluorescence imaging as described in the experimental procedures. Briefly, filamentous actin staining is colored Red, Bip (an ER marker) staining is colored Blue, and STAS domain staining is colored green. (A) Empty Vector, (B) wild type SLC26A3, (C) $\Delta Y526/7$ SLC26A3, (D) I544N SLC26A3, (E) I675/6ins SLC26A3, (F) G702Tins SLC26A3.

the STAS domain to interact with the transmembrane spans of the transporter.

HEK 293 cells transiently transfected with wild type or mutant SLC26A3 proteins were lysed by sonication. Increasing concentrations of trypsin were incubated with the lysates at room temperature for 12 min, and the digests were stopped by the addition of sample buffer. The tryptic fragments were detected by western blot analysis using the STAS domain antibody, detecting the full length protein (FL) and three predominant proteolytic fragments labeled 1-3 (Figure 5-14A).

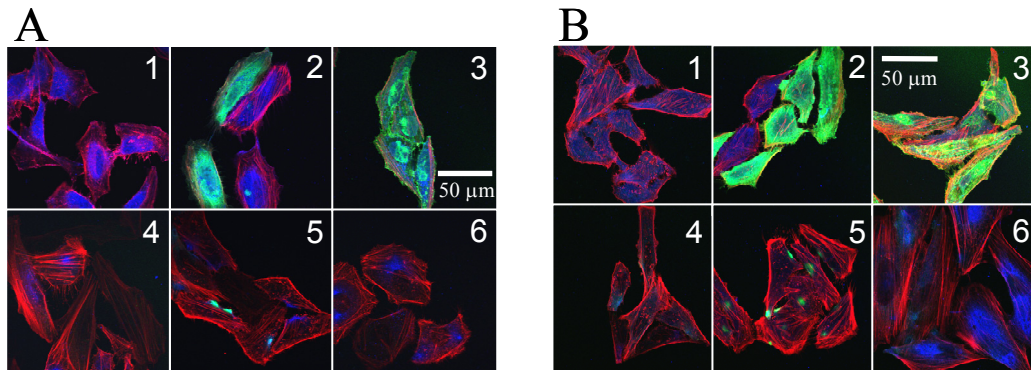


FIGURE 5-13: The termini of SLC26A3 are located in the cytosol. (A) HeLa cells transfected with wild type SLC26A3, the STAS domain or empty vector were either permeabilized using Triton X 100 (panels 1-3) or were not permeabilized (panels 4-6). The cells were stained using TRITC conjugated Phalloidin (Red), the anti-BIP antibody (colored blue) and the anti-c-Myc antibody (colored green). (1 and 4) Vector control, (2 and 5) wild type STAS domain, (3 and 6) wild type SLC26A3. (B) HeLa cells transfected with the wild type transporter, STAS domain or empty vector were either permeabilized using Triton X 100 (panels 1-3) or were not permeabilized (panels 4-6). The cells were stained using TRITC conjugated Phalloidin (Red), the anti-BIP antibody (colored blue) and the anti-STAS antibody (colored green). (1 and 4) Vector control, (2 and 5) wild type STAS domain, (3 and 6) wild type SLC26A3.

The I675/6ins and G702Tins transporters were more susceptible to proteolysis than the wild type, $\Delta Y526/7$ and I544N transporters, consistent with their STAS domains being misfolded and more readily degraded. Subtle conformational differences between the wild type and the $\Delta Y526/7$ and I544N transporters were also observed, but were less dramatic than those of the I675/6ins and G702Tins mutants. Quantifying the degradation of the full-length transporter (Figure 5-14B) reveals significant differences between the wild type and the I544N transporters at the 30 and 100 $\mu\text{g/ml}$ trypsin concentrations. The quantity of the

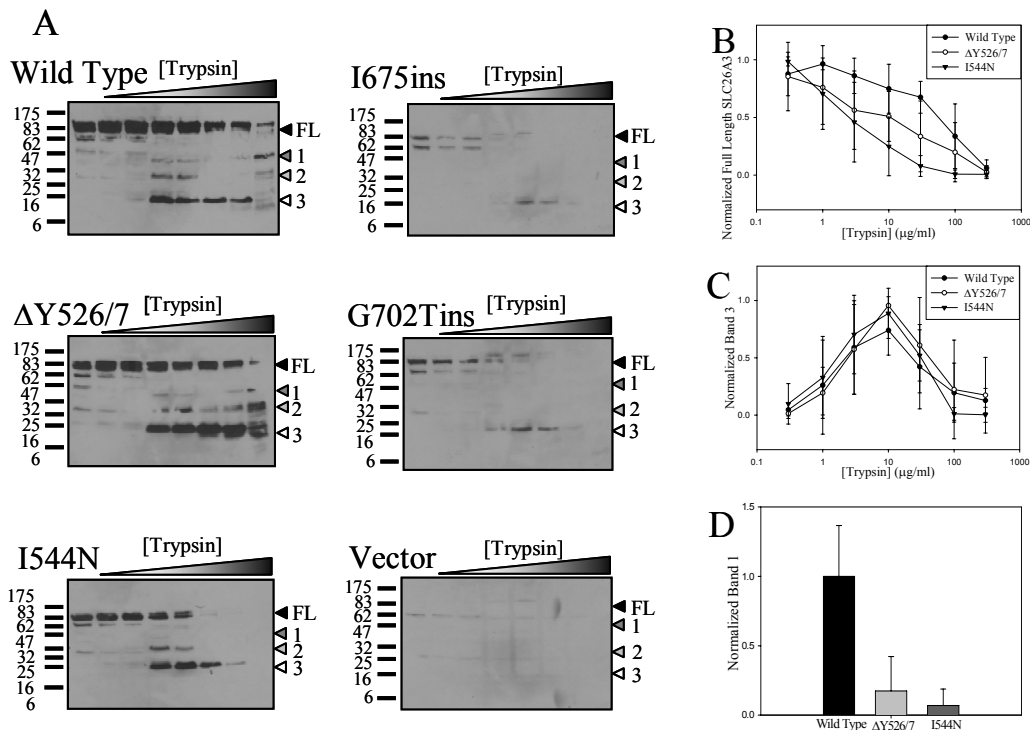


FIGURE 5-14: Trypsin digest of SLC26A3. (A) Wild type and mutant SLC26A3 proteins were transiently transfected in HEK 293 cells and were harvested 48 hr post transfection. The cells were lysed via sonication and then incubated with increasing concentrations of trypsin (from left to right: 0, 0.3, 1, 3, 10, 30, 100, 300 μg/ml) at room temperature for 12 min. The digest reactions were stopped by the addition of sample buffer, and the samples were visualized via western blot analysis using the anti-STAS domain antibody. Triangles indicate the full length (FL) protein and consistently observed tryptic fragments (1, 2, and 3). (B) Quantification of the loss of the wild type, ΔY526/7 and I544N SLC26A3 full-length (FL) protein species at various trypsin concentrations. Each experiment was quantified and normalized independently of one another, and the mean and standard deviation were calculated from all experiments (wild type n=5, ΔY526/7 n=5, I544N n=7). (C) Quantification of tryptic fragment 3 from the wild type, ΔY526/7 and I544N SLC26A3 trypsin digest experiments. The relative amounts of tryptic fragment 3 have been normalized to the most prevalent band within each individual experiment, and means and standard deviations were calculated from all experiments (wild type n=5, ΔY526/7 n=5, I544N n=7). (D) Quantification of tryptic fragment 1 from the wild type, ΔY526/7 and I544N SLC26A3 trypsin digest experiments. The amount of tryptic fragment 1 in the 300 μg/ml trypsin lane was quantified via densitometry and then normalized to the average amount of wild type tryptic fragment.

$\Delta Y526/7$ transporter is reduced compared to the wild type transporter at the 30 and 100 $\mu\text{g/ml}$ trypsin concentrations, but the changes were not significant. No differences were observed between the wild type and the $\Delta Y526/7$ and I544N transporters when proteolytic fragment 3 was quantitated, however, a significant difference was observed in proteolytic fragment 1 between the wild type and the $\Delta Y526/7$ and I544N mutant transporters. This data taken together suggests that the $\Delta Y526/7$ and I544N mutations may be causing subtle conformational changes in the transporter that may be caused by altered domain-domain interactions.

Discussion:

I set out to test what effect that the CLD causing mutations which reside in the STAS domain have on SLC26A3, and to identify the molecular mechanism by which they cause disease. The $\text{Cl}^-/\text{HCO}_3^-$ exchange rates of the wild type and mutant SLC26A3 transporters were measured, and the results suggested that the I544N, I675/6ins and G702Tins mutations result in a loss of functional protein at the plasma membrane. This is consistent with previous reports showing an observed loss of Cl^- transport activity for the I544N and I675/6ins mutant transporters (Chernova et al., 2003; Ko et al., 2002). The $\Delta\text{Y526/7}$ mutation caused an approximate 50% decrease in the $\text{Cl}^-/\text{HCO}_3^-$ exchange activity, suggesting that some functional protein resides in the plasma membrane. A number of disease-causing mutations in SLC26A2 and SLC26A4 have also been previously reported to have intermediate function similar to that of the $\Delta\text{Y526/7}$ mutation in SLC26A3, consistent with the notion that reduction, but not ablation, of transport activity can lead to the disease state (Karniski, 2001; Karniski, 2004; Taylor et al., 2002).

Western blots were performed to test for any differences in protein expression levels which could explain the lack of measurable transport activity. The band intensity for the I675/6ins and G702Tins mutants was consistently observed to be about two fold lower than those of the wild type, $\Delta\text{Y526/7}$ and I544N mutant transporters under steady state conditions (Figure 5-2A, untreated

lanes). However, these differences in protein expression levels cannot explain the complete loss of transport activity. In addition the $\Delta Y526/7$ and I544N proteins are expressed to similar levels when compared to the wild type protein, so a loss of protein expression cannot account for the reduction and complete loss of $\text{Cl}^-/\text{HCO}_3^-$ exchange activity of these two mutants. Interestingly, previous reports have suggested that the N-terminus of SLC26A3 may be proteolytically cleaved *in vivo* (Byeon et al., 1996; Lohi et al., 2002b). In our experiments we observed the same banding pattern of our N-terminal myc-tagged SLC26A3 constructs regardless of the antibody used for protein detection (anti-c-Myc or anti-STAS domain, data not shown), suggesting that our SLC26A3 construct is not undergoing any significant proteolysis under our experimental conditions.

The reduction or complete loss of measurable $\text{Cl}^-/\text{HCO}_3^-$ exchange activity for the four mutant SLC26A3 transporters suggested that the mutations may be affecting the transport cycle of the protein, or they may be affecting the ability of the transporters to fold and traffic properly. Previously the SLC26A3 protein has been demonstrated to be glycosylated (Byeon et al., 1998; Byeon et al., 1996), as have a number of other SLC26A family members (Karniski, 2004; Karniski et al., 1998; Toure et al., 2001). By utilizing the endoglycosydase enzyme EndoH, which cleave only the core glycosylated sugar moieties from a protein but do not remove the complexly glycosylated sugars, or PNGaseF, which remove all sugar moieties from a protein, the core and complexly glycosylated SLC26A3 species

could be distinguished from one another (Maley et al., 1989). Since core glycosylation occurs co-translationally in the ER, and complex glycosylation occurs in a post ER compartment, the different glycosylation states of the protein can be used to monitor protein trafficking out of the ER (Helenius and Aebi, 2001; Helenius and Aebi, 2004). The glycosylation data suggests that the I544N, I675/6ins and G702Tins transporters are accumulating in the ER and are not trafficking to the plasma membrane (Figure 5-2A), which is consistent with the lack of observed function for these three mutations. The $\Delta Y526/7$ transporter does show some complexly glycosylated protein, but the intensity of the complexly glycosylated species is significantly reduced compared to the wild type protein. This brings up the intriguing possibility that the $\Delta Y526/7$ mutant transporter may be hyperactive compared to the wild type transporter. In addition, the amount of complexly glycosylated $\Delta Y526/7$ transporter could be increased by reducing the expression temperature from 37°C to 30°C, however, no significant increases of the complexly glycosylated species of the other mutants were observed (Figure 5-2B). This data taken together suggests that the $\Delta Y526/7$ mutation may be less severe than the I544N, I675/6ins and G702Tins mutations.

By directly monitoring the subcellular localization of the wild type and mutant transporters by immunofluorescence I found the wild type SLC26A3 to be expressed and localized to the plasma membrane, while the I544N, I675/6ins and G702Tins mutant proteins accumulated in the ER (Figure 5-3). The analogous

mutation to I675/6ins has been previously made and in the mouse SLC26A3 protein, which showed plasma membrane localization by immunofluorescence (Ko et al., 2002). The finding that human I675/6ins SLC26A3 accumulates in the ER suggests that there are differential effects of this mutation on the mouse and human SLC26A3 proteins. The $\Delta Y526/7$ transporter was observed as a mixed population with some cells having the mutant transporter at the plasma membrane, while a larger percentage of the cells had retained the transporter in the ER with no detectable protein at the plasma membrane (Figure 5-3C1 and C2). This is consistent with the glycosylation data which showed only a small amount of the total protein expressed being complexly glycosylated (Figure 5-2A). The functional, glycosylation and immunofluorescence data suggest that the I544N, I675/6ins and G702Tins mutations result in disease due to a loss of functional protein at the plasma membrane caused by the mutant proteins being retained in the ER. This seems to be a common mechanism among the SLC26A protein family members as a number of disease causing mutations in SLC26A2 and SLC26A4 have also been studied by immunofluorescence, and ER retention has been observed for a number of these mutant transporters (Karniski, 2004; Ko et al., 2002; Rotman-Pikielny et al., 2002; Taylor et al., 2002).

The disease causing mechanism of the $\Delta Y526/7$ transporter is a bit less clear since it does retain some $\text{Cl}^-/\text{HCO}_3^-$ exchange activity, and hence some functional protein at the plasma membrane. The glycosylation data along with the

immunofluorescence results suggests that the $\Delta Y526/7$ mutation is extremely sensitive to its cellular environment and is possibly a less severe mutation than the I544N, I675/6ins or G702Tins. It should be noted that the $\Delta Y526/7$ mutant was identified in a patient who had the I675/6ins mutation in the other SLC26A3 allele (Hoglund et al., 1998), and perhaps if a person was homozygous for the $\Delta Y526/7$ mutation they would present with a less severe form of CLD, or may not present with any CLD symptoms since the $\Delta Y526/7$ mutant transporter retains some plasma membrane $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. Conversely, a person homozygous for $\Delta Y526/7$ SLC26A3 may still present with CLD if the transport function of $\Delta Y526/7$ SLC26A3 is reduced to levels which significantly perturb the ability of the tissue to properly absorb Cl^- and secrete HCO_3^- . A similar phenomenon has been observed in the various chondrodysplasias which are caused by mutations in the SLC26A2 gene, where the severity of the disease phenotype correlates with the amount of residual sulfate transport activity of the mutant SLC26A2 protein (Karniski, 2004).

Cell surface labeling of the wild type and mutant transporters were consistently observed in the biotin-streptavidin pull-down experiments (Figure 5-4). A number of control experiments were performed to validate the assay. CFTR was transfected into HEK 293 cells and used for cell surface biotinylation reactions, and following the pull-down and western blot analysis only the

complexly glycosylated form of CFTR (Band C) was observed, consistent with band C being the mature form of the protein present on the cell surface (Figure 5-4D). Cell surface biotinylation reactions and subsequent pull-down reactions were performed to test the integrity of the plasma membrane by using a mixture of the anti-c-Myc and anti-actin antibodies to develop the western blots. If some of the cell's plasma membranes were broken or somehow permeable to biotin, labeling of intracellular proteins during the course of my "cell surface" biotinylation experiment could be occurring. Actin was readily detected in the whole cell lysates but was not detectable to any significant level after the pull-down reaction was completed, suggesting that the vast majority of the cell's plasma membranes were still intact and not permeable to biotin under these experimental conditions (Figure 5-4A and B). Finally, cell surface biotinylation reactions were performed in the absence of any biotin to control for non-specific binding of the SLC26A3 transporters to the streptavidin agarose beads. These control experiments indicated that very little non-specific binding of the SLC26A3 transporters was occurring with the agarose beads (Figure 5-4C). Interestingly, unlike the SLC26A3 transporters, the wild type STAS domain showed some non-specific binding to the streptavidin agarose beads in the absence of biotin (Figure 5-4C), although it was pulled down to a greater extent when the cells were treated with biotin. This data suggests that part of the STAS domain may be extracellularly exposed (Figure 5-4B and C). The results from the

biotinylation experiments taken together suggested that the wild type and mutant SLC26A3 transporters may be present at the plasma membrane. However, it is unclear as to why the core glycosylated species of the SLC26A3 proteins would be biotinylated since these proteins should be retained in the ER and never exposed to the extracellular environment. This is precisely what was observed for the control experiments performed with CFTR, where the core glycosylated band B was not biotinylated while the complexly glycosylated band C was (Figure 5-4D). This combined with the biotinylation of the STAS domain suggests that a technical problem may exist in the biotinylation experiments with the SLC26A3 transporters even though the control experiments suggest the biotinylation procedure is working as expected.

The cell surface biotinylation data contradicts the glycosylation and immunofluorescence data previously collected. In addition, immunofluorescence experiments suggest that the STAS domain is intracellularly located as an isolated domain or as part of the intact SLC26A3 transporter (Figure 5-13). One possible mechanism that may explain the observed results is that the wild type and mutant SLC26A3 transporters reside in a macromolecular complex in which some of these proteins are at least partially exposed to the extracellular environment. These additional proteins could be specifically labeled with biotin and could be pulled down with the streptavidin agarose carrying along with them their binding partners including the SLC26A3 transporters. To test this possibility, cell surface

biotinylation experiments were carried out with a non-reversible binding biotin. Immunoprecipitation reactions of the wild type and mutant SLC26A3 transporters were performed, and the proteins separated by SDS-PAGE and blotted for biotin in an attempt to observe any cell surface proteins which may be co-precipitating with the SLC26A3 transporters. The results from these experiments showed no specific bands were co-immunoprecipitated with the SLC26A3 transporters (data not shown). Unfortunately, the results from these experiments did not provide a satisfactory explanation for the unexpected cell surface biotinylation results. Therefore, it still remains an open question as to what is happening, and how to interpret the cell surface biotinylation data.

To better understand the molecular mechanism(s) by which the STAS domain residing CLD causing mutations cause ER retention, the SLC26A3 STAS domain was studied *in vitro*. The SLC26A3 STAS domain was defined in chapter 4, and this construct was used to study the CLD causing mutations. The wild type and mutant SLC26A3 STAS domains were expressed as Smt3 fusion proteins in *E. coli*, and the solubly produced protein was monitored. The wild type, $\Delta Y526/7$ and I544N STAS domains were all produced in a soluble form, whereas the I675/6ins and G702Tins STAS domains were expressed almost exclusively as insoluble aggregates (Figure 5-5A). A similar phenomenon was also observed when the wild type and mutant STAS domains were expressed as myc-tagged fusions in HEK 293 cells (Figure 5-5B). Since two different expression systems

yielded very similar results, it was concluded that the I675/6ins and G702Tins mutations are either causing a dramatic loss in solubility, or they are causing the STAS domain to misfold. Since the I675/6ins and G702Tins mutations result in the transporter to be retained in the ER, which is consistent with a misfolding event, we favor the hypothesis that the mutations are causing the STAS domains to misfold. Nonetheless, it is still a formal possibility that the I675/6ins and G702Tins mutations are causing a dramatic loss in STAS domain solubility, and these mutations are not causing a misfolding event. However, a reduction in the solubility of a membrane protein domain does not seem to be a likely disease causing mechanism, whereas protein misfolding is a well established mechanism for disease (Chaudhuri and Paul, 2006; Forman et al., 2004; Lee and Yu, 2005; Thomas et al., 1995).

The solubly produced STAS domains (wild type, $\Delta Y526/7$ and I544N) were purified to homogeneity for comparison of their biochemical and biophysical properties (Figure 5-6 and 5-7A). The wild type and mutant STAS domains purified in the same manner and had similar biochemical properties to one another, suggesting that the mutations are not dramatically changing the properties of the domains. This was further confirmed by TROSY-HSQC NMR spectroscopy which revealed that the wild type and $\Delta Y526/7$ STAS domains are well folded proteins showing approximately 190 backbone proton-nitrogen cross peaks with significant spectral dispersion out of a possible 223 (Figure 5-7B).

This suggests that the STAS domain well folded and that the $\Delta Y526/7$ mutation is not dramatically affecting the global fold of the domain.

The CD spectrum of the wild type, $\Delta Y526/7$ and I544N STAS domains were indistinguishable from one another (Figure 5-7C), consistent with the CLD causing mutation not dramatically perturbing the secondary structure of the STAS domain. Similarly, thermal denaturation experiments showed no difference between the wild type and $\Delta Y526/7$ and I544N mutant STAS domains as measured by CD spectroscopy at 222 nm (Figure 5-6D). The proteins also had similar tryptophan fluorescence spectra when compared to one another (Figure 5-8). This data taken together suggests that the $\Delta Y526/7$ and I544N mutations are not causing major structural or biochemical changes in the STAS domain, and it is likely that these domains have a very similar structure to the wild type protein.

Unlike the I675/6ins and G702Tins mutations which appeared to cause the STAS domain to misfold, which the quality control machinery of the cell recognizes as non-native and retains these transporters in the ER, the $\Delta Y526/7$ and I544N STAS domains appear to be well folded and indistinguishable from the wild type protein. However, the quality control machinery of the cell clearly recognizes the $\Delta Y526/7$ and I544N mutant transporters as different from the wild type transporter as evident by the mistrafficking of these two proteins. One possible mechanism by which the overall structure of a domain can be unaltered

yet it still can be recognized as non-native in the context of the full-length protein is if the mutations are disrupting domain-domain interactions. It is possible that the $\Delta Y526/7$ and I544N mutations disrupt an interaction between the STAS domain and the transmembrane domain of the SLC26A3 transporter. To test this hypothesis co-transfection experiments were performed with the transmembrane spans of SLC26A3 (Δ STAS) along with the STAS domain. The rationale for these experiments was that the Δ STAS protein would be recognized as misfolded by the quality control machinery of the cell, and upon addition of the STAS domain they might assemble into a native like structure which would be recognized by the quality control machinery as properly folded and permitted to traffic out of the ER to the plasma membrane. Thus, the glycosylation state of the Δ STAS construct could be used as a marker to monitor trafficking out of the ER upon co-transfection with the STAS domain.

When expressed in HEK 293 cells the Δ STAS construct was found to show some complexly glycosylated protein, which made interpretation of the STAS and Δ STAS co-transfection experiment difficult since a dramatic change in the complexly glycosylated state of the Δ STAS protein was not observed (Figure 5-9). As another means to monitor the potential interaction between the STAS domain and the transmembrane spans, and the effect that the STAS domain CLD

causing mutations may have on this putative interaction, limited proteolysis experiments were performed on the SLC26A3 transporter using trypsin.

Limited proteolysis experiments were performed on SLC26A3 using trypsin, and the production of proteolytic fragments was monitored on western blots by probing for the myc tag. Unfortunately, no proteolytic fragments were observed using this method, potentially indicating that the myc tag was being cleaved off of the rest of the protein by trypsin. There are eleven putative trypsin cleavage sites between the N-terminal myc tag and the first predicted transmembrane span of SLC26A3, which is consistent with the interpretation that the myc tag was being proteolytically removed from the transporter during our limited proteolysis experiments. As a potential work around for this problem, a polyclonal antibody was raised against the STAS domain allowing for the direct monitoring of the domain of interest. In addition, the STAS domain polyclonal antibody likely recognizes multiple epitopes in the STAS domain, which potentially allows for more proteolytically cleaved STAS domain fragments to be detected.

The rabbit polyclonal antibody that was produced is capable of detecting SLC26A proteins via western blot analysis (Figure 5-10), and is useful for immunoprecipitation (Figure 5-11) and immunofluorescence experiments (Figure 5-12). Performing immunofluorescence experiments with and without cell permeabilization while using the anti-c-Myc antibody to recognize the N-terminal

myc tag, or the anti-STAS antibody to detect the C-terminal STAS domain, the N and C-termini were both found to be located in the cytosol (Figure 5-13A and B). This is consistent with the topology of SLC26A5 and the proposed models for many of the SLC26A family members which have an even number of transmembrane spans and intracellularly located N and C-termini (Navaratnam et al., 2005; Zheng et al., 2001).

Using our STAS domain antibody, limited proteolysis experiments were performed using the enzyme trypsin. When increasing concentrations of trypsin were incubated with HEK 293 cell lysates containing wild type or mutant SLC26A3 proteins, three consistent proteolytic fragments were observed when western blots were probed with the anti-STAS antibody (Figure 5-14). Quantification of the full length or proteolytic fragments 1 and 3 suggested that there may be subtle differences in the conformations of the wild type, $\Delta Y526/7$ and I544N mutant transporters. The full-length protein seems to be degraded most quickly for the I544N > $\Delta Y526/7$ > wild type transporter, however, no difference is observed in proteolytic fragment 3. Nonetheless, there is a significant difference in the amount of proteolytic fragment 1 detected in the trypsin digest experiments. The observed differences between the wild type and the $\Delta Y526/7$ and I544N mutants in the full-length protein as well as proteolytic fragment 1, suggest that there are subtle conformational changes between the three proteins which are being revealed by limited proteolysis.

A recent publication of a study which randomly mutated the *Arabidopsis thaliana* Sultr1.2 STAS domain identified a number of mutations which were critical for function as well as proper trafficking (Shibagaki and Grossman, 2006). Y523 and I541 are the Sultr1.2 residues analogous to Y526 and I544 in the human SLC26A3 protein. Y523H of Sultr1.2 was identified in the mutagenic screen as a mutation which had no sulfate transport activity, yet it had wild type levels of protein expression and membrane integration. Similarly, the Δ Y526/7 mutation in SLC26A3 does not alter the protein expression levels in the full-length transporter or in the mutant STAS domain. However, unlike the Y523H mutation in Sultr1.2 the Δ Y526/7 mutation in SLC26A3 retained some $\text{Cl}^-/\text{HCO}_3^-$ exchange activity. There are a number of different possible explanations for the observed differences in the behavior of mutations at this position, the first being that they are not the same mutation. If Y523 in Sultr1.2 was deleted, then perhaps some residual transport activity would remain. Contrary to this idea, deleting Y523 in Sultr1.2 may cause a loss in transport activity similar to the Y523H mutation found in the mutagenic screen. It is possible that the observed function in the Δ Y526/7 SLC26A3 protein is due the fact that the human SLC26A3 sequence has two Tyr residues in a row at positions 526 and 527, and that deletion of one Tyr residue is not enough to eliminate $\text{Cl}^-/\text{HCO}_3^-$ exchange activity due to compensation by the adjacent Tyr residue.

The Sultr1.2 mutagenic screen also identified A540S and Y542C as having reduced and no sulfate activity respectively. Both proteins were shown to be expressed and the Y542C mutant Sultr1.2 transporter was found in the plasma membrane (Shibagaki and Grossman, 2006). These two residues are predicted to be the two adjacent residues of I544 in SLC26A3. Functionally the A540S mutation is more similar to the I544N mutation of SLC26A3 than is Y542C since A540S results in immeasurable transport activity. Although plasma membrane localization was not determined for the A540S mutation, the Y542C mutation was found to be in the plasma membrane unlike the I544N mutation. However, these mutations in Sultr1.2 are only adjacent to the analogous I544N position and are not the same mutation, so it may not be surprising to see different mutant phenotypes. Interestingly, when the Y523H, A540S and Y542C Sultr1.2 mutations are mapped onto the SpoIIAA protein structure they all reside on the same protein face that is important for SpoIIAB binding. Since all three of these mutations alter sulfate transport activity, and they reside on the same SpoIIAA surface, Shibagaki and colleagues speculate that molecular interactions on this surface may be important for proper Sultr1.2 transport activity. Since the $\Delta Y526/7$ and I544N mutations in SLC26A3 are analogous to Y523 and I541, they are also predicted to be on the surface of the SpoIIAA like fold near the SpoIIAB binding site (Shibagaki and Grossman, 2006). This suggests that the $\Delta Y526/7$ and I544N mutations may be resulting in CLD due to improper domain-domain

interaction, either with the transmembrane domain of SLC26A3 which is consistent with our limited proteolysis data, or other functionally important interactions such as with chaperones or other regulatory proteins.

Our data taken together suggests a model by which the CLD causing mutations in the STAS domain of SLC26A3 result in disease by causing a loss in wild type levels of functional protein at the plasma membrane (Figure 5-15). The wild type SLC26A3 transporter is synthesized and folded in the ER where it reaches a native like structure that the quality control machinery recognizes as folded. The quality control machinery then allows the transporter to traffic out of the ER through the secretory pathway where it is complexly glycosylated and is eventually shuttled to the plasma membrane (Figure 5-15, upper third). The CLD causing mutations which reside in the STAS domain of the SLC26A3 protein result in protein mistrafficking and ER retention by at least two distinct mechanisms. The I675/6ins and G702Tins mutations both seem to destabilize the STAS domains themselves. This likely results in improper transporter folding and or assembly, which the quality control machinery of the cell recognizes as non-native and retains these misfolded proteins in the ER (Figure 5-15, lower third). This is in contrast to the $\Delta Y526/7$ and I544N mutations which do not cause any significant changes to the STAS domain's biochemical or biophysical properties that have been tested, however, they still cause CLD. The $\Delta Y526/7$ mutation seems to be exquisitely sensitive to its cellular environment as

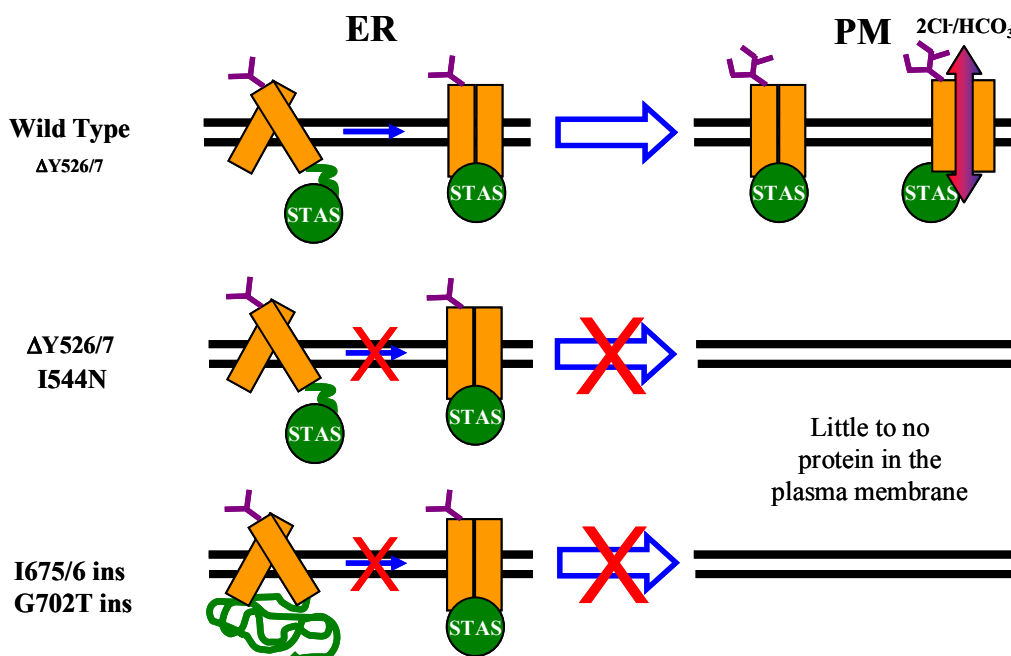


FIGURE 5-15: A model for wild type and mutant SLC26A3 folding and trafficking. A protein folding and trafficking model for the wild type and mutant SLC26A3 proteins is presented with proper folding of both the STAS domain and the transmembrane spans being required for transport from the ER to the plasma membrane. The circle labeled STAS denote properly folded STAS domain while the undulating lines represent improperly folded STAS domain. The vertical rectangles represent properly folded and assembled transmembrane spans while the skewed rectangles symbolize non-native transmembrane structure. The horizontal black lines represent the ER and plasma membranes and the branched structures on the transmembrane domain represent the glycosylation state of the protein. The wild type transporter and a portion of the $\Delta Y526/7$ transporters can adopt the appropriate structure which is recognized by the quality control machinery of the ER as native, and is then allowed to proceed through the secretory pathway where it is complexly glycosylated and eventually reaches the plasma membrane (PM). A large portion of the $\Delta Y526/7$ transporters are retained in the ER and not trafficked to the plasma membrane as are the I544N, I675/6ins and G702Tins mutant transporters. The large and small font type for the $\Delta Y526/7$ labels represents the larger and smaller population of cells which have native like or non-native like transporter. This conceptual model has no information regarding the order to which the STAS and transmembrane domains fold and are assembled in the ER, rather it highlights the different mechanisms by which the STAS domain mutations bring about protein misfolding and the absence of active protein at the plasma membrane.

demonstrated by our glycosylation data and the observed mixed population of cells showing plasma membrane localization or ER retention in the immunofluorescence experiments. The model reflects this sensitivity in that a small amount of $\Delta Y526/7$ SLC26A3 protein is found to properly traffic to the plasma membrane (smaller font of $\Delta Y526/7$ in the upper third of the model), while a larger portion of the expressed protein is found to accumulate in the ER which is reflected by the larger font type of $\Delta Y526/7$ in the middle third of the model. Subtle changes were also observed in our limited proteolysis experiments between the wild type, $\Delta Y526/7$ and I544N mutant transporters, possibly suggesting that these mutations effect domain-domain interactions or the conformational dynamics of the STAS domain itself. Indeed, the recent publication by Shibagaki and Grossman suggests that when the $\Delta Y526/7$ and I544N mutations are mapped onto the SpoIIAA structure they are found to be at the SpoIIAA-SpoIIAB binding interface, suggesting that this surface of the STAS domain forms molecular interactions which are important for proper trafficking and sulfate transporter function (Shibagaki and Grossman, 2006). While these data suggest that the $\Delta Y526/7$ and I544N mutations may be disrupting important molecular interactions, they do not rule out the possibility that the $\Delta Y526/7$ and I544N mutations may be causing disease by an alternative mechanism. These

formal possibilities while important, are outside the scope of the current study, and will have to be experimentally tested in the future.

Chapter Six:

Structural Studies of the Human SLC26A3 and *Aquifex* Sultr

STAS Domains

Introduction:

One of the hallmarks of the SLC26A family of proteins is that they all have a C-terminally conserved domain that is homologous to the SpoIIAA anti-sigma factor antagonists (Aravind and Koonin, 2000) (see Figure 2-18). The SLC26A proteins were originally predicted to be a family of sulfate transporters and because of this, their C-terminally conserved domain was named STAS for sulfate transporters and anti-sigma factor antagonists. The function of the STAS domain in the SLC26A transporters is not well understood, however, the function of the SpoIIAA proteins is to help regulate gene transcription during spore formation in bacteria (Errington, 2003). This regulatory function of SpoIIAA is achieved through a somewhat complex four component signaling circuit that includes the anti-sigma factor kinase SpoIIAB, a phosphatase SpoIIE and sigma factor F (σ^F) (see chapter 2 for more details).

A great deal of structural work has been done on this signaling circuit which has resulted in a number of crystal and NMR structures of the SpoIIAA protein alone or in complex with SpoIIAB (Clarkson et al., 2001; Clarkson et al.,

2003; Kovacs et al., 1998; Kovacs et al., 2001; Masuda et al., 2004; Seavers et al., 2001a; Seavers et al., 2001b). The SpoIIAA structures solved to date show a mixed α/β protein containing a central four stranded β -sheet and four surrounding α -helices (Clarkson et al., 2001; Clarkson et al., 2003; Kovacs et al., 1998; Masuda et al., 2004; Seavers et al., 2001a) (see Figure 2-20). Since the SpoIIAA proteins were identified as homologous to the C-terminal STAS domains of the SLC26A transporters, researchers studying this family of transporters have used the SpoIIAA protein as a structural model to aid in interpreting experimental results (Rouached et al., 2005; Shibagaki and Grossman, 2004; Shibagaki and Grossman, 2006). However, close inspection of the multiple sequence alignment between the SLC26A transporters and the SpoIIAA proteins reveals regions of poor sequence identity (see Figure 2-18). The biggest difference between the SLC26A STAS domains and the SpoIIAA proteins is that the SLC26A3 STAS domains have a large insertion between the first α -helix and the third β -strand of the SpoIIAA fold (Aravind and Koonin, 2000). We have named this insertion the intervening sequence (IVS) which varies in length between the different SLC26A transporters from 0 to 140 residues for SLC26A11 and SLC26A8 respectively. In addition to this large insertion, the N and C-terminal sequences of the STAS domains do not align well with the SpoIIAA termini. In addition, the highly conserved STAS domain N-terminal sequence $G\Phi XI\Phi XXXSPIYFAN$ and C-terminal sequence $F\Phi S/TVXD/EA\Phi$ (where X is any amino acid and Φ is a

hydrophobic amino acid) are not as highly conserved in the SpoIIAA proteins, yet the high degree of conservation of these sequences suggests that they are critical for the function of the SLC26A STAS domains. As a result of these observations, and the fact that the STAS domains are critical for the activity of the SLC26A transporters, I decided to structurally characterize the SLC26A3 STAS domain or a bacterial STAS domain from *Aquifex aeolicus* VF5, with the intent of testing the notion that the SpoIIAA proteins are a good structural model for STAS domains.

Experimental Procedures:

STAS Domain Expression Plasmid Construction: The human SLC26A3 STAS domain construct was generated with domain boundaries encompassing amino acid residues 510-741, and were cloned into the BamHI site of the Smt3-pET28 vector (Mossessova and Lima, 2000). The Δ IVS STAS domain construct was created by deleting amino acid residues 565-639 from the human SLC26A3 STAS domain in the Smt3-pET28 vector (Mossessova and Lima, 2000). STAS domains from two bacterium *Aquifex aeolicus* VF5 and *Desulfovibrio vulgaris* Hildenborough (gi:15606490 and gi:46448107 respectively) were also cloned into the Smt3-pET28 vector with the amino acid boundaries 436-605 and 429-568 respectively. All protein expression constructs were verified by DNA sequencing. Additional *Aquifex aeolicus* VF5 STAS domains were constructed using three N-terminal boundaries (436, 450, and 459) and three C-terminal boundaries (605, 588, and 575) and the additional constructs were cloned into the Smt3 pET 28 vector (Mossessova and Lima, 2000).

Human SLC26A3 STAS Expression and Purification: Five liters of *E.coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene) containing the wild type or mutant Smt3-STAS domain plasmids were grown at 37°C in LB containing 50 µg/L Kanamycin and 34 µg/L Chloramphenicol to an O.D.₆₀₀ of 0.8. The cells were then induced with 750 µM IPTG (Research Products International) and shifted to

15°C for overnight expression. The following day the cells were pelleted at 3000 rcf and then resuspended in 70 ml of buffer A (20 mM Tris pH 7.9, 200 mM NaCl, 1 mM BME). The resuspended cells were lysed via sonication, and the resulting lysates cleared by centrifugation at 40,000 rcf. Standard Ni chromatography was performed on the supernatant fractions using 10 ml bed volume of resin (Novagen). Buffer A was used as the equilibration/binding buffer while the wash buffer was buffer A containing 40 mM imidazole. The elution buffer was buffer A containing 400 mM imidazole, and fractions containing the Smt3-STAS fusion protein were pooled together and digested overnight with a 1:1000 dilution of the Ulp1 protease at 4°C.

The following day 750 mM Ammonium Sulfate was added to the cleaved protein solution and then subjected to Butyl hydrophobic interaction chromatography (Amersham Biosciences). The equilibration/binding buffer contained 20 mM Na₂PO₄ pH 5.9, 750 mM Ammonium Sulfate, 1 mM DTT and the elution buffer contained 20 mM Na₂PO₄ pH 6.2, 1 mM DTT. Fractions containing the STAS protein were pooled together and 350 mM NaCl was added to the solution. The protein was then concentrated to 2 ml and run over a HiPrep 16/60 Sephacryl S-200 column (Amersham Biosciences) in 20 mM Na₂PO₄ pH 6.2, 350 mM NaCl, 1 mM DTT.

Aquifex STAS Expression and Purification: The *Aquifex* STAS domain was expressed and purified from *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene) grown at 37°C in LB containing 50 µg/L Kanamycin to an O.D.₆₀₀ of 1.0. The cells were then induced with 750 µM IPTG (Research Products International) and allowed to express protein for 6 hr. Cells were collected via centrifugation and were resuspended and lysed in 30 ml of buffer A via sonication. The resulting lysates were cleared by centrifugation at 40,000 rcf and standard Ni chromatography was performed on the resulting supernatant. The equilibration/binding buffer (buffer A) contained 20 mM Tris pH 7.9, 200 mM NaCl, 1 mM BME, the column was washed using buffer A containing 40 mM imidazole. The protein was eluted from the column using buffer A containing 400 mM imidazole and fractions containing the Smt3-*Aquifex* STAS fusion protein were pooled together and digested overnight with a 1:1000 dilution of the Ulp1 protease at 4°C.

The following day 750 mM Ammonium Sulfate was added to the cleaved protein solution and then subjected to butyl hydrophobic interaction chromatography (Amersham Biosciences). The equilibration/binding buffer contained 20 mM Na₂PO₄ pH 6.0, 750 mM Ammonium Sulfate, 1 mM DTT and the elution buffer contained 20 mM Na₂PO₄ pH 6.0, 1 mM DTT. Fractions containing the STAS protein were pooled together and the protein was

concentrated to 2 ml and run over a HiPrep 16/60 Sephacryl S-200 column (Amersham Biosciences) in 50 mM Na₂PO₄ pH 6.0, 50 mM NaCl, 1 mM DTT.

Optimum Solubility Screening and Dynamic Light Scattering: The optimum solubility (OS) screening method was applied to the wild type SLC26A3 STAS domain to help determine the most suitable buffer for crystallization (Jancarik et al., 2004). Vapor-diffusion hanging-drops were set up with wild type SLC26A3 STAS domain at 170 μ M in 20 mM Na₂PO₄ pH 6.2, 350 mM NaCl, 1 mM DTT. Conditions 6-24 (see Table 6-1) were screened for homogeneity and monodispersity by dynamic light scattering with a final STAS concentration of 28 μ M diluted into the OS buffers.

Protein Crystallization Screening: Protein crystallization conditions for the wild type SLC26A3 STAS (510-741) domain were performed using the vapor-diffusion hanging-drop method with a protein concentration of 10 mg/ml in 20 mM CAPS pH 10.0, 10 mM DTT. The Hampton Screen I, (NH₄)₂SO₄, Light, SaltRx, and Index Screens were set up and incubated at 20°C.

The vapor-diffusion hanging-drop method was used to screen for crystallization conditions for the *Aquifex* STAS domain. The *Aquifex* STAS domain was screened at 9.2 mg/ml in 10 mM Tris pH 7.0, 50 mM NaCl, 1 mM

DTT with the following Hampton screens: screen I and II, PEG Ion, Light, SaltRx, Low Ionic Strength and Index.

SLC26A3 STAS NMR: A 1D proton NMR spectra was taken of 150 μM unlabeled purified STAS domain in 20 mM Na_2PO_4 pH 6.8, 350 mM NaCl and 2 mM DTT. A TROSY-HSQC spectra of 90 μM ^{15}N labeled purified STAS domain in 50 mM K_2PO_4 pH 6.8, 400 mM KCl, 100 mM Arginine, 2% glucose, 2 mM DTT was collected at 30°C in a 600 MHz spectrometer.

Aquifex STAS NMR: A HSQC of 60 μM ^{15}N labeled *Aquifex* 436-605 STAS domain was collected on a 500 MHz spectrometer for 2 hr at room temperature in 50 mM Na_2PO_4 pH 6.0, 50 mM NaCl, 1 mM DTT. The *Aquifex* 450-588 STAS domain HSQC spectra was collected 500 MHz spectrometer fitted with a cold probe for 2 hr at 25°C. The *Aquifex* 450-588 STAS domain was 250 μM and the buffer conditions were 50 mM Na_2PO_4 pH 6.0, 50 mM NaCl, 1 mM DTT in the presence or absence of 1 mM ATP, or 1 mM ATP + 1 mM GDP- γS .

Mass Spectrometry and N-terminal Sequencing: Purified *Aquifex* STAS domain was given to the UT Southwestern Biopolymer Laboratory for electrospray mass spectrometry analysis and by Edman Degradation N-terminal sequencing.

Results:

To test the notion that the SpoIIAA proteins are structural homologs of the STAS domains, as well as to better understand the structural nature of the intervening sequence (IVS), I used the SLC26A3 STAS domain 510-741 protein for initial crystallization screening. I began by setting up vapor-diffusion hanging-drops of the Hampton Screen I, $(\text{NH}_4)_2\text{SO}_4$, Light, SaltRx, and Index Screens with STAS 510-741 at 10 mg/ml at 20°C. Unfortunately, all of the drops precipitated almost immediately indicating that under these screening conditions our protein construct was unsuitable for crystallization.

Subsequently, I decided to put the STAS domain through the optimization screen of Jancarik *et al.* to determine if buffer conditions more amenable to crystallization could be found for the STAS domain (Jancarik *et al.*, 2004). Vapor-diffusion hanging-drops were set up of the STAS domain at 4.5 mg/ml and incubated overnight at 20°C. The following day the hanging drops for buffer conditions 6-24 had remained clear (Table 1), and the STAS domain protein was then diluted directly into buffers 6-24 and dynamic light scattering (DLS) experiments were performed on the 19 samples. Buffer conditions 6-23 all showed signs of aggregation by dynamic light scattering as did the phosphate buffer condition used for the initial protein crystallization screening. However, the STAS domain appeared monomeric by DLS in buffer condition 24 (100 mM CAPS buffer pH 10.0) (Figure 6-1). Various additives were also screened in

No.	Buffer (100 mM)	pH
1	Glycine	3
2	Citric acid	3.2
3	PIPPS	3.7
4	Citric acid	4
5	Sodium acetate	4.5
6	Sodium/potassium phosphate	5
7	Sodium citrate	5.5
8	Sodium/potassium phosphate	6
9	Bis-tris	6
10	MES	6.2
11	ADA	6.5
12	Bis-tris propane	6.5
13	Cacodylate	6.5
14	Ammonium acetate	7
15	MOPS	7
16	Sodium/potassium phosphate	7
17	HEPES	7.5
18	Tris	7.5
19	EPPS	8
20	Imidazole	8
21	Tris	8.5
22	CHES	9
23	CHES	9.5
24	CAPS	10

Table 6-1: Listed are the buffer conditions from the Jancarick optimization screen.

combination with the CAPS buffer condition in an attempt to reduce the level of polydispersity however, none of the additives tried significantly reduced the polydispersity of the STAS domain under the CAPS buffer condition (Table 2).

Crystallization screens were set up of the human STAS domain 510-741 protein in the CAPS buffer and many of the crystal drops precipitated, while in contrast, a number of drops remained clear after a few days in conditions containing high concentrations of ammonium or sodium nitrate (1.5-6.0 M) at pH 7.0 or 8.5, and

remained clear for up to three months. However, none of the conditions which were screened resulted in the production of crystals and at this point we began to evaluate other SLC26A3 STAS domain homologs for structural studies of the STAS domain.

Two bacterial sulfate transporter homologs were chosen for study because they both contained a putative STAS domain fused to a transmembrane domain and had higher sequence identity to the SLC26A STAS domains than did the

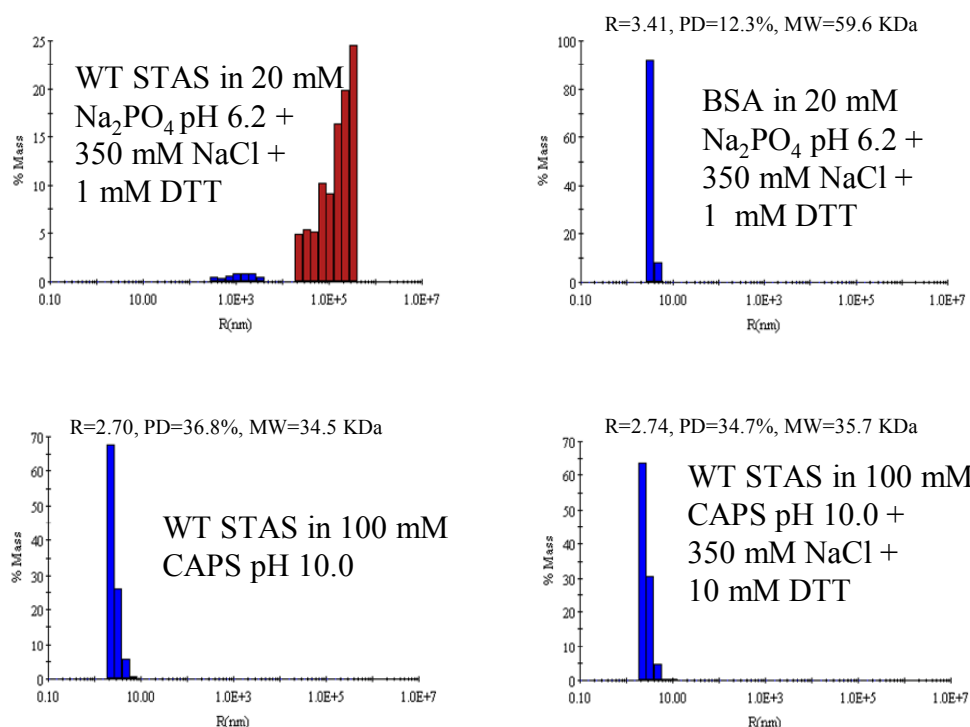


FIGURE 6-1: Dynamic light scattering of STAS 510-741. Dynamic light scattering experiments were performed on the wild type STAS 510-741 protein in various buffer conditions along with BSA as a positive control. The computed radius (R), polydispersity (PD) and the calculated molecular weight (MW) are shown above each graph where applicable.

SpoIIAA proteins. The two sulfate transporter (Sultr) homologs are from *Aquifex aeolicus* VF5 and *Desulfovibrio vulgaris* Hildenborough (gi:15606490 and gi:46448107 respectively). The STAS domains of these two proteins were cloned into the Smt3 expression vector and their soluble expression tested along with a new SLC26A3 STAS construct which lacked the intervening sequence (IVS). All three of these constructs were expressed, however, only the *Aquifex* Sultr (amino acids 436-605) and *Desulfovibrio* Sultr (amino acids 429-568) constructs

	Radius (nm)	Polydispersity (%)	Molecular Weight (kDa)
CAPS Buffer Alone	0.11	26.5	0.02
BSA	3.41	12.3	59.6
STAS	2.67 ± 0.36	39.3 ± 13.4	34.4 ± 10.1
STAS + 50 mM NaCl	2.99	44.3	44.0
STAS + 350 mM NaCl	2.48	45.6	28.3
STAS + 10 mM DTT	2.77	26.5	36.5
STAS + 350 mM NaCl + 10 mM DTT	2.74	34.7	35.7
STAS + 10% Glycerol	3.90	37.7	81.7
STAS + 0.1% O β G	4.64	38.8	122
STAS + 1.0% O β G	22.2	41.8	4771
STAS + 0.1% DDM	4.27	39.0	101
STAS + 1.0% DDM	2.34	44.0	24.7

Table 6-2: DLS estimates of the radius, polydispersity and molecular weight of the STAS domain in CAPS buffer with various additives.

produced any significant amounts of soluble protein (Figure 6-2A). The *Desulfovibrio* Sultr STAS was highly expressed but showed small amounts of degradation while the *Aquifex* Sultr STAS construct was similarly expressed; it did not show any obvious signs of degradation. For this reason the *Aquifex* Sultr STAS domain was chosen for additional structural studies.

The *Aquifex* STAS domain was purified to homogeneity and submitted to the UT Southwestern Biopolymer Laboratory for mass spectrometry and N-terminal sequencing analysis (Figure 6-2B). The observed molecular weight of the *Aquifex* STAS domain from the mass spectrometry was 19,930 Da which is 1 Da lower than the predicted molecular weight. The N-terminal sequencing returned the amino acid sequence SEFEL, which are the first five amino acids of

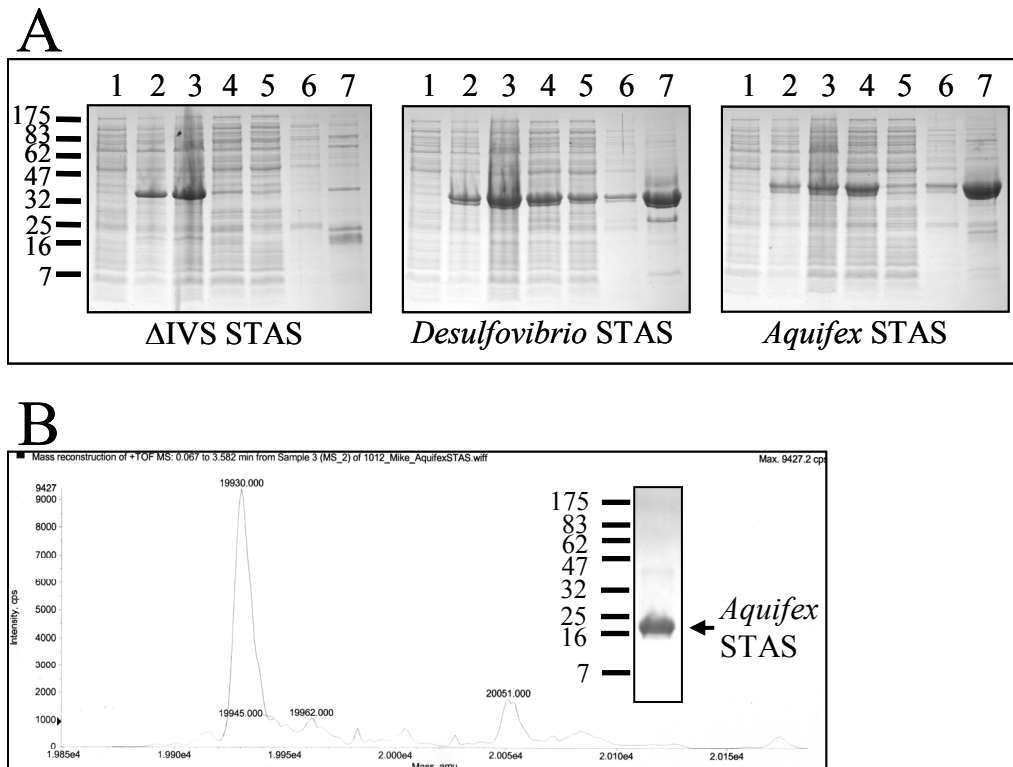


FIGURE 6-2: Additional STAS domain constructs for structural studies. (A) Three additional STAS domain constructs were produced as Smt3 fusions in *E. coli*, Δ IVS STAS (amino acids boundaries 510-741 with a deletion of amino acids 565-639) along with two additional STAS domains from *Desulfovibrio vulgaris* Hildenborough (gi:46448107, amino acid boundaries 429-568) and *Aquifex aeolicus* VF5 (gi:15606490, amino acid boundaries 436-605). Three Coomassie-stained gels showing the expression and purification of the three new constructs are shown with molecular weight markers shown in kDa. The following proteins samples are loaded on the gel: lane 1) uninduced cell lysate, 2) induced cell lysate, 3) pellet fraction of induced cell lysate, 4) supernatant fraction of induced cell lysate, 5) Ni column flow through, 6) Ni column wash, 7) Ni column elution. (B) A Coomassie-stained gel of the *Aquifex* STAS domain which was purified to homogeneity (inset) and subjected to mass spectrometry which yielded a mass of 19930 Da, and N-terminal sequencing returned the sequence SEFEL.

the *Aquifex* STAS construct. These two pieces of data suggest that the *Aquifex* STAS domain is full length polypeptide and it is not being degraded to any significant extent.

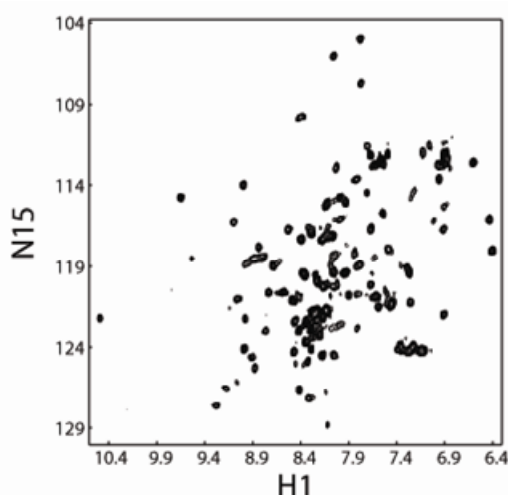


FIGURE 6-3: *Aquifex* STAS NMR HSQC. ^{15}N labeled *Aquifex* STAS 436-605 was expressed and purified as described in the experimental procedures and a HSQC spectra was collected on 60 mM labeled STAS on a 500 MHz spectrometer at room temperature for two hours in 50 mM Na_2PO_4 pH 6.0, 50 mM NaCl, 1 mM DTT.

A ^{15}N - ^1H HSQC experiment was performed on the *Aquifex* STAS 436-605 protein before any crystal screening was performed to help determine if this construct might be suitable for crystallization. A ^{15}N labeled *Aquifex* 436-605 sample was expressed and purified, then given to Dr. Carlos Amezcua who collected an HSQC spectrum of the labeled *Aquifex* STAS domain 436-605 at 1.1 mg/ml (60 μM). The resulting HSQC spectrum showed only about half of

the proton-nitrogen coupled cross peaks predicted from the amino acid sequence (Figure 6-3). In addition to a large number of unobserved cross peaks, there were a number of very intense cross peaks in the spectrum, indicative of highly mobile residues. As a result of this data, I decided to try and refine the domain boundaries of the *Aquifex* STAS domain.

Six additional *Aquifex* STAS domain constructs were produced and their soluble expression and Ulp1 cleavage efficiency was tested (Figure 6-4). The shortest *Aquifex* STAS construct that was produced solubly and was efficiently

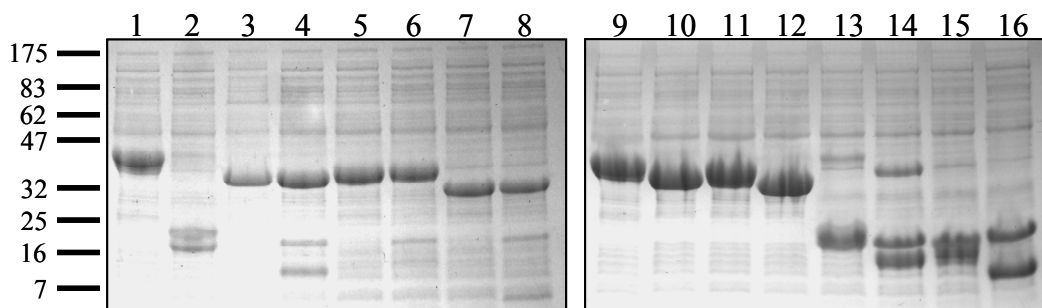


FIGURE 6-4: *Aquifex* STAS domain boundary refinement. Six additional *Aquifex* STAS constructs were produced to help refine the N and C-terminal boundaries. Coomassie-stained SDS-PAGE gels of Ulp1 cleaved and uncleaved supernatant samples are shown. Lane 1) 436-650 uncleaved, 2) 436-650 Ulp1 cleaved, 3) 436-575 uncleaved, 4) 436-575 Ulp1 cleaved, 5) 459-605 uncleaved, 6) 459-605 Ulp1 cleaved, 7) 459-575 uncleaved, 8) 459-575 Ulp1 cleaved, 9) 436-650 uncleaved, 10) 436-588 uncleaved, 11) 450-605 uncleaved, 12) 450-588 uncleaved, 13) 436-650 Ulp1 cleaved, 14) 436-588 Ulp1 cleaved, 15) 450-605 Ulp1 cleaved, 16) 450-588 Ulp1 cleaved. The molecular weight markers are measured in kDa.

cleaved by Ulp1, had N and C-terminal boundaries of 450-588. The *Aquifex* STAS domain 450-588 was purified to homogeneity and submitted to the UT Southwestern Biopolymer Laboratory for mass spectrometry and N-terminal sequencing analysis (Figure 6-5). The mass spectrometry results showed a molecular weight of 15,952 Da which is 20 Da higher than the predicted mass from the amino acid sequence. N-terminal sequencing returned the predicted N-terminal amino acid sequence of SEKRG, suggesting that the *Aquifex* 450-588 protein contains all the predicted amino acids.

The *Aquifex* STAS domain 450-588 was then produced as a ^{15}N labeled sample and a NMR HSQC spectra was collected (Figure 6-6A). After the initial HSQC spectrum was collected 1 mM ATP was added to the protein to see if

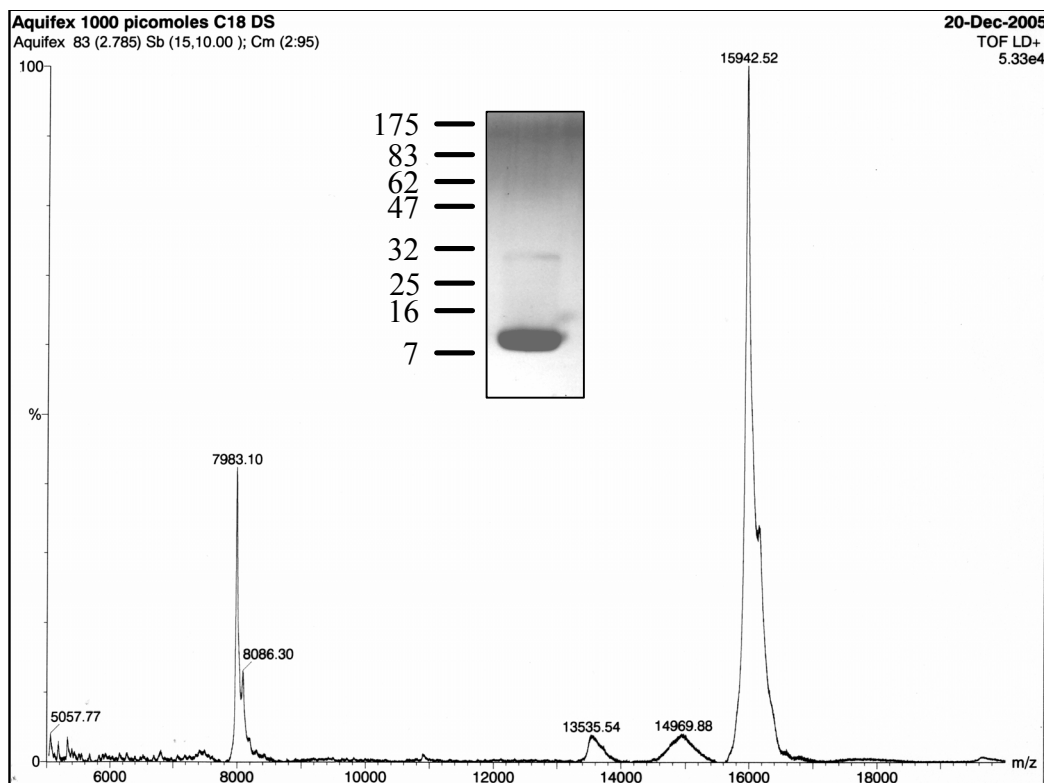


FIGURE 6-5: *Aquifex* STAS 450-588 mass spectrometry and N-terminal sequencing. Purified *Aquifex* STAS 450-588 protein was used for MALDI-TOF mass spectrometry and N-terminal sequencing yielding a mass of 15942 Da and the amino acid sequence SEKRG. The purity of the *Aquifex* STAS domain is shown in the Coomassie-stained gel. The molecular weight markers to the left are measured in kDa.

movement in any proton-nitrogen cross peaks could be detected as a function of ATP concentration (Figure 6-6B). After the *Aquifex* STAS + ATP spectrum was collected, 1 mM GDP- γ S was added to test if any cross peaks moved due to the presence of GDP- γ S (Figure 6-6C). By overlaying the three *Aquifex* STAS domain 450-588 HSQC spectra, it was clear that there are no significant changes in the proton-nitrogen coupled cross peak chemical shifts.

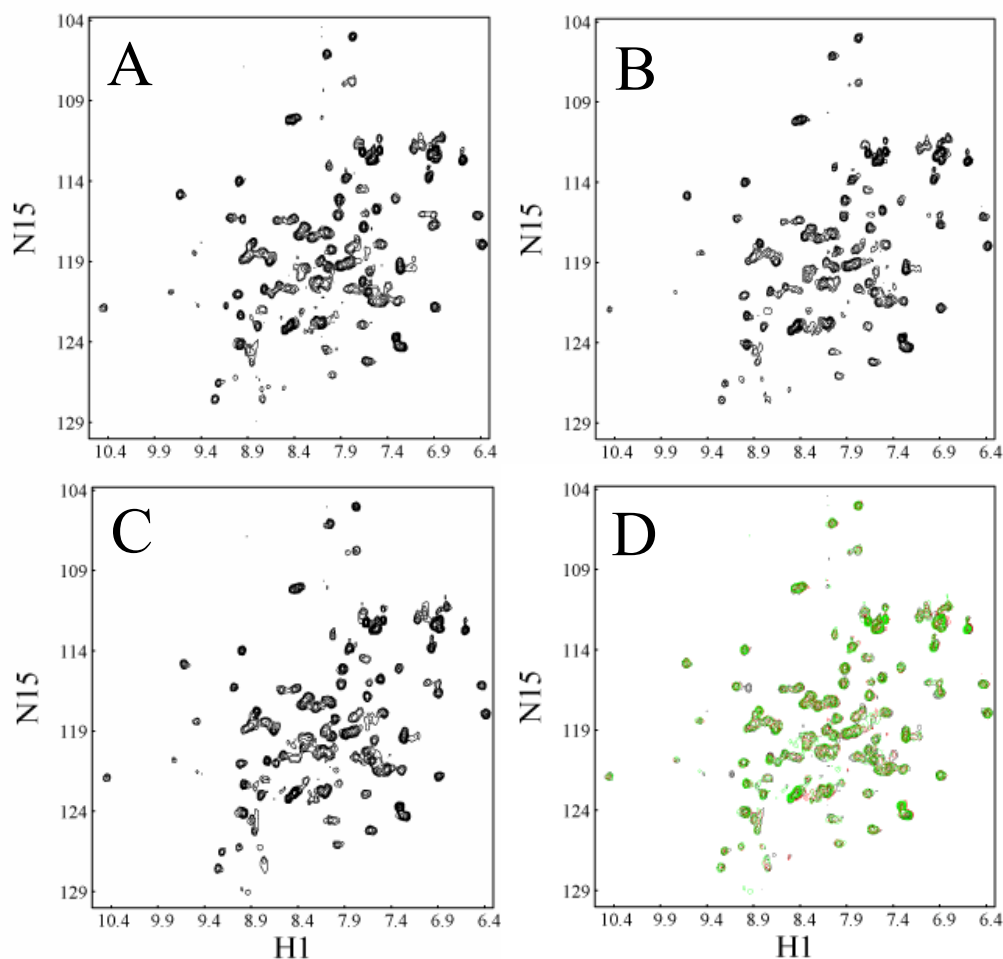


FIGURE 6-6: HSQC spectrum of *Aquifex* STAS 450-588. ^{15}N labeled *Aquifex* STAS 450-588 was expressed and purified as described in the experimental procedures, and spectra were collected in 50 mM Na_2PO_4 pH 6.0, 50 mM NaCl, 1 mM DTT (A) A HSQC spectra was collected of 250 μM ^{15}N labeled *Aquifex* STAS 450-588 protein on a 500 MHz spectrometer fitted with a cold probe at 25°C. (B) After the first HSQC spectrum was collected, 1 mM ATP was added and a subsequent HSQC spectrum was collected of the *Aquifex* STAS domain. (C) A third HSQC spectrum was collected on the *Aquifex* STAS 450-588 protein in the presence of 1 mM ATP and 1 mM $\text{GDP}\gamma\text{S}$. (D) An overlay of the nucleotide free (black spectra), ATP (red spectra) and ATP + $\text{GDP}\gamma\text{S}$ (green spectra) *Aquifex* 450-588 HSQC spectrum.

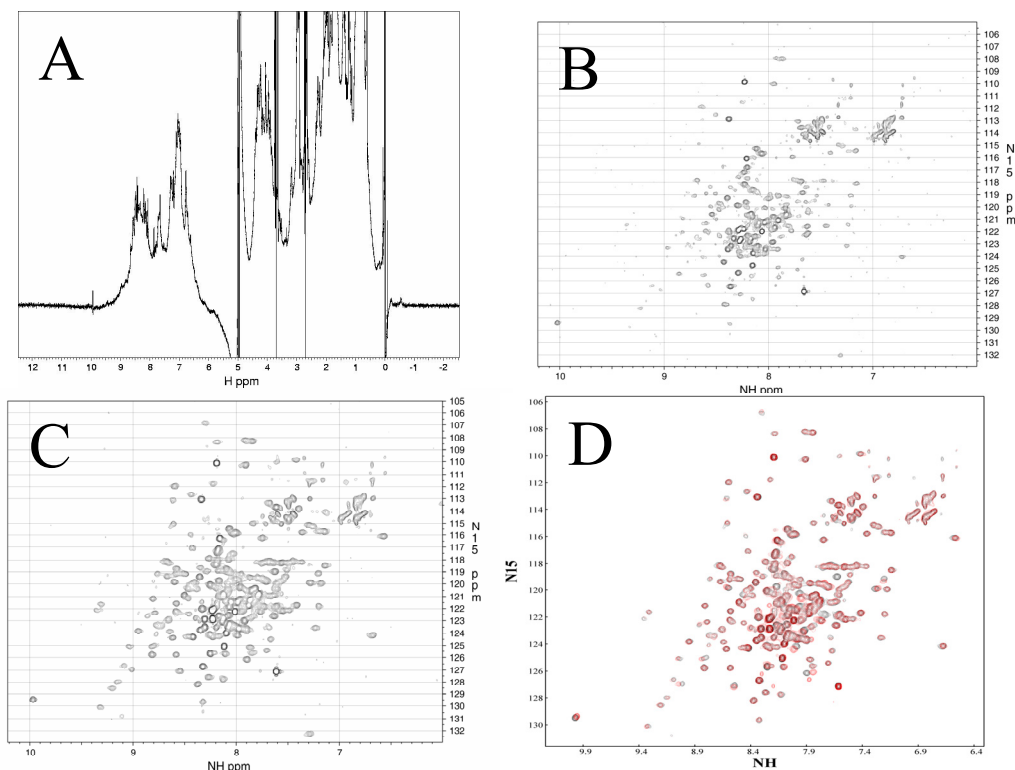


FIGURE 6-7: NMR spectra of SLC26A3 STAS 510-741. (A) A 1D NMR spectrum of unlabeled STAS showing methyl peaks at -1 to 0 ppm indicative of folded protein along with sharp peaks in the 6-9 ppm range. (B) A TROSY-HSQC spectrum of ^{15}N -labeled STAS in the presence of 400 mM Arginine. (C) A TROSY-HSQC spectrum of 90 μM ^{15}N -labeled STAS in 50 mM K_2PO_4 pH6.8, 400 mM KCl, 100 mM Arg, 2% glucose and 2 mM DTT. (D) TROSY-HSQC spectrum of ^{15}N labeled 100 μM wild type (black peaks) and $\Delta\text{Y526/7}$ (red peaks) SLC26A3 STAS at 30°C for 15 hr in the same buffer conditions as in (C).

With a reasonable looking HSQC spectrum of the *Aquifex* STAS domain 450-588 I screened for crystallization conditions again using the Hampton screens I and II, SaltRX, Low Ionic Strength, PEG Ion, Light and Index screens. Unfortunately, no crystallization conditions were found even though approximately 1/3 of the drops remained clear after two weeks.

While screening the various STAS domain constructs for protein crystallization conditions, Dr. Julie Forman-Kay's laboratory was trying to optimize the buffer conditions for the human SLC26A3 STAS domain for HSQC analysis. They found buffer conditions which allowed for HSQC spectra to be collected on the SLC26A3 STAS domain revealing 190 backbone proton-nitrogen cross peaks out of a possible 223 (Figure 6-7).

Discussion:

The SpoIIAA proteins have been suggested to be structural models for the SLC26A STAS domains based upon sequence homology (Aravind and Koonin, 2000) (see Figure 2-18). A number of interesting observations were made from this alignment, many of which have previously been described in the discussion section of chapter 4. An observation that was made from the multiple sequence alignment not previously discussed is that the highly conserved SLC26A STAS N and C-terminal sequences GΦXIΦXXXSPIYFAN and FΦS/TVXD/EAΦ (where X is any amino acid and Φ is a hydrophobic amino acid) are not well conserved in the SpoIIAA proteins (see Figure 2-18). The high degree of conservation of these sequences suggests that they are important for the function of the STAS domains, and the absence of these sequences in the SpoIIAA proteins suggests that this function may not be conserved in the SpoIIAA proteins.

Since there were a number of structural questions regarding the SLC26A STAS domains, and to date there is no high resolution structural information for any STAS domain, I set out to crystallize the SLC26A3 STAS domain. Vapor-diffusion hanging-drops of the human SLC26A3 STAS 510-741 protein were set up using the Hampton screen I, (NH₄)₂SO₄, Light, SaltRx, and Index Screens and almost every crystal drop set up immediately precipitated. This suggested that our protein construct was not suitable for crystallization under our current experimental conditions. Two factors that influence a protein's ability to

crystallize are the domain boundaries of the protein itself and the buffer it is in. Previously, nine different protein constructs had been screened to determine the N and C-terminal boundaries, and the conclusion of those experiments was that the 510-741 construct likely encompassed the entire STAS domain based upon its biochemical and functional properties (see chapter 4 for details). As a result, the buffer conditions of our SLC26A3 STAS domain protein were changed with the hopes that new buffer conditions would aid us in crystallizing the SLC26A3 STAS domain.

As a general rule, proteins that crystallize have to be relatively soluble, stable and typically exist as a uniform population. The SLC26A3 STAS domain 510-741 was soluble to at least 10 mg/ml, was stable for two weeks at 4°C, and was monomeric as judged by gel filtration chromatography and analytical ultracentrifugation (see chapter 4 for details). In spite of this, new buffer conditions for the STAS domain were screened for using dynamic light scattering (DLS), following the procedure of Jancarik *et al.* (Jancarik et al., 2004). A number of conditions from the initial Jancarik screen remained clear after 24 hr and the protein in these buffer conditions was then screened by DLS for its estimated molecular weight and polydispersity. Interestingly, the protein buffer which the initial crystallization screens were performed in (20 mM Na₂PO₄ pH 6.2, 350 mM NaCl, 1 mM DTT) seemed to contain mostly aggregated protein in solution as measured by DLS (Figure 6-1A). As a control, DLS was also

performed on BSA which appeared monomeric in solution with an estimate molecular weight of 59.6 kDa and a polydispersity of 12.3% (Figure 6-1B). The DLS data on the SLC26A3 STAS domain is inconsistent with the gel filtration chromatography and analytical ultracentrifugation data previously collected which suggest that the STAS domain is monomeric in solution (see chapter 4 for details). However, the DLS readout also enabled the quick screening of a number of other buffer conditions to determine what effect various buffers had on the STAS domain. 100 mM CAPS buffer at pH 10.0 yielded an estimated molecular weight for the STAS domain to be 34.5 kDa which is close to the calculated molecular weight of 29.9 kDa (Figure 6-1C).

Crystal drops were set up of 10 mg/ml SLC26A3 STAS domain 510-741 in 20 mM CAPS pH 10.0, 10 mM DTT at 20°C using the Hampton Screen I, $(\text{NH}_4)_2\text{SO}_4$, Light, SaltRx, and Index Screens. 10 mM DTT was included in the protein buffer because it had previously been determined that the STAS domain readily forms disulfide bonds in the absence of reductant. In this experiment a number of crystal drops remained clear for long periods of time or never precipitated at all, suggesting that the CAPS buffer condition may be more amenable to protein crystallization than was the phosphate buffer condition. However, no crystallization conditions were identified under the CAPS buffer conditions.

Since the SLC26A3 STAS 510-741 construct did not yield protein crystals, it was decided that altering the protein construct to make it more amenable to protein crystallization was the logical next step. The *Aquifex* STAS domain was chosen for further purification and crystallization screening in favor of the *Desulfovibrio* STAS domain because the *Desulfovibrio* STAS domain appeared to have a degradation product after the Ni column purification, suggesting that this domain may not be as stable as the *Aquifex* STAS domain. N-terminal sequencing and mass spectrometry analysis of the purified *Aquifex* STAS domain indicated that the purified protein was not proteolytically cleaved during expression and purification, suggesting that the domain is well folded (Figure 6-2B).

A ^{15}N labeled sample of the *Aquifex* STAS domain 436-605 was expressed and purified so a NMR HSQC spectrum of the protein could be collected before we began screening for crystallization conditions. The *Aquifex* STAS domain HSQC spectrum indicated that roughly half of the predicted cross peaks were present in the spectrum, suggesting that there were a large number of residues which were highly mobile in our protein construct (Figure 6-3). One possible explanation for this was that the N and C-termini of the *Aquifex* STAS construct were too long. As a result six new *Aquifex* STAS domain constructs were produced with the hopes that one of these constructs would be more amenable to crystallization.

The refinement of the *Aquifex* STAS domain boundaries suggested that amino acids 450-588 was the shortest domain that was solubly expressed and cleavable by Ulp1 protease, and therefore was chosen for further purification and structural studies (Figure 6-4). When compared to the original *Aquifex* STAS domain this construct had 14 amino acids removed from the N-terminus and 16 from the C-terminus, which potentially could improve the quality of the NMR HSQC spectrum.

To test this a HSQC spectrum was collected on purified ^{15}N labeled *Aquifex* STAS 450-588 protein and indeed the spectrum showed greater chemical peak dispersion than the *Aquifex* STAS 436-605 HSQC spectrum (Figure 6-3 and 6-6A). This data suggests that the *Aquifex* STAS 450-588 protein is better behaved than the *Aquifex* 436-605 construct, and therefore may be more amenable to crystallization.

A previous report in the literature suggested that the *B. subtilis* SpoIIAA protein had GTP binding and hydrolysis properties (Najafi et al., 1996), so I decided to test the *Aquifex* STAS domain's ability to bind nucleotide. No significant chemical shift perturbations were observed in the HSQC spectra upon addition of ATP (Figure 6-6B) or the GTP non-hydrolysable analog GDP γ S (Figure 6-6C) to the *Aquifex* 450-588 protein. The overlay of the three *Aquifex* STAS 450-588 HSQC spectra show little to no change in the three spectra; this indicates that the *Aquifex* STAS 450-588 does not significantly interact with

nucleotide under these experimental conditions (Figure 6-6D). The *B. subtilis* SpoIIAA was reported to bind to GTP with a 20 μ M affinity as determined by photocrosslinking (Najafi et al., 1996). Perhaps using NMR, which is an equilibrium method, is insufficient to detect nucleotide binding, or maybe the STAS domains have lost the ability to bind nucleotide throughout the course of evolution. ATP and GTP hydrolysis measurements were not made on the *Aquifex* STAS domain, so it is not known if this domain can hydrolyze either of these nucleotides. However, the highly conserved and phosphorylatable Ser58 in the SpoIIAA proteins was also demonstrated to play a role in the GTP binding and hydrolysis reaction (Najafi et al., 1996). When Ser58 was mutated to Asp, the GTPase activity was reduced to background levels, while the S58A mutation reduced the GTPase activity about 75% (Najafi et al., 1996). The analogous residue to Ser58 in the *Aquifex* STAS domain is Ala510, so perhaps it is not surprising that the *Aquifex* STAS domain does not have any ATP or GTP binding ability if it is lacking a residue critical for this function (see Figure 2-18).

With the *Aquifex* STAS 450-588 HSQC spectrum indicating a well folded protein, screening for protein crystallization conditions was carried out using the hanging-drop vapor-diffusion method. Approximately 550 unique crystallization conditions were tested and no conditions yielded protein crystals. About this time, Dr. Julie Forman-Kay's laboratory had made some progress in collecting HSQC spectra of the SLC26A3 STAS domain. Previously, a collaboration with

Dr. Forman-Kay's laboratory had been formed to study the STAS and R-domains interaction in greater detail. One problem that they had been trying to overcome was finding conditions where the STAS domain appeared to be well folded and monomeric under the experimental conditions required for NMR.

A one dimensional proton NMR spectrum of unlabeled STAS showed upfield shifted methyl peaks between 0 and -1 H ppm, indicating a folded protein (Figure 6-7A). However, when HSQC-TROSY experiments were performed on ^{15}N labeled SLC26A3 STAS domain at high protein concentrations (200-300 μM), the resulting spectrum were of poor quality for the size and concentration of the protein and indicated that some protein aggregation may be present. As a result they screened various buffer additives and found that arginine and glucose help to improve protein solubility. TROSY-HSQC experiments performed on the SLC26A3 STAS domain at lower protein concentration and in the presence of arginine and glucose increased the number of peaks visible in their spectra (Figure 6-7B and C). Arginine has been shown in many instances to improve the biochemical behavior of proteins, which may arise due to the hydrophobic and ionic nature of the arginine side chain. The addition of glucose and arginine, as well as the reduction of the protein concentration to 100 μM likely reduced the amount of aggregation over the time course of the TROSY-HSQC experiment which significantly increased the number of visible cross peaks in the spectrum. Using these conditions and the HSQC-TROSY NMR technique approximately

190 backbone H-N cross peaks are visible out of a possible 223 (Figure 6-7C). Of these peaks, approximately 120 are reasonably dispersed, indicating that the STAS domain is well folded. Approximately 70 residues have limited chemical shift dispersion with sharp lineshapes, indicating the presence of some disordered protein segments. This disordered region of the STAS domain may potentially correspond to the IVS. The backbone cross-peaks not observed in the TROSY-HSQC spectra are likely overlapped with the 70 sharp resonances and a three dimensional NMR experiment would likely resolve these peaks. Thus, the 100 or so resonances with limited dispersion likely correspond to the STAS IVS, unstructured loops within the folded domain and any disordered segments of the N and C-termini. The NMR data taken together support the notion that the SpoIIAA proteins are a good structural model of the STAS domains.

With the newly identified buffer conditions allowing high quality TROSY-HSQC spectrum to be collected, we were able to monitor the effects that the CLD causing mutations had on the domain. Jennifer Baker in the Forman-Kay laboratory expressed and purified the $\Delta Y526/7$ SLC26A3 STAS domain and collected a TROSY-HSQC spectrum under the same conditions as the wild type STAS domain. When the wild type and $\Delta Y526/7$ STAS domain spectra are overlaid, very few changes in cross peak positions are observed (Figure 6-7D). This suggests that only very minor structural changes are occurring to the mutant

STAS domain, consistent with residues in close proximity to Y526/7 re-orienting to accommodate the deletion.

The new buffer conditions identified by Jennifer Baker in the Forman-Kay laboratory have put us in a position to once again screen for crystallization conditions for the SLC26A3 STAS domain. However, these experiments are outside the scope of this dissertation and will have to be completed in the future and described elsewhere.

Chapter Seven

Future Directions

Over the last few years, remarkable progress has been made in understanding the structure, function and regulation of CFTR and the SLC26A family of proteins. Nevertheless, additional studies and continued research in this area are needed to fully understand the functions and physiological roles these molecules play in the body. CF research has been going on for decades now, and the understanding of this disease is more advanced than any of the diseases associated with the SLC26A transporters. Nevertheless, a number of structural and mechanistic questions still remain regarding CFTR. To better understand the gating mechanism of CFTR, the proposed NBD1-NBD2 heterodimer needs to be biochemically and structurally characterized (Basso et al., 2003; Berger et al., 2005; Vergani et al., 2005; Vergani et al., 2003). To accomplish this feat, milligram quantities of soluble and pure CFTR NBD2 need to be produced. Exploring different expression conditions with different growth medias, fusion partners and construct boundaries may help facilitate the production of a protein construct that is amenable to x-ray crystallography or NMR experiments.

In addition to structural studies on CFTR NBD2, understanding the ATPase cycle of the NBDs is critical for understanding CFTR channel gating. Utilization of the Walker B glutamate to glutamine mutation may aid in observing

the proposed NBD1-NBD2 dimer in solution. In addition, NBD1 also lacks the conserved histidine which has been suggested to help coordinate residues important for ATP binding, dimerization and catalysis (Zaitseva et al., 2005a). Introducing the conserved histidine into NBD1 may also aid in observing dimers in solution, as well as furthering our understanding of the role this position plays in CFTR NBD dimerization and channel activity.

CFTR has been shown to activate the transport activity of a number of the SLC26A family members (Ko et al., 2002; Ko et al., 2004). In addition, a number of other regulatory mechanisms have been suggested for the SLC26A transporters including binding to carbonic anhydrase II and MAPK (Alvarez et al., 2005; Xu et al., 2006). A regulatory mechanism involving a kinase for SLC26A7 trafficking suggests that other SLC26A family members may also be regulated by phosphorylation. The conserved serine residue in the SpoIIAA proteins, which is phosphorylated by SpoIIAB, is conserved in most of the SLC26A family members. From the multiple sequence alignment only SLC26A1 and SLC26A4 do not have either a serine or a threonine within one residue of the phosphorylatable SpoIIAA serine. The *Arabidopsis thaliana* sulfate transporters also have a conserved threonine at this position, suggesting that this residue may also be phosphorylated in the SLC26A transporters.

To test the phosphorylation hypothesis, cells transfected with various SLC26A family members can be loaded with radioactive phosphate, and

immunoprecipitation reactions can be performed. Separating the immunoprecipitated proteins by SDS-PAGE and performing autoradiography will allow for the detection of any phosphorylated SLC26A protein. Similar experiments can be performed by stimulating cells with various kinase activators such as forskolin to help identify kinases which may be involved in a potential phosphorylation reaction. In addition, the use of mass spectrometry would be a valuable tool in identifying the sites of phosphorylation. From the sequence(s) identified by mass spectrometry, a kinase phosphorylation consensus site may be apparent which would also help identify the kinase(s) responsible for phosphorylation.

Identifying the molecular interactions between the STAS domain of the SLC26A transporters and the R-domain of CFTR is critical for our understanding of the molecular mechanisms that lead to the reciprocal activation of these protein's activities. Using NMR to identify residues in each of the domains critical for the interaction will be a powerful tool in understanding this molecular interaction and its regulation. In addition, mutating the PKA phosphorylated serine residues to alanine will help identify which phosphorylated serines play a major, or minor, role in the binding interaction between the STAS and R-domains. Inserting disease causing mutations into the STAS domain, and monitoring the impact these mutations have on the interaction between the STAS

and R-domains may lead to a better understanding of the residues involved in this interaction, as well as potentially providing a molecular mechanism(s) for disease.

Thus far SLC26A3, SLC26A4 and SLC26A6 have been demonstrated to be activated by CFTR (Ko et al., 2002). Testing the ability of CFTR to activate the other SLC26A family members would be of considerable interest to determine if the activation is specific to certain family members, or if the activation is a general phenomenon for the entire protein family. Determining if CFTR activated SLC26A1 and SLC26A7 would be interesting since they have been reported to reside in the basolateral membrane of epithelial cells, whereas CFTR is localized to the apical membrane. In addition, determining if SLC26A5 has the ability to activate CFTR's chloride channel activity would also be of interest since transport activity has not yet been described for SLC26A5. The results from these experiments would help determine if the activation of CFTR by the SLC26A transporters is a general phenomenon or not, as well as help to locate common sequences between the transporters which are activated by CFTR. These sequences could then be mutated or chimeric STAS domains created to identify residues critical for their activation. It will be particularly interesting to see if the activation sequence of the SLC26A proteins lies in the highly divergent IVS.

Searching for additional binding partners of the SLC26A transporters may also be useful in uncovering new modes of regulation. Using standard genetic approaches such as the yeast two-hybrid or split ubiquitin systems may identify

new proteins which associate with the SLC26A transporters *in vivo*. Identifying these proteins will further our understanding of the regulation of the SLC26A transporters and may reveal additional physiological processes that the SLC26A transporters are involved in.

In addition to understanding the regulatory mechanisms by which the SLC26A proteins are regulated, identifying the ion transport pathway and beginning to understand the transport cycle of the SLC26A proteins is also important. Conserved positively charged lysine and arginine residues residing in the transmembrane spans of the transporter are potential candidates for the anion binding sites. Alanine mutations of these residues can be made to monitor their effect on the transport properties of the protein. In addition to using residue conservation, mapping disease causing mutations onto the transmembrane spans of the SLC26A transporters may help identify residues that are important for anion binding as well as those critical for the conformation changes which presumably accompany anion translocation.

Implicit in the strategy outlined above is the knowledge of where the transmembrane spans are located. Sequence homology and hydrophobicity can predict the location of the transmembrane spans. However, experimental data testing these predictions should be collected. Inserting antibody epitopes such as a FLAG tag into the predicted intracellular and extracellular loops and performing immunofluorescence studies in the presence or absence of membrane

permeabilization one can determine if this sequence of protein is located inside or outside of the cell. Controlling for proper membrane targeting and function would be necessary for these insertion mutants. Nevertheless, this would provide an experimentally derived topology map of the SLC26A proteins which would aid in the mapping of residues important for anion translocation in the context of the plasma membrane.

Knowing the spatial organization of the transmembrane spans in relation to the STAS domain is also important for understanding the transport mechanism and regulation of the SLC26A transporters. With the STAS domains being important for proper transporter trafficking, it is possible that the STAS domain makes critical contacts with the transmembrane domain in the native state. Testing this hypothesis is important for our understanding of how the transporter is regulated. By expressing the STAS and transmembrane domain (TMD) as separate polypeptides, co-immunoprecipitation reactions could be performed to identify a potential STAS-TMD interaction. To increase the sensitivity of this experiment, the TMD could also be translated in an *in vitro* system where ³⁵S labeled cystine and methionine could be added to the translation reaction. Alternatively, an association between the STAS domain and the TMD could be monitored by the glycosylation state of the TMD. If a TMD construct could be found which does not traffic to the plasma membrane, and is therefore only core glycosylated, the STAS domain could be expressed in trans to see if it might

restore the trafficking defect of the TMD. To increase the local concentration of the STAS domain with the TMD, the STAS domain could be tethered to a TM span and localized to the ER membrane. A genetic approach, such the split ubiquitin assay, could also be used to try and detect an interaction between the TMD and the STAS domain.

High resolution structural information of the STAS domain and the TMD would provide tremendous insight into the function and transport mechanism of the SLC26A transporters. Crystallizing an intact SLC26A transporter would identify the topology of the transmembrane spans as well as determine if the STAS domain makes significant contacts with the intracellular loops of the TMD. The structure may also help elucidate the ion binding sites as well as a potential ion transport pathway. The structure of the STAS domain alone would also be informative as it would help to identify the fold of the domain as well as if the IVS is structured or not. In addition, the structure of the STAS domain would allow for disease causing mutations to be mapped onto a true STAS domain structure as opposed to the SpoIIAA homologs. This would allow for better predictions of the structural and functional consequences of these mutations.

A great deal of work still remains before a complete understanding of the molecular mechanisms behind anion transport of the SLC26A family of proteins is known. A number of the experiments suggested aim to further characterize the overall structure and function of these transporters. As more information is

learned about this important family of transporters, more specific questions can be asked about the ion transport and regulatory mechanisms of these transporters. With multiple laboratories working on various aspects of the SLC26A transporter's structure and function, it won't be long before many of the experiments outlined above have been completed. These results will provide a better understanding of the physiological roles the SLC26A transporters play in the human body, and how their critical functions are regulated.

Bibliography

- Abrahams, J.P., Leslie, A.G., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria. *Nature*, **370**, 621-628.
- Ahmed, N., Corey, M., Forstner, G., Zielenski, J., Tsui, L.C., Ellis, L., Tullis, E. and Durie, P. (2003) Molecular consequences of cystic fibrosis transmembrane regulator (CFTR) gene mutations in the exocrine pancreas. *Gut*, **52**, 1159-1164.
- Ahn, W., Kim, K.H., Lee, J.A., Kim, J.Y., Choi, J.Y., Moe, O.W., Milgram, S.L., Muallem, S. and Lee, M.G. (2001) Regulatory interaction between the cystic fibrosis transmembrane conductance regulator and HCO₃⁻ salvage mechanisms in model systems and the mouse pancreatic duct. *J Biol Chem*, **276**, 17236-17243.
- Aleksandrov, L., Aleksandrov, A.A., Chang, X.B. and Riordan, J.R. (2002) The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction, whereas the Second Is a Site of Rapid Turnover. *J Biol Chem*, **277**, 15419-15425.
- Alvarez, B.V., Kieller, D.M., Quon, A.L., Markovich, D. and Casey, J.R. (2004) Slc26a6: a cardiac chloride-hydroxyl exchanger and predominant chloride-bicarbonate exchanger of the mouse heart. *J Physiol*, **561**, 721-734.
- Alvarez, B.V., Vilas, G.L. and Casey, J.R. (2005) Metabolon disruption: a mechanism that regulates bicarbonate transport. *Embo J*, **24**, 2499-2511.
- Anderson, M.P., Berger, H.A., Rich, D.P., Gregory, R.J., Smith, A.E. and Welsh, M.J. (1991a) Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell*, **67**, 775-784.
- Anderson, M.P., Gregory, R.J., Thompson, S., Souza, D.W., Paul, S., Mulligan, R.C., Smith, A.E. and Welsh, M.J. (1991b) Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science*, **253**, 202-205.
- Anderson, M.P. and Welsh, M.J. (1992) Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains. *Science*, **257**, 1701-1704.
- Annereau, J.P., Ko, Y.H. and Pedersen, P.L. (2003) Cystic fibrosis transmembrane conductance regulator: the NBF1+R (nucleotide-binding fold 1 and regulatory domain) segment acting alone catalyses a Co²⁺/Mn²⁺/Mg²⁺-ATPase activity markedly inhibited by both Cd²⁺ and the transition-state analogue orthovanadate. *Biochem J*, **371**, 451-462.

- Antalis, T.M., Reeder, J.A., Gotley, D.C., Byeon, M.K., Walsh, M.D., Henderson, K.W., Papas, T.S. and Schweinfest, C.W. (1998) Down-regulation of the down-regulated in adenoma (DRA) gene correlates with colon tumor progression. *Clin Cancer Res*, **4**, 1857-1863.
- Aparicio, G., Buche, A., Mendez, C. and Salas, J.A. (1996) Characterization of the ATPase activity of the N-terminal nucleotide binding domain of an ABC transporter involved in oleandomycin secretion by *Streptomyces antibioticus*. *FEMS Microbiol Lett*, **141**, 157-162.
- Aravind, L. and Koonin, E.V. (2000) The STAS domain - a link between anion transporters and antisigma-factor antagonists. *Curr Biol*, **10**, R53-55.
- Ashmore, J.F. (1987) A fast motile response in guinea-pig outer hair cells: the cellular basis of the cochlear amplifier. *J Physiol*, **388**, 323-347.
- Bai, J.P., Navaratnam, D., Samaranayake, H. and Santos-Sacchi, J. (2006) En block C-terminal charge cluster reversals in prestin (SLC26A5): Effects on voltage-dependent electromechanical activity. *Neurosci Lett*, **404**, 270-275.
- Bakos, E., Klein, I., Welker, E., Szabo, K., Muller, M., Sarkadi, B. and Varadi, A. (1997) Characterization of the human multidrug resistance protein containing mutations in the ATP-binding cassette signature region. *Biochem J*, **323 (Pt 3)**, 777-783.
- Barone, S., Amlal, H., Xu, J., Kujala, M., Kere, J., Petrovic, S. and Soleimani, M. (2004) Differential regulation of basolateral Cl⁻/HCO₃⁻ exchangers SLC26A7 and AE1 in kidney outer medullary collecting duct. *J Am Soc Nephrol*, **15**, 2002-2011.
- Basso, C., Vergani, P., Nairn, A.C. and Gadsby, D.C. (2003) Prolonged nonhydrolytic interaction of nucleotide with CFTR's NH₂-terminal nucleotide binding domain and its role in channel gating. *J Gen Physiol*, **122**, 333-348.
- Berger, A.L., Ikuma, M. and Welsh, M.J. (2005) Normal gating of CFTR requires ATP binding to both nucleotide-binding domains and hydrolysis at the second nucleotide-binding domain. *Proc Natl Acad Sci U S A*, **102**, 455-460.
- Berger, H.A., Anderson, M.P., Gregory, R.J., Thompson, S., Howard, P.W., Maurer, R.A., Mulligan, R., Smith, A.E. and Welsh, M.J. (1991) Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J Clin Invest*, **88**, 1422-1431.
- Bhaskar, K.R., Gong, D.H., Bansil, R., Pajevic, S., Hamilton, J.A., Turner, B.S. and LaMont, J.T. (1991) Profound increase in viscosity and aggregation of pig gastric mucin at low pH. *Am J Physiol*, **261**, G827-832.

- Bianchet, M.A., Ko, Y.H., Amzel, L.M. and Pedersen, P.L. (1997) Modeling of nucleotide binding domains of ABC transporter proteins based on a F1-ATPase/recA topology: structural model of the nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator (CFTR). *J Bioenerg Biomembr*, **29**, 503-524.
- Bissig, M., Hagenbuch, B., Stieger, B., Koller, T. and Meier, P.J. (1994) Functional expression cloning of the canalicular sulfate transport system of rat hepatocytes. *J Biol Chem*, **269**, 3017-3021.
- Bliss, J.M., Garon, C.F. and Silver, R.P. (1996) Polysialic acid export in *Escherichia coli* K1: the role of KpsT, the ATP-binding component of an ABC transporter, in chain translocation. *Glycobiology*, **6**, 445-452.
- Browne, B.L., McClendon, V. and Bedwell, D.M. (1996) Mutations within the first LSGGQ motif of Ste6p cause defects in a-factor transport and mating in *Saccharomyces cerevisiae*. *J Bacteriol*, **178**, 1712-1719.
- Byeon, M.K., Frankel, A., Papas, T.S., Henderson, K.W. and Schweinfest, C.W. (1998) Human DRA functions as a sulfate transporter in Sf9 insect cells. *Protein Expr Purif*, **12**, 67-74.
- Byeon, M.K., Westerman, M.A., Maroulakou, I.G., Henderson, K.W., Suster, S., Zhang, X.K., Papas, T.S., Vesely, J., Willingham, M.C., Green, J.E. and Schweinfest, C.W. (1996) The down-regulated in adenoma (DRA) gene encodes an intestine-specific membrane glycoprotein. *Oncogene*, **12**, 387-396.
- Campbell, C., Cucci, R.A., Prasad, S., Green, G.E., Edeal, J.B., Galer, C.E., Karniski, L.P., Sheffield, V.C. and Smith, R.J. (2001) Pendred syndrome, DFNB4, and PDS/SLC26A4 identification of eight novel mutations and possible genotype-phenotype correlations. *Hum Mutat*, **17**, 403-411.
- Canani, R.B., Terrin, G., Cirillo, P., Castaldo, G., Salvatore, F., Cardillo, G., Coruzzo, A. and Troncone, R. (2004) Butyrate as an effective treatment of congenital chloride diarrhea. *Gastroenterology*, **127**, 630-634.
- Carson, M.R., Travis, S.M. and Welsh, M.J. (1995) The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *J Biol Chem*, **270**, 1711-1717.
- Chan, K.W., Csanady, L., Seto-Young, D., Nairn, A.C. and Gadsby, D.C. (2000) Severed molecules functionally define the boundaries of the cystic fibrosis transmembrane conductance regulator's NH(2)-terminal nucleotide binding domain. *J Gen Physiol*, **116**, 163-180.
- Chang, G. (2003) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *J Mol Biol*, **330**, 419-430.

- Chang, G. and Roth, C.B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science*, **293**, 1793-1800.
- Chapman, J.M., Knoepp, S.M., Byeon, M.K., Henderson, K.W. and Schweinfest, C.W. (2002) The colon anion transporter, down-regulated in adenoma, induces growth suppression that is abrogated by E1A. *Cancer Res*, **62**, 5083-5088.
- Chappe, V., Irvine, T., Liao, J., Evagelidis, A. and Hanrahan, J.W. (2005) Phosphorylation of CFTR by PKA promotes binding of the regulatory domain. *Embo J*, **24**, 2730-2740.
- Chaudhuri, T.K. and Paul, S. (2006) Protein-misfolding diseases and chaperone-based therapeutic approaches. *Febs J*, **273**, 1331-1349.
- Chen, J., Lu, G., Lin, J., Davidson, A.L. and Quijcho, F.A. (2003) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle. *Mol Cell*, **12**, 651-661.
- Cheng, S.H., Fang, S.L., Zabner, J., Marshall, J., Piraino, S., Schiavi, S.C., Jefferson, D.M., Welsh, M.J. and Smith, A.E. (1995) Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression. *Am J Physiol*, **268**, L615-624.
- Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R. and Smith, A.E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*, **63**, 827-834.
- Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J. and Smith, A.E. (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*, **66**, 1027-1036.
- Chernova, M.N., Jiang, L., Shmukler, B.E., Schweinfest, C.W., Blanco, P., Freedman, S.D., Stewart, A.K. and Alper, S.L. (2003) Acute regulation of the SLC26A3 congenital chloride diarrhoea anion exchanger (DRA) expressed in *Xenopus* oocytes. *J Physiol*, **549**, 3-19.
- Cheung, M. and Akabas, M.H. (1996) Identification of cystic fibrosis transmembrane conductance regulator channel-lining residues in and flanking the M6 membrane-spanning segment. *Biophys J*, **70**, 2688-2695.
- Cheung, M. and Akabas, M.H. (1997) Locating the anion-selectivity filter of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. *J Gen Physiol*, **109**, 289-299.
- Choi, J.Y., Lee, M.G., Ko, S. and Muallem, S. (2001a) Cl(-)-dependent HCO₃⁻ transport by cystic fibrosis transmembrane conductance regulator. *Jop*, **2**, 243-246.

- Choi, J.Y., Muallem, D., Kiselyov, K., Lee, M.G., Thomas, P.J. and Muallem, S. (2001b) Aberrant CFTR-dependent HCO₃⁻ transport in mutations associated with cystic fibrosis. *Nature*, **410**, 94-97.
- Clarkson, J., Campbell, I.D. and Yudkin, M.D. (2001) NMR studies of the interactions of SpoIIAA with its partner proteins that regulate sporulation in *Bacillus subtilis*. *J Mol Biol*, **314**, 359-364.
- Clarkson, J., Campbell, I.D. and Yudkin, M.D. (2003) Phosphorylation induces subtle structural changes in SpoIIAA, a key regulator of sporulation. *Biochem J*, **372**, 113-119.
- Coakley, R.D., Grubb, B.R., Paradiso, A.M., Gatzky, J.T., Johnson, L.G., Kreda, S.M., O'Neal, W.K. and Boucher, R.C. (2003) Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proc Natl Acad Sci U S A*, **100**, 16083-16088.
- Cotten, J.F. and Welsh, M.J. (1998) Covalent modification of the nucleotide binding domains of cystic fibrosis transmembrane conductance regulator. *J Biol Chem*, **273**, 31873-31879.
- Coyle, B., Coffey, R., Armour, J.A., Gausden, E., Hochberg, Z., Grossman, A., Britton, K., Pembrey, M., Reardon, W. and Trembath, R. (1996) Pendred syndrome (goitre and sensorineural hearing loss) maps to chromosome 7 in the region containing the nonsyndromic deafness gene DFNB4. *Nat Genet*, **12**, 421-423.
- Dallos, P. (1992) The active cochlea. *J Neurosci*, **12**, 4575-4585.
- Davidson, A.L., Laghaeian, S.S. and Mannering, D.E. (1996) The maltose transport system of *Escherichia coli* displays positive cooperativity in ATP hydrolysis. *J Biol Chem*, **271**, 4858-4863.
- Dawson, P.A. and Markovich, D. (2005) Pathogenetics of the human SLC26 transporters. *Curr Med Chem*, **12**, 385-396.
- Dean, M., Hamon, Y. and Chimini, G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res*, **42**, 1007-1017.
- Delepelaire, P. (1994) PrtD, the integral membrane ATP-binding cassette component of the *Erwinia chrysanthemi* metalloprotease secretion system, exhibits a secretion signal-regulated ATPase activity. *J Biol Chem*, **269**, 27952-27957.
- Devor, D.C., Singh, A.K., Lambert, L.C., DeLuca, A., Frizzell, R.A. and Bridges, R.J. (1999) Bicarbonate and chloride secretion in Calu-3 human airway epithelial cells. *J Gen Physiol*, **113**, 743-760.
- Diederichs, K., Diez, J., Greller, G., Muller, C., Breed, J., Schnell, C., Vonnrhein, C., Boos, W. and Welte, W. (2000) Crystal structure of MalK, the ATPase subunit of the trehalose/maltose ABC transporter of the archaeon *Thermococcus litoralis*. *Embo J*, **19**, 5951-5961.

- Dudas, P.L., Mentone, S., Greineder, C.F., Biemesderfer, D. and Aronson, P.S. (2006) Immunolocalization of anion transporter Slc26a7 in mouse kidney. *Am J Physiol Renal Physiol*, **290**, F937-945.
- Duffieux, F., Annereau, J.P., Boucher, J., Miclet, E., Pamard, O., Schneider, M., Stoven, V. and Lallemand, J.Y. (2000) Nucleotide-binding domain 1 of cystic fibrosis transmembrane conductance regulator production of a suitable protein for structural studies. *Eur J Biochem*, **267**, 5306-5312.
- Dulhanty, A.M. and Riordan, J.R. (1994) A two-domain model for the R domain of the cystic fibrosis transmembrane conductance regulator based on sequence similarities. *FEBS Lett*, **343**, 109-114.
- Duncan, L., Alper, S. and Losick, R. (1996) SpoIIAA governs the release of the cell-type specific transcription factor sigma F from its anti-sigma factor SpoIIAB. *J Mol Biol*, **260**, 147-164.
- Duncan, L. and Losick, R. (1993) SpoIIAB is an anti-sigma factor that binds to and inhibits transcription by regulatory protein sigma F from *Bacillus subtilis*. *Proc Natl Acad Sci U S A*, **90**, 2325-2329.
- Errington, J. (2003) Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol*, **1**, 117-126.
- Everett, L.A., Glaser, B., Beck, J.C., Idol, J.R., Buchs, A., Heyman, M., Adawi, F., Hazani, E., Nassir, E., Baxevanis, A.D., Sheffield, V.C. and Green, E.D. (1997) Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet*, **17**, 411-422.
- Forlino, A., Piazza, R., Tiveron, C., Della Torre, S., Tatangelo, L., Bonafè, L., Gualeni, B., Romano, A., Pecora, F., Superti-Furga, A., Cetta, G. and Rossi, A. (2005) A diastrophic dysplasia sulfate transporter (SLC26A2) mutant mouse: morphological and biochemical characterization of the resulting chondrodysplasia phenotype. *Hum Mol Genet*, **14**, 859-871.
- Forman, M.S., Trojanowski, J.Q. and Lee, V.M. (2004) Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. *Nat Med*, **10**, 1055-1063.
- Fraser, G.R. (1965) Association of Congenital Deafness with Goitre (Pendred's Syndrome) a Study of 207 Families. *Ann Hum Genet*, **28**, 201-249.
- Frelet, A. and Klein, M. (2006) Insight in eukaryotic ABC transporter function by mutation analysis. *FEBS Lett*, **580**, 1064-1084.
- Gadsby, D.C., Vergani, P. and Csanady, L. (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature*, **440**, 477-483.
- Garsin, D.A., Paskowitz, D.M., Duncan, L. and Losick, R. (1998) Evidence for common sites of contact between the antisigma factor SpoIIAB and its partners SpoIIAA and the developmental transcription factor sigmaF in *Bacillus subtilis*. *J Mol Biol*, **284**, 557-568.

- Gaudet, R. and Wiley, D.C. (2001) Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. *Embo J*, **20**, 4964-4972.
- Gottesman, M.M. and Ambudkar, S.V. (2001) Overview: ABC transporters and human disease. *J Bioenerg Biomembr*, **33**, 453-458.
- Gray, M.A., Pollard, C.E., Harris, A., Coleman, L., Greenwell, J.R. and Argent, B.E. (1990) Anion selectivity and block of the small-conductance chloride channel on pancreatic duct cells. *Am J Physiol*, **259**, C752-761.
- Greeley, T., Shumaker, H., Wang, Z., Schweinfest, C.W. and Soleimani, M. (2001) Downregulated in adenoma and putative anion transporter are regulated by CFTR in cultured pancreatic duct cells. *Am J Physiol Gastrointest Liver Physiol*, **281**, G1301-1308.
- Gregory, R.J., Cheng, S.H., Rich, D.P., Marshall, J., Paul, S., Hehir, K., Ostedgaard, L., Klinger, K.W., Welsh, M.J. and Smith, A.E. (1990) Expression and characterization of the cystic fibrosis transmembrane conductance regulator. *Nature*, **347**, 382-386.
- Gregory, R.J., Rich, D.P., Cheng, S.H., Souza, D.W., Paul, S., Manavalan, P., Anderson, M.P., Welsh, M.J. and Smith, A.E. (1991) Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Mol Cell Biol*, **11**, 3886-3893.
- Guggino, W.B. (1999) Cystic fibrosis and the salt controversy. *Cell*, **96**, 607-610.
- Guggino, W.B. (2001) Cystic fibrosis salt/fluid controversy: in the thick of it. *Nat Med*, **7**, 888-889.
- Guzman, P., Westpheling, J. and Youngman, P. (1988) Characterization of the promoter region of the *Bacillus subtilis* spoIIIE operon. *J Bacteriol*, **170**, 1598-1609.
- Haila, S., Hastbacka, J., Bohling, T., Karjalainen-Lindsberg, M.L., Kere, J. and Saarialho-Kere, U. (2001) SLC26A2 (diastrophic dysplasia sulfate transporter) is expressed in developing and mature cartilage but also in other tissues and cell types. *J Histochem Cytochem*, **49**, 973-982.
- Hastbacka, J., de la Chapelle, A., Mahtani, M.M., Clines, G., Reeve-Daly, M.P., Daly, M., Hamilton, B.A., Kusumi, K., Trivedi, B., Weaver, A. and et al. (1994) The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. *Cell*, **78**, 1073-1087.
- Hastbacka, J., Superti-Furga, A., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Lander, E.S. (1996a) Atelosteogenesis type II is caused by mutations in the diastrophic dysplasia sulfate-transporter gene (DTDST): evidence for a phenotypic series involving three chondrodysplasias. *Am J Hum Genet*, **58**, 255-262.

- Hastbacka, J., Superti-Furga, A., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Lander, E.S. (1996b) Sulfate transport in chondrodysplasia. *Ann N Y Acad Sci*, **785**, 131-136.
- Helenius, A. and Aebi, M. (2001) Intracellular functions of N-linked glycans. *Science*, **291**, 2364-2369.
- Helenius, A. and Aebi, M. (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem*, **73**, 1019-1049.
- Hemminki, A., Hoglund, P., Pukkala, E., Salovaara, R., Jarvinen, H., Norio, R. and Aaltonen, L.A. (1998) Intestinal cancer in patients with a germline mutation in the down-regulated in adenoma (DRA) gene. *Oncogene*, **16**, 681-684.
- Henriksnas, J., Phillipson, M., Storm, M., Engstrand, L., Soleimani, M. and Holm, L. (2006) Impaired mucus-bicarbonate barrier in *Helicobacter pylori*-infected mice. *Am J Physiol Gastrointest Liver Physiol*, **291**, G396-403.
- Higgins, C.F. (1992) ABC transporters: from microorganisms to man. *Annu Rev Cell Biol*, **8**, 67-113.
- Higgins, C.F., Hiles, I.D., Whalley, K. and Jamieson, D.J. (1985) Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems. *Embo J*, **4**, 1033-1039.
- Hihnala, S., Hoglund, P., Lammi, L., Kokkonen, J., Ormala, T. and Holmberg, C. (2006a) Long-term clinical outcome in patients with congenital chloride diarrhea. *J Pediatr Gastroenterol Nutr*, **42**, 369-375.
- Hihnala, S., Kujala, M., Toppari, J., Kere, J., Holmberg, C. and Hoglund, P. (2006b) Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea. *Mol Hum Reprod*.
- Ho, M.S., Carniol, K. and Losick, R. (2003) Evidence in support of a docking model for the release of the transcription factor sigma F from the antisigma factor SpoIIAB in *Bacillus subtilis*. *J Biol Chem*, **278**, 20898-20905.
- Hoglund, P., Auranen, M., Socha, J., Popinska, K., Nazer, H., Rajaram, U., Al Sanie, A., Al-Ghanim, M., Holmberg, C., de la Chapelle, A. and Kere, J. (1998) Genetic background of congenital chloride diarrhea in high-incidence populations: Finland, Poland, and Saudi Arabia and Kuwait. *Am J Hum Genet*, **63**, 760-768.
- Hoglund, P., Haila, S., Scherer, S.W., Tsui, L.C., Green, E.D., Weissenbach, J., Holmberg, C., de la Chapelle, A. and Kere, J. (1996a) Positional candidate genes for congenital chloride diarrhea suggested by high-resolution physical mapping in chromosome region 7q31. *Genome Res*, **6**, 202-210.
- Hoglund, P., Haila, S., Socha, J., Tomaszewski, L., Saarialho-Kere, U., Karjalainen-Lindsberg, M.L., Airola, K., Holmberg, C., de la Chapelle, A.

- and Kere, J. (1996b) Mutations of the Down-regulated in adenoma (DRA) gene cause congenital chloride diarrhoea. *Nat Genet*, **14**, 316-319.
- Hoglund, P., Hihnala, S., Kujala, M., Tiitinen, A., Dunkel, L. and Holmberg, C. (2006) Disruption of the SLC26A3-mediated anion transport is associated with male subfertility. *Fertil Steril*, **85**, 232-235.
- Hoglund, P., Holmberg, C., Sherman, P. and Kere, J. (2001) Distinct outcomes of chloride diarrhoea in two siblings with identical genetic background of the disease: implications for early diagnosis and treatment. *Gut*, **48**, 724-727.
- Holmberg, C. (1986) Congenital chloride diarrhoea. *Clin Gastroenterol*, **15**, 583-602.
- Hoof, T., Demmer, A., Hadam, M.R., Riordan, J.R. and Tummeler, B. (1994) Cystic fibrosis-type mutational analysis in the ATP-binding cassette transporter signature of human P-glycoprotein MDR1. *J Biol Chem*, **269**, 20575-20583.
- Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P. and Tainer, J.A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell*, **101**, 789-800.
- Horn, C., Bremer, E. and Schmitt, L. (2003) Nucleotide dependent monomer/dimer equilibrium of OpuAA, the nucleotide-binding protein of the osmotically regulated ABC transporter OpuA from *Bacillus subtilis*. *J Mol Biol*, **334**, 403-419.
- Howell, L.D., Borchardt, R. and Cohn, J.A. (2000) ATP hydrolysis by a CFTR domain: pharmacology and effects of G551D mutation. *Biochem Biophys Res Commun*, **271**, 518-525.
- Hung, L.W., Wang, I.X., Nikaido, K., Liu, P.Q., Ames, G.F. and Kim, S.H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature*, **396**, 703-707.
- Hwang, T.C., Wang, F., Yang, I.C. and Reenstra, W.W. (1997) Genistein potentiates wild-type and delta F508-CFTR channel activity. *Am J Physiol*, **273**, C988-998.
- Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature*, **346**, 362-365.
- Jacob, P., Rossmann, H., Lamprecht, G., Kretz, A., Neff, C., Lin-Wu, E., Gregor, M., Groneberg, D.A., Kere, J. and Seidler, U. (2002) Down-regulated in adenoma mediates apical Cl⁻/HCO₃⁻ exchange in rabbit, rat, and human duodenum. *Gastroenterology*, **122**, 709-724.
- Jancarik, J., Pufan, R., Hong, C., Kim, S.H. and Kim, R. (2004) Optimum solubility (OS) screening: an efficient method to optimize buffer

- conditions for homogeneity and crystallization of proteins. *Acta Crystallogr D Biol Crystallogr*, **60**, 1670-1673.
- Jiang, Z., Grichtchenko, II, Boron, W.F. and Aronson, P.S. (2002) Specificity of anion exchange mediated by mouse Slc26a6. *J Biol Chem*, **277**, 33963-33967.
- Johansen, P.G., Anderson, C.M. and Hadorn, B. (1968) Cystic fibrosis of the pancreas. A generalised disturbance of water and electrolyte movement in exocrine tissues. *Lancet*, **1**, 455-460.
- Jones, P.M. and George, A.M. (1999) Subunit interactions in ABC transporters: towards a functional architecture. *FEMS Microbiol Lett*, **179**, 187-202.
- Karniski, L.P. (2001) Mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene: correlation between sulfate transport activity and chondrodysplasia phenotype. *Hum Mol Genet*, **10**, 1485-1490.
- Karniski, L.P. (2004) Functional expression and cellular distribution of diastrophic dysplasia sulfate transporter (DTDST) gene mutations in HEK cells. *Hum Mol Genet*, **13**, 2165-2171.
- Karniski, L.P., Lotscher, M., Fucntese, M., Hilfiker, H., Biber, J. and Murer, H. (1998) Immunolocalization of sat-1 sulfate/oxalate/bicarbonate anion exchanger in the rat kidney. *Am J Physiol*, **275**, F79-87.
- Karow, M. and Georgopoulos, C. (1993) The essential *Escherichia coli* msbA gene, a multicopy suppressor of null mutations in the htrB gene, is related to the universally conserved family of ATP-dependent translocators. *Mol Microbiol*, **7**, 69-79.
- Karpowich, N., Martsinkevich, O., Millen, L., Yuan, Y.R., Dai, P.L., MacVey, K., Thomas, P.J. and Hunt, J.F. (2001) Crystal structures of the MJ1267 ATP binding cassette reveal an induced-fit effect at the ATPase active site of an ABC transporter. *Structure*, **9**, 571-586.
- Kartner, N., Hanrahan, J.W., Jensen, T.J., Naismith, A.L., Sun, S.Z., Ackerley, C.A., Reyes, E.F., Tsui, L.C., Rommens, J.M., Bear, C.E. and et al. (1991) Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. *Cell*, **64**, 681-691.
- Katoh, F., Tresguerres, M., Lee, K.M., Kaneko, T., Aida, K. and Goss, G.G. (2006) Cloning of rainbow trout SLC26A1: involvement in renal sulfate secretion. *Am J Physiol Regul Integr Comp Physiol*, **290**, R1468-1478.
- Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science*, **245**, 1073-1080.
- Kidd, J.F., Ramjeesingh, M., Stratford, F., Huan, L.J. and Bear, C.E. (2004) A heteromeric complex of the two nucleotide binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) mediates ATPase activity. *J Biol Chem*, **279**, 41664-41669.

- Kim, K.H., Shcheynikov, N., Wang, Y. and Muallem, S. (2005) SLC26A7 is a Cl⁻-channel regulated by intracellular pH. *J Biol Chem*, **280**, 6463-6470.
- Knauf, F., Yang, C.L., Thomson, R.B., Mentone, S.A., Giebisch, G. and Aronson, P.S. (2001) Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells. *Proc Natl Acad Sci U S A*, **98**, 9425-9430.
- Ko, S.B., Shcheynikov, N., Choi, J.Y., Luo, X., Ishibashi, K., Thomas, P.J., Kim, J.Y., Kim, K.H., Lee, M.G., Naruse, S. and Muallem, S. (2002) A molecular mechanism for aberrant CFTR-dependent HCO₃⁻ transport in cystic fibrosis. *Embo J*, **21**, 5662-5672.
- Ko, S.B., Zeng, W., Dorwart, M.R., Luo, X., Kim, K.H., Millen, L., Goto, H., Naruse, S., Soyombo, A., Thomas, P.J. and Muallem, S. (2004) Gating of CFTR by the STAS domain of SLC26 transporters. *Nat Cell Biol*, **6**, 343-350.
- Ko, Y.H., Delannoy, M. and Pedersen, P.L. (1997) Cystic fibrosis transmembrane conductance regulator: the first nucleotide binding fold targets the membrane with retention of its ATP binding function. *Biochemistry*, **36**, 5053-5064.
- Ko, Y.H. and Pedersen, P.L. (1995) The first nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator can function as an active ATPase. *J Biol Chem*, **270**, 22093-22096.
- Ko, Y.H., Thomas, P.J., Delannoy, M.R. and Pedersen, P.L. (1993) The cystic fibrosis transmembrane conductance regulator. Overexpression, purification, and characterization of wild type and delta F508 mutant forms of the first nucleotide binding fold in fusion with the maltose-binding protein. *J Biol Chem*, **268**, 24330-24338.
- Kopelman, H., Corey, M., Gaskin, K., Durie, P., Weizman, Z. and Forstner, G. (1988) Impaired chloride secretion, as well as bicarbonate secretion, underlies the fluid secretory defect in the cystic fibrosis pancreas. *Gastroenterology*, **95**, 349-355.
- Koronakis, E., Hughes, C., Milisav, I. and Koronakis, V. (1995) Protein exporter function and in vitro ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol Microbiol*, **16**, 87-96.
- Kovacs, H., Comfort, D., Lord, M., Campbell, I.D. and Yudkin, M.D. (1998) Solution structure of SpoIIAA, a phosphorylatable component of the system that regulates transcription factor sigmaF of *Bacillus subtilis*. *Proc Natl Acad Sci U S A*, **95**, 5067-5071.
- Kovacs, H., Comfort, D., Lord, M., Yudkin, M., Campbell, I.D. and Nilges, M. (2001) NMR studies of the sporulation protein SpoIIAA: implications for the regulation of the transcription factor sigmaF in *Bacillus subtilis*. *J Biomol NMR*, **19**, 293-304.

- Kujala, M., Tienari, J., Lohi, H., Elomaa, O., Sariola, H., Lehtonen, E. and Kere, J. (2005) SLC26A6 and SLC26A7 anion exchangers have a distinct distribution in human kidney. *Nephron Exp Nephrol*, **101**, e50-58.
- Kulczycki, L.L., Kostuch, M. and Bellanti, J.A. (2003) A clinical perspective of cystic fibrosis and new genetic findings: relationship of CFTR mutations to genotype-phenotype manifestations. *Am J Med Genet A*, **116**, 262-267.
- Lamprecht, G., Heil, A., Baisch, S., Lin-Wu, E., Yun, C.C., Kalbacher, H., Gregor, M. and Seidler, U. (2002) The down regulated in adenoma (dra) gene product binds to the second PDZ domain of the NHE3 kinase A regulatory protein (E3KARP), potentially linking intestinal Cl-/HCO₃-exchange to Na⁺/H⁺ exchange. *Biochemistry*, **41**, 12336-12342.
- Lebowitz, J., Lewis, M.S. and Schuck, P. (2002) Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Sci*, **11**, 2067-2079.
- Lee, A., Beck, L. and Markovich, D. (2003) The mouse sulfate anion transporter gene *Sat1* (*Slc26a1*): cloning, tissue distribution, gene structure, functional characterization, and transcriptional regulation thyroid hormone. *DNA Cell Biol*, **22**, 19-31.
- Lee, C. and Yu, M.H. (2005) Protein folding and diseases. *J Biochem Mol Biol*, **38**, 275-280.
- Lee, C.S., Lucet, I. and Yudkin, M.D. (2000) Fate of the SpoIIAB*-ADP liberated after SpoIIAB phosphorylates SpoIIAA of *Bacillus subtilis*. *J Bacteriol*, **182**, 6250-6253.
- Lee, M.C., Penland, C.M., Widdicombe, J.H. and Wine, J.J. (1998) Evidence that Calu-3 human airway cells secrete bicarbonate. *Am J Physiol*, **274**, L450-453.
- Lewis, H.A., Buchanan, S.G., Burley, S.K., Connors, K., Dickey, M., Dorwart, M., Fowler, R., Gao, X., Guggino, W.B., Hendrickson, W.A., Hunt, J.F., Kearins, M.C., Lorimer, D., Maloney, P.C., Post, K.W., Rajashankar, K.R., Rutter, M.E., Sauder, J.M., Shriver, S., Thibodeau, P.H., Thomas, P.J., Zhang, M., Zhao, X. and Emtage, S. (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *Embo J*, **23**, 282-293.
- Lewis, H.A., Zhao, X., Wang, C., Sauder, J.M., Rooney, I., Noland, B.W., Lorimer, D., Kearins, M.C., Connors, K., Condon, B., Maloney, P.C., Guggino, W.B., Hunt, J.F. and Emtage, S. (2005) Impact of the deltaF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. *J Biol Chem*, **280**, 1346-1353.

- Li, X.C., Everett, L.A., Lalwani, A.K., Desmukh, D., Friedman, T.B., Green, E.D. and Wilcox, E.R. (1998) A mutation in PDS causes non-syndromic recessive deafness. *Nat Genet*, **18**, 215-217.
- Liberman, M.C., Gao, J., He, D.Z., Wu, X., Jia, S. and Zuo, J. (2002) Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. *Nature*, **419**, 300-304.
- Linsdell, P., Tabcharani, J.A., Rommens, J.M., Hou, Y.X., Chang, X.B., Tsui, L.C., Riordan, J.R. and Hanrahan, J.W. (1997) Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions. *J Gen Physiol*, **110**, 355-364.
- Liu, C.E., Liu, P.Q. and Ames, G.F. (1997) Characterization of the adenosine triphosphatase activity of the periplasmic histidine permease, a traffic ATPase (ABC transporter). *J Biol Chem*, **272**, 21883-21891.
- Liu, X.Z., Ouyang, X.M., Xia, X.J., Zheng, J., Pandya, A., Li, F., Du, L.L., Welch, K.O., Petit, C., Smith, R.J., Webb, B.T., Yan, D., Arnos, K.S., Corey, D., Dallos, P., Nance, W.E. and Chen, Z.Y. (2003) Prestin, a cochlear motor protein, is defective in non-syndromic hearing loss. *Hum Mol Genet*, **12**, 1155-1162.
- Locher, K.P., Lee, A.T. and Rees, D.C. (2002) The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science*, **296**, 1091-1098.
- Lohi, H., Kujala, M., Kerkela, E., Saarialho-Kere, U., Kestila, M. and Kere, J. (2000) Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. *Genomics*, **70**, 102-112.
- Lohi, H., Kujala, M., Makela, S., Lehtonen, E., Kestila, M., Saarialho-Kere, U., Markovich, D. and Kere, J. (2002a) Functional characterization of three novel tissue-specific anion exchangers SLC26A7, -A8, and -A9. *J Biol Chem*, **277**, 14246-14254.
- Lohi, H., Lamprecht, G., Markovich, D., Heil, A., Kujala, M., Seidler, U. and Kere, J. (2003) Isoforms of SLC26A6 mediate anion transport and have functional PDZ interaction domains. *Am J Physiol Cell Physiol*, **284**, C769-779.
- Lohi, H., Makela, S., Pulkkinen, K., Hoglund, P., Karjalainen-Lindsberg, M.L., Puolakkainen, P. and Kere, J. (2002b) Upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol*, **283**, G567-575.
- Lu, N.T. and Pedersen, P.L. (2000) Cystic fibrosis transmembrane conductance regulator: the purified NBF1+R protein interacts with the purified NBF2 domain to form a stable NBF1+R/NBF2 complex while inducing a

- conformational change transmitted to the C-terminal region. *Arch Biochem Biophys*, **375**, 7-20.
- Macias-Gomez, N.M., Megarbane, A., Leal-Ugarte, E., Rodriguez-Rojas, L.X. and Barros-Nunez, P. (2004) Diastrophic dysplasia and atelosteogenesis type II as expression of compound heterozygosis: first report of a Mexican patient and genotype-phenotype correlation. *Am J Med Genet A*, **129**, 190-192.
- Maeda, K., Miyamoto, Y., Sawai, H., Karniski, L.P., Nakashima, E., Nishimura, G. and Ikegawa, S. (2006) A compound heterozygote harboring novel and recurrent DTDST mutations with intermediate phenotype between atelosteogenesis type II and diastrophic dysplasia. *Am J Med Genet A*, **140**, 1143-1147.
- Magnin, T., Lord, M., Errington, J. and Yudkin, M.D. (1996) Establishing differential gene expression in sporulating *Bacillus subtilis*: phosphorylation of SpoIIAA (anti-anti-sigmaF) alters its conformation and prevents formation of a SpoIIAA/SpoIIAB/ADP complex. *Mol Microbiol*, **19**, 901-907.
- Magnin, T., Lord, M. and Yudkin, M.D. (1997) Contribution of partner switching and SpoIIAA cycling to regulation of sigmaF activity in sporulating *Bacillus subtilis*. *J Bacteriol*, **179**, 3922-3927.
- Makela, S., Eklund, R., Lahdetie, J., Mikkola, M., Hovatta, O. and Kere, J. (2005) Mutational analysis of the human SLC26A8 gene: exclusion as a candidate for male infertility due to primary spermatogenic failure. *Mol Hum Reprod*, **11**, 129-132.
- Makela, S., Kere, J., Holmberg, C. and Hoglund, P. (2002) SLC26A3 mutations in congenital chloride diarrhea. *Hum Mutat*, **20**, 425-438.
- Maley, F., Trimble, R.B., Tarentino, A.L. and Plummer, T.H., Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem*, **180**, 195-204.
- Mansoura, M.K., Smith, S.S., Choi, A.D., Richards, N.W., Strong, T.V., Drumm, M.L., Collins, F.S. and Dawson, D.C. (1998) Cystic fibrosis transmembrane conductance regulator (CFTR) anion binding as a probe of the pore. *Biophys J*, **74**, 1320-1332.
- Masmoudi, S., Charfedine, I., Hmani, M., Grati, M., Ghorbel, A.M., Elgaied-Boulila, A., Drira, M., Hardelin, J.P. and Ayadi, H. (2000) Pendred syndrome: phenotypic variability in two families carrying the same PDS missense mutation. *Am J Med Genet*, **90**, 38-44.
- Masuda, S., Murakami, K.S., Wang, S., Anders Olson, C., Donigian, J., Leon, F., Darst, S.A. and Campbell, E.A. (2004) Crystal structures of the ADP and ATP bound forms of the *Bacillus* anti-sigma factor SpoIIAB in complex with the anti-anti-sigma SpoIIAA. *J Mol Biol*, **340**, 941-956.

- McDonough, S., Davidson, N., Lester, H.A. and McCarty, N.A. (1994) Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron*, **13**, 623-634.
- Meier, P.J., Knickelbein, R., Moseley, R.H., Dobbins, J.W. and Boyer, J.L. (1985) Evidence for carrier-mediated chloride/bicarbonate exchange in canalicular rat liver plasma membrane vesicles. *J Clin Invest*, **75**, 1256-1263.
- Meier, P.J., Valantinas, J., Hugentobler, G. and Rahm, I. (1987) Bicarbonate sulfate exchange in canalicular rat liver plasma membrane vesicles. *Am J Physiol*, **253**, G461-468.
- Melin, P., Thoreau, V., Norez, C., Bilan, F., Kitzis, A. and Becq, F. (2004) The cystic fibrosis mutation G1349D within the signature motif LSHGH of NBD2 abolishes the activation of CFTR chloride channels by genistein. *Biochem Pharmacol*, **67**, 2187-2196.
- Moody, J.E., Millen, L., Binns, D., Hunt, J.F. and Thomas, P.J. (2002) Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. *J Biol Chem*, **277**, 21111-21114.
- Morbach, S., Tebbe, S. and Schneider, E. (1993) The ATP-binding cassette (ABC) transporter for maltose/maltodextrins of *Salmonella typhimurium*. Characterization of the ATPase activity associated with the purified MalK subunit. *J Biol Chem*, **268**, 18617-18621.
- Moseley, R.H., Hoglund, P., Wu, G.D., Silberg, D.G., Haila, S., de la Chapelle, A., Holmberg, C. and Kere, J. (1999) Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea. *Am J Physiol*, **276**, G185-192.
- Moskowitz, S.M., Gibson, R.L. and Effmann, E.L. (2005) Cystic fibrosis lung disease: genetic influences, microbial interactions, and radiological assessment. *Pediatr Radiol*, **35**, 739-757.
- Mossessova, E. and Lima, C.D. (2000) Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell*, **5**, 865-876.
- Mount, D.B. and Romero, M.F. (2004) The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch*, **447**, 710-721.
- Muallem, D. and Ashmore, J. (2006) An anion antiporter model of prestin, the outer hair cell motor protein. *Biophys J*, **90**, 4035-4045.
- Najafi, S.M., Harris, D.A. and Yudkin, M.D. (1996) The SpoIIAA protein of *Bacillus subtilis* has GTP-binding properties. *J Bacteriol*, **178**, 6632-6634.
- Najafi, S.M., Harris, D.A. and Yudkin, M.D. (1997) Properties of the phosphorylation reaction catalyzed by SpoIIAB that help to regulate sporulation of *Bacillus subtilis*. *J Bacteriol*, **179**, 5628-5631.

- Navaratnam, D., Bai, J.P., Samaranayake, H. and Santos-Sacchi, J. (2005) N-terminal-mediated homomultimerization of prestin, the outer hair cell motor protein. *Biophys J*, **89**, 3345-3352.
- Neville, D.C., Rozanas, C.R., Price, E.M., Gruis, D.B., Verkman, A.S. and Townsend, R.R. (1997) Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci*, **6**, 2436-2445.
- Nikaido, K., Liu, P.Q. and Ames, G.F. (1997) Purification and characterization of HisP, the ATP-binding subunit of a traffic ATPase (ABC transporter), the histidine permease of *Salmonella typhimurium*. Solubility, dimerization, and ATPase activity. *J Biol Chem*, **272**, 27745-27752.
- Oliver, D., He, D.Z., Klocker, N., Ludwig, J., Schulte, U., Waldegger, S., Ruppersberg, J.P., Dallos, P. and Fakler, B. (2001) Intracellular anions as the voltage sensor of prestin, the outer hair cell motor protein. *Science*, **292**, 2340-2343.
- Ostedgaard, L.S., Baldursson, O., Vermeer, D.W., Welsh, M.J. and Robertson, A.D. (2000) A functional R domain from cystic fibrosis transmembrane conductance regulator is predominantly unstructured in solution. *Proc Natl Acad Sci U S A*, **97**, 5657-5662.
- Ostedgaard, L.S., Baldursson, O. and Welsh, M.J. (2001) Regulation of the cystic fibrosis transmembrane conductance regulator Cl⁻ channel by its R domain. *J Biol Chem*, **276**, 7689-7692.
- Panagiotidis, C.H., Reyes, M., Sievertsen, A., Boos, W. and Shuman, H.A. (1993) Characterization of the structural requirements for assembly and nucleotide binding of an ATP-binding cassette transporter. The maltose transport system of *Escherichia coli*. *J Biol Chem*, **268**, 23685-23696.
- Park, M., Ko, S.B., Choi, J.Y., Muallem, G., Thomas, P.J., Pushkin, A., Lee, M.S., Kim, J.Y., Lee, M.G., Muallem, S. and Kurtz, I. (2002) The cystic fibrosis transmembrane conductance regulator interacts with and regulates the activity of the HCO₃⁻ salvage transporter human Na⁺-HCO₃⁻ cotransport isoform 3. *J Biol Chem*, **277**, 50503-50509.
- Petrovic, S., Amlal, H., Sun, X., Karet, F., Barone, S. and Soleimani, M. (2006) Vasopressin induces expression of the Cl⁻/HCO₃⁻ exchanger SLC26A7 in kidney medullary collecting ducts of Brattleboro rats. *Am J Physiol Renal Physiol*, **290**, F1194-1201.
- Petrovic, S., Barone, S., Xu, J., Conforti, L., Ma, L., Kujala, M., Kere, J. and Soleimani, M. (2004) SLC26A7: a basolateral Cl⁻/HCO₃⁻ exchanger specific to intercalated cells of the outer medullary collecting duct. *Am J Physiol Renal Physiol*, **286**, F161-169.
- Petrovic, S., Ju, X., Barone, S., Seidler, U., Alper, S.L., Lohi, H., Kere, J. and Soleimani, M. (2003a) Identification of a basolateral Cl⁻/HCO₃⁻

- exchanger specific to gastric parietal cells. *Am J Physiol Gastrointest Liver Physiol*, **284**, G1093-1103.
- Petrovic, S., Ma, L., Wang, Z. and Soleimani, M. (2003b) Identification of an apical Cl⁻/HCO₃⁻ exchanger in rat kidney proximal tubule. *Am J Physiol Cell Physiol*, **285**, C608-617.
- Petrovic, S., Wang, Z., Ma, L., Seidler, U., Forte, J.G., Shull, G.E. and Soleimani, M. (2002) Colocalization of the apical Cl⁻/HCO₃⁻ exchanger PAT1 and gastric H-K-ATPase in stomach parietal cells. *Am J Physiol Gastrointest Liver Physiol*, **283**, G1207-1216.
- Picciotto, M.R., Cohn, J.A., Bertuzzi, G., Greengard, P. and Nairn, A.C. (1992) Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem*, **267**, 12742-12752.
- Pier, G.B. (2002) CFTR mutations and host susceptibility to *Pseudomonas aeruginosa* lung infection. *Curr Opin Microbiol*, **5**, 81-86.
- Polissi, A. and Georgopoulos, C. (1996) Mutational analysis and properties of the *msbA* gene of *Escherichia coli*, coding for an essential ABC family transporter. *Mol Microbiol*, **20**, 1221-1233.
- Poulsen, J.H., Fischer, H., Illek, B. and Machen, T.E. (1994) Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A*, **91**, 5340-5344.
- Prasad, S., Kolln, K.A., Cucci, R.A., Trembath, R.C., Van Camp, G. and Smith, R.J. (2004) Pendred syndrome and DFNB4-mutation screening of SLC26A4 by denaturing high-performance liquid chromatography and the identification of eleven novel mutations. *Am J Med Genet A*, **124**, 1-9.
- Qu, B.H., Strickland, E.H. and Thomas, P.J. (1997) Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. *J Biol Chem*, **272**, 15739-15744.
- Qu, B.H. and Thomas, P.J. (1996) Alteration of the cystic fibrosis transmembrane conductance regulator folding pathway. *J Biol Chem*, **271**, 7261-7264.
- Quinton, P.M. (1983) Chloride impermeability in cystic fibrosis. *Nature*, **301**, 421-422.
- Quondamatteo, F., Krick, W., Hagos, Y., Kruger, M.H., Neubauer-Saile, K., Herken, R., Ramadori, G., Burckhardt, G. and Burckhardt, B.C. (2006) Localization of the sulfate/anion exchanger in the rat liver. *Am J Physiol Gastrointest Liver Physiol*, **290**, G1075-1081.
- Ramjeesingh, M., Kidd, J.F., Huan, L.J., Wang, Y. and Bear, C.E. (2003a) Dimeric cystic fibrosis transmembrane conductance regulator exists in the plasma membrane. *Biochem J*, **374**, 793-797.
- Ramjeesingh, M., Li, C., Kogan, I., Wang, Y., Huan, L.J. and Bear, C.E. (2001) A monomer is the minimum functional unit required for channel and ATPase

- activity of the cystic fibrosis transmembrane conductance regulator. *Biochemistry*, **40**, 10700-10706.
- Ramjeesingh, M., Ugwu, F., Li, C., Dhani, S., Huan, L.J., Wang, Y. and Bear, C.E. (2003b) Stable dimeric assembly of the second membrane-spanning domain of CFTR (cystic fibrosis transmembrane conductance regulator) reconstitutes a chloride-selective pore. *Biochem J*, **375**, 633-641.
- Regeer, R.R., Lee, A. and Markovich, D. (2003) Characterization of the human sulfate anion transporter (hsat-1) protein and gene (SAT1; SLC26A1). *DNA Cell Biol*, **22**, 107-117.
- Regeer, R.R. and Markovich, D. (2004) A dileucine motif targets the sulfate anion transporter sat-1 to the basolateral membrane in renal cell lines. *Am J Physiol Cell Physiol*, **287**, C365-372.
- Reuter, G., Janvilisri, T., Venter, H., Shahi, S., Balakrishnan, L. and van Veen, H.W. (2003) The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities. *J Biol Chem*, **278**, 35193-35198.
- Reyes, C.L. and Chang, G. (2005) Structure of the ABC transporter MsbA in complex with ADP.vanadate and lipopolysaccharide. *Science*, **308**, 1028-1031.
- Rich, D.P., Anderson, M.P., Gregory, R.J., Cheng, S.H., Paul, S., Jefferson, D.M., McCann, J.D., Klinger, K.W., Smith, A.E. and Welsh, M.J. (1990) Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature*, **347**, 358-363.
- Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L. and et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245**, 1066-1073.
- Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N. and et al. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, **245**, 1059-1065.
- Rosing, J., Harris, D.A., Kemp, A., Jr. and Slater, E.C. (1975) Nucleotide-binding properties of native and cold-treated mitochondrial ATPase. *Biochim Biophys Acta*, **376**, 13-26.
- Rossi, A. and Superti-Furga, A. (2001) Mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene (SLC26A2): 22 novel mutations, mutation review, associated skeletal phenotypes, and diagnostic relevance. *Hum Mutat*, **17**, 159-171.
- Rossmann, H., Jacob, P., Baisch, S., Hassoun, R., Meier, J., Natour, D., Yahya, K., Yun, C., Biber, J., Lackner, K.J., Fiehn, W., Gregor, M., Seidler, U.

- and Lamprecht, G. (2005) The CFTR associated protein CAP70 interacts with the apical Cl-/HCO₃- exchanger DRA in rabbit small intestinal mucosa. *Biochemistry*, **44**, 4477-4487.
- Rotman-Pikielny, P., Hirschberg, K., Maruvada, P., Suzuki, K., Royaux, I.E., Green, E.D., Kohn, L.D., Lippincott-Schwartz, J. and Yen, P.M. (2002) Retention of pendrin in the endoplasmic reticulum is a major mechanism for Pendred syndrome. *Hum Mol Genet*, **11**, 2625-2633.
- Rouached, H., Berthomieu, P., El Kassis, E., Cathala, N., Catherinot, V., Labesse, G., Davidian, J.C. and Fourcroy, P. (2005) Structural and functional analysis of the C-terminal STAS (sulfate transporter and anti-sigma antagonist) domain of the Arabidopsis thaliana sulfate transporter SULTR1.2. *J Biol Chem*, **280**, 15976-15983.
- Rowntree, R.K. and Harris, A. (2003) The phenotypic consequences of CFTR mutations. *Ann Hum Genet*, **67**, 471-485.
- Royaux, I.E., Suzuki, K., Mori, A., Katoh, R., Everett, L.A., Kohn, L.D. and Green, E.D. (2000) Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. *Endocrinology*, **141**, 839-845.
- Santos-Sacchi, J. (1991) Reversible inhibition of voltage-dependent outer hair cell motility and capacitance. *J Neurosci*, **11**, 3096-3110.
- Santos-Sacchi, J. and Huang, G. (1998) Temperature dependence of outer hair cell nonlinear capacitance. *Hear Res*, **116**, 99-106.
- Sato, R., Iida, S., Noji, A., Murasima, S. and Kondo, Y. (1995) [A study on health and caregiving status of the elderly over age 75 living in urban Tokyo--comparing 1987 with 1993]. *Seiroka Kango Daigaku Kiyo*, **21**, 1-13.
- Satoh, H., Susaki, M., Shukunami, C., Iyama, K., Negoro, T. and Hiraki, Y. (1998) Functional analysis of diastrophic dysplasia sulfate transporter. Its involvement in growth regulation of chondrocytes mediated by sulfated proteoglycans. *J Biol Chem*, **273**, 12307-12315.
- Schillers, H., Danker, T., Madeja, M. and Oberleithner, H. (2001) Plasma membrane protein clusters appear in CFTR-expressing *Xenopus laevis* oocytes after cAMP stimulation. *J Membr Biol*, **180**, 205-212.
- Schillers, H., Shahin, V., Albermann, L., Schafer, C. and Oberleithner, H. (2004) Imaging CFTR: a tail to tail dimer with a central pore. *Cell Physiol Biochem*, **14**, 1-10.
- Schmees, G., Stein, A., Hunke, S., Landmesser, H. and Schneider, E. (1999) Functional consequences of mutations in the conserved 'signature sequence' of the ATP-binding-cassette protein MalK. *Eur J Biochem*, **266**, 420-430.
- Schmitt, L., Benabdelhak, H., Blight, M.A., Holland, I.B. and Stubbs, M.T. (2003) Crystal structure of the nucleotide-binding domain of the ABC-

- transporter haemolysin B: identification of a variable region within ABC helical domains. *J Mol Biol*, **330**, 333-342.
- Schneider, E. and Hunke, S. (1998) ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol Rev*, **22**, 1-20.
- Schneider, E., Wilken, S. and Schmid, R. (1994) Nucleotide-induced conformational changes of MalK, a bacterial ATP binding cassette transporter protein. *J Biol Chem*, **269**, 20456-20461.
- Schweinfest, C.W., Henderson, K.W., Suster, S., Kondoh, N. and Papas, T.S. (1993) Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. *Proc Natl Acad Sci U S A*, **90**, 4166-4170.
- Scott, D.A. and Karniski, L.P. (2000) Human pendrin expressed in *Xenopus laevis* oocytes mediates chloride/formate exchange. *Am J Physiol Cell Physiol*, **278**, C207-211.
- Seavers, P.R., Lewis, R.J., Brannigan, J.A., Verschueren, K.H., Murshudov, G.N. and Wilkinson, A.J. (2001a) Structure of the *Bacillus* cell fate determinant SpoIIAA in phosphorylated and unphosphorylated forms. *Structure*, **9**, 605-614.
- Seavers, P.R., Lewis, R.J., Brannigan, J.A. and Wilkinson, J.A. (2001b) Crystallization and preliminary X-ray analysis of the sporulation factor SpoIIAA in its native and phosphorylated forms. *Acta Crystallogr D Biol Crystallogr*, **57**, 292-295.
- Shcheynikov, N., Kim, K.H., Kim, K.M., Dorwart, M.R., Ko, S.B., Goto, H., Naruse, S., Thomas, P.J. and Muallem, S. (2004) Dynamic control of cystic fibrosis transmembrane conductance regulator Cl(-)/HCO₃(-) selectivity by external Cl(-). *J Biol Chem*, **279**, 21857-21865.
- Shcheynikov, N., Wang, Y., Park, M., Ko, S.B., Dorwart, M., Naruse, S., Thomas, P.J. and Muallem, S. (2006) Coupling modes and stoichiometry of Cl-/HCO₃- exchange by slc26a3 and slc26a6. *J Gen Physiol*, **127**, 511-524.
- Sheffield, V.C., Kraiem, Z., Beck, J.C., Nishimura, D., Stone, E.M., Salameh, M., Sadeh, O. and Glaser, B. (1996) Pendred syndrome maps to chromosome 7q21-34 and is caused by an intrinsic defect in thyroid iodine organification. *Nat Genet*, **12**, 424-426.
- Shibagaki, N. and Grossman, A.R. (2004) Probing the function of STAS domains of the *Arabidopsis* sulfate transporters. *J Biol Chem*, **279**, 30791-30799.
- Shibagaki, N. and Grossman, A.R. (2006) The role of the stas domain in the function and biogenesis of a sulfate transporter as probed by random mutagenesis. *J Biol Chem*.

- Shumaker, H., Amlal, H., Frizzell, R., Ulrich, C.D., 2nd and Soleimani, M. (1999) CFTR drives Na^+ -nHCO₃ cotransport in pancreatic duct cells: a basis for defective HCO₃ secretion in CF. *Am J Physiol*, **276**, C16-25.
- Shyamala, V., Baichwal, V., Beall, E. and Ames, G.F. (1991) Structure-function analysis of the histidine permease and comparison with cystic fibrosis mutations. *J Biol Chem*, **266**, 18714-18719.
- Silberg, D.G., Wang, W., Moseley, R.H. and Traber, P.G. (1995) The Down regulated in Adenoma (dra) gene encodes an intestine-specific membrane sulfate transport protein. *J Biol Chem*, **270**, 11897-11902.
- Simpson, J.E., Gawenis, L.R., Walker, N.M., Boyle, K.T. and Clarke, L.L. (2005) Chloride conductance of CFTR facilitates basal Cl⁻/HCO₃⁻ exchange in the villous epithelium of intact murine duodenum. *Am J Physiol Gastrointest Liver Physiol*, **288**, G1241-1251.
- Smit, L.S., Wilkinson, D.J., Mansoura, M.K., Collins, F.S. and Dawson, D.C. (1993) Functional roles of the nucleotide-binding folds in the activation of the cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A*, **90**, 9963-9967.
- Smith, P.C., Karpowich, N., Millen, L., Moody, J.E., Rosen, J., Thomas, P.J. and Hunt, J.F. (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol Cell*, **10**, 139-149.
- Soleimani, M., Greeley, T., Petrovic, S., Wang, Z., Amlal, H., Kopp, P. and Burnham, C.E. (2001) Pendrin: an apical Cl⁻/OH⁻/HCO₃⁻ exchanger in the kidney cortex. *Am J Physiol Renal Physiol*, **280**, F356-364.
- Soleimani, M. and Ulrich, C.D., 2nd. (2000) How cystic fibrosis affects pancreatic ductal bicarbonate secretion. *Med Clin North Am*, **84**, 641-655, x.
- Song, Y., Thiagarajah, J. and Verkman, A.S. (2003) Sodium and chloride concentrations, pH, and depth of airway surface liquid in distal airways. *J Gen Physiol*, **122**, 511-519.
- Story, R.M. and Steitz, T.A. (1992) Structure of the recA protein-ADP complex. *Nature*, **355**, 374-376.
- Story, R.M., Weber, I.T. and Steitz, T.A. (1992) The structure of the E. coli recA protein monomer and polymer. *Nature*, **355**, 318-325.
- Strickland, E., Qu, B.H., Millen, L. and Thomas, P.J. (1997) The molecular chaperone Hsc70 assists the in vitro folding of the N-terminal nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem*, **272**, 25421-25424.
- Sun, F., Hug, M.J., Lewarchik, C.M., Yun, C.H., Bradbury, N.A. and Frizzell, R.A. (2000) E3KARP mediates the association of ezrin and protein kinase

- A with the cystic fibrosis transmembrane conductance regulator in airway cells. *J Biol Chem*, **275**, 29539-29546.
- Superti-Furga, A., Hastbacka, J., Rossi, A., van der Harten, J.J., Wilcox, W.R., Cohn, D.H., Rimoin, D.L., Steinmann, B., Lander, E.S. and Gitzelmann, R. (1996a) A family of chondrodysplasias caused by mutations in the diastrophic dysplasia sulfate transporter gene and associated with impaired sulfation of proteoglycans. *Ann N Y Acad Sci*, **785**, 195-201.
- Superti-Furga, A., Hastbacka, J., Wilcox, W.R., Cohn, D.H., van der Harten, H.J., Rossi, A., Blau, N., Rimoin, D.L., Steinmann, B., Lander, E.S. and Gitzelmann, R. (1996b) Achondrogenesis type IB is caused by mutations in the diastrophic dysplasia sulphate transporter gene. *Nat Genet*, **12**, 100-102.
- Superti-Furga, A., Neumann, L., Riebel, T., Eich, G., Steinmann, B., Spranger, J. and Kunze, J. (1999) Recessively inherited multiple epiphyseal dysplasia with normal stature, club foot, and double layered patella caused by a DTDST mutation. *J Med Genet*, **36**, 621-624.
- Superti-Furga, A., Rossi, A., Steinmann, B. and Gitzelmann, R. (1996c) A chondrodysplasia family produced by mutations in the diastrophic dysplasia sulfate transporter gene: genotype/phenotype correlations. *Am J Med Genet*, **63**, 144-147.
- Tarran, R., Grubb, B.R., Parsons, D., Picher, M., Hirsh, A.J., Davis, C.W. and Boucher, R.C. (2001) The CF salt controversy: in vivo observations and therapeutic approaches. *Mol Cell*, **8**, 149-158.
- Taylor, J.P., Metcalfe, R.A., Watson, P.F., Weetman, A.P. and Trembath, R.C. (2002) Mutations of the PDS gene, encoding pendrin, are associated with protein mislocalization and loss of iodide efflux: implications for thyroid dysfunction in Pendred syndrome. *J Clin Endocrinol Metab*, **87**, 1778-1784.
- Thomas, P.J., Qu, B.H. and Pedersen, P.L. (1995) Defective protein folding as a basis of human disease. *Trends Biochem Sci*, **20**, 456-459.
- Toure, A., Morin, L., Pineau, C., Becq, F., Dorseuil, O. and Gacon, G. (2001) Tat1, a novel sulfate transporter specifically expressed in human male germ cells and potentially linked to rhogtpase signaling. *J Biol Chem*, **276**, 20309-20315.
- Townsend, R.R., Lipniunas, P.H., Tulk, B.M. and Verkman, A.S. (1996) Identification of protein kinase A phosphorylation sites on NBD1 and R domains of CFTR using electrospray mass spectrometry with selective phosphate ion monitoring. *Protein Sci*, **5**, 1865-1873.
- Tuo, B., Riederer, B., Wang, Z., Colledge, W.H., Soleimani, M. and Seidler, U. (2006) Involvement of the anion exchanger SLC26A6 in prostaglandin

- E2- but not forskolin-stimulated duodenal HCO_3^- secretion. *Gastroenterology*, **130**, 349-358.
- Verdon, G., Albers, S.V., Dijkstra, B.W., Driessen, A.J. and Thunnissen, A.M. (2003a) Crystal structures of the ATPase subunit of the glucose ABC transporter from *Sulfolobus solfataricus*: nucleotide-free and nucleotide-bound conformations. *J Mol Biol*, **330**, 343-358.
- Verdon, G., Albers, S.V., van Oosterwijk, N., Dijkstra, B.W., Driessen, A.J. and Thunnissen, A.M. (2003b) Formation of the productive ATP-Mg²⁺-bound dimer of GlcV, an ABC-ATPase from *Sulfolobus solfataricus*. *J Mol Biol*, **334**, 255-267.
- Vergani, P., Lockless, S.W., Nairn, A.C. and Gadsby, D.C. (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature*, **433**, 876-880.
- Vergani, P., Nairn, A.C. and Gadsby, D.C. (2003) On the mechanism of MgATP-dependent gating of CFTR Cl⁻ channels. *J Gen Physiol*, **121**, 17-36.
- Vincourt, J.B., Jullien, D., Amalric, F. and Girard, J.P. (2003) Molecular and functional characterization of SLC26A11, a sodium-independent sulfate transporter from high endothelial venules. *Faseb J*, **17**, 890-892.
- Vincourt, J.B., Jullien, D., Kossida, S., Amalric, F. and Girard, J.P. (2002) Molecular cloning of SLC26A7, a novel member of the SLC26 sulfate/anion transporter family, from high endothelial venules and kidney. *Genomics*, **79**, 249-256.
- Waldegger, S., Moschen, I., Ramirez, A., Smith, R.J., Ayadi, H., Lang, F. and Kubisch, C. (2001) Cloning and characterization of SLC26A6, a novel member of the solute carrier 26 gene family. *Genomics*, **72**, 43-50.
- Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J*, **1**, 945-951.
- Wang, W., He, Z., O'Shaughnessy, T.J., Rux, J. and Reenstra, W.W. (2002a) Domain-domain associations in cystic fibrosis transmembrane conductance regulator. *Am J Physiol Cell Physiol*, **282**, C1170-1180.
- Wang, Z., Petrovic, S., Mann, E. and Soleimani, M. (2002b) Identification of an apical Cl⁻/HCO₃⁻ exchanger in the small intestine. *Am J Physiol Gastrointest Liver Physiol*, **282**, G573-579.
- Ward, C.L. and Kopito, R.R. (1994) Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J Biol Chem*, **269**, 25710-25718.
- Wilkinson, D.J., Mansoura, M.K., Watson, P.Y., Smit, L.S., Collins, F.S. and Dawson, D.C. (1996) CFTR: the nucleotide binding folds regulate the

- accessibility and stability of the activated state. *J Gen Physiol*, **107**, 103-119.
- Wilkinson, D.J., Strong, T.V., Mansoura, M.K., Wood, D.L., Smith, S.S., Collins, F.S. and Dawson, D.C. (1997) CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *Am J Physiol*, **273**, L127-133.
- Xie, Q., Welch, R., Mercado, A., Romero, M.F. and Mount, D.B. (2002) Molecular characterization of the murine Slc26a6 anion exchanger: functional comparison with Slc26a1. *Am J Physiol Renal Physiol*, **283**, F826-838.
- Xu, J., Henriksnas, J., Barone, S., Witte, D., Shull, G.E., Forte, J.G., Holm, L. and Soleimani, M. (2005) SLC26A9 is expressed in gastric surface epithelial cells, mediates Cl⁻/HCO₃⁻ exchange, and is inhibited by NH₄⁺. *Am J Physiol Cell Physiol*, **289**, C493-505.
- Xu, J., Worrell, R.T., Li, H.C., Barone, S.L., Petrovic, S., Amlal, H. and Soleimani, M. (2006) Chloride/bicarbonate exchanger SLC26A7 is localized in endosomes in medullary collecting duct cells and is targeted to the basolateral membrane in hypertonicity and potassium depletion. *J Am Soc Nephrol*, **17**, 956-967.
- Yike, I., Ye, J., Zhang, Y., Manavalan, P., Gerken, T.A. and Dearborn, D.G. (1996) A recombinant peptide model of the first nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator: comparison of wild-type and delta F508 mutant forms. *Protein Sci*, **5**, 89-97.
- Yuan, Y.R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P.J. and Hunt, J.F. (2001) The crystal structure of the MJ0796 ATP-binding cassette. Implications for the structural consequences of ATP hydrolysis in the active site of an ABC transporter. *J Biol Chem*, **276**, 32313-32321.
- Zabner, J., Smith, J.J., Karp, P.H., Widdicombe, J.H. and Welsh, M.J. (1998) Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. *Mol Cell*, **2**, 397-403.
- Zaitseva, J., Jenewein, S., Wiedenmann, A., Benabdelhak, H., Holland, I.B. and Schmitt, L. (2005) Functional characterization and ATP-induced dimerization of the isolated ABC-domain of the haemolysin B transporter. *Biochemistry*, **44**, 9680-9690.
- Zeng, W., Lee, M.G., Yan, M., Diaz, J., Benjamin, I., Marino, C.R., Kopito, R., Freedman, S., Cotton, C., Muallem, S. and Thomas, P. (1997) Immuno and functional characterization of CFTR in submandibular and pancreatic acinar and duct cells. *Am J Physiol*, **273**, C442-455.
- Zhang, X.M., Wang, X.T., Yue, H., Leung, S.W., Thibodeau, P.H., Thomas, P.J. and Guggino, S.E. (2003) Organic solutes rescue the functional defect in

- delta F508 cystic fibrosis transmembrane conductance regulator. *J Biol Chem*, **278**, 51232-51242.
- Zhang, Z.R., McDonough, S.I. and McCarty, N.A. (2000) Interaction between permeation and gating in a putative pore domain mutant in the cystic fibrosis transmembrane conductance regulator. *Biophys J*, **79**, 298-313.
- Zhang, Z.R., Zeltwanger, S., Smith, S.S., Dawson, D.C. and McCarty, N.A. (2002) Voltage-sensitive gating induced by a mutation in the fifth transmembrane domain of CFTR. *Am J Physiol Lung Cell Mol Physiol*, **282**, L135-145.
- Zheng, J., Du, G.G., Matsuda, K., Orem, A., Aguinaga, S., Deak, L., Navarrete, E., Madison, L.D. and Dallos, P. (2005) The C-terminus of prestin influences nonlinear capacitance and plasma membrane targeting. *J Cell Sci*, **118**, 2987-2996.
- Zheng, J., Long, K.B., Shen, W., Madison, L.D. and Dallos, P. (2001) Prestin topology: localization of protein epitopes in relation to the plasma membrane. *Neuroreport*, **12**, 1929-1935.
- Zheng, J., Shen, W., He, D.Z., Long, K.B., Madison, L.D. and Dallos, P. (2000) Prestin is the motor protein of cochlear outer hair cells. *Nature*, **405**, 149-155.
- Zhou, Z., White, K.A., Polissi, A., Georgopoulos, C. and Raetz, C.R. (1998) Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J Biol Chem*, **273**, 12466-12475.
- Zielenski, J. (2000) Genotype and phenotype in cystic fibrosis. *Respiration*, **67**,

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