Epitope-Tagging of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) for the Detection of Interacting Proteins During its Biosynthesis

by

Kathy Chen

A Thesis Submitted to the Faculty of Graduate Studies

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Table of Contents

Index of Figures

Index of Tables

List of Abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
ALLN	N-acetyl-Leu-Leu-norleucinal
BCA	bicinchoninic acid
BFA	brefeldin A
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFTR-flag	flag epitope inserted at the carboxyl terminus of CFTR
CHO	chinese hamster ovary
ΔF508	deletion of Phenylalaine codon at amino acid position 508 of CFTR
ΔF508-flag	flag epitope inserted at the carboxyl terminus of ΔF508
DTT	dithiothreitol
endoH	endoglycosidase H
ER	endoplasmic reticulum
FBS	fetal bovine serum
G418	geneticin
HBS	hepes-buffered saline
Hsc70	constitutively expressed heat shock protein 70
Hsp70	heat shock protein, molecular weight 70 kDa

Hsp90	heat shock protein, molecular weight 90 kDa
IRES	internal ribosome entry site
kb	kilobase
kDa	kilodalton
Met	methionine
mA	milliamps
mM	millimolar
NBD	nucleotide biniding domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI	pancreatic insufficient
PS	pancreatic sufficient
PVDF	polyvinylidene difluoride
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulfate
TMD	transmembrane domain
µg	microgram
αMEM	minimal eagles medium, alpha modification
V	voltage
Wt	wild type

ABSTRACT

Cystic Fibrosis (CF) represents the most common life-threatening recessive genetic trait among the caucasion population, especially those of northern European descent. The disease is caused by the functional absence of a plasma membrane chloride channel, designated as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The majority of the severe CF cases are linked to the deletion of a phenylalanine residue at position 508 (ΔF508), which interferes with the folding of the newly synthesized CFTR polypeptide. Newly synthesized ΔF508 molecules fail to traffic to the plasma membrane and are retained in the endoplasmic reticulum by quality control mechanisms that prevent unfolded or misfolded proteins from exiting the ER. Three chaperone proteins, Hsp70, calnexin and Hsp90 have been shown to interact with both wild-type and the mutant CFTR. These associations have been postulated to be responsible for the retention of mutant proteins in the ER and their ultimate degradation. However, the exact mechanism by which misfolded polypeptides are recognized and the number of players involved in this process are not known.

We are interested in investigating which proteins associate with CFTR during its biosynthesis in a manner that may determine whether or not the CFTR protein would mature into a functional plasma membrane channel. Co-immunoprecipitation procedures have been previously applied in detecting such protein-protein interactions. In these experiments, antibodies to known chaperone proteins were assessed for their ability to co-immunoprecipitate newly synthesized CFTR. While such trials successfully identified interactions with Hsp70, calnexin and Hsp90, they do not assess the issue of whether any other, perhaps unknown, proteins associate with CFTR. We wish to do the converse of

the previous experiments and immunoprecipitate CFTR and characterize the proteins found in these co-precipitates. A lack of suitable, high affinity antibodies to CFTR led us to explore the use of short polypeptide tags for affinity purification. The tags were well characterized epitopes or ligands for affinity matrices and were added to the carboxyl-terminus of the CFTR coding sequence. Our results showed that the eight amino acid 'Flag' epitope (DYKDDDK) was the best-suited tag for our purposes. Of particular importance was the finding that a rabbit antibody to this epitope could be used for specific and near quantitative immunoprecipitation of the CFTR-flag constructs. Stable cell lines have been generated that express wt CFTR-flag or ΔF508 CFTR-flag in HeLa cells. They were employed in long-term labeling experiments to identify the proteins interacting with newly synthesized CFTR. With this system, we were able to confirm the interaction of CFTR with Hsc70, Hsp90 and calnexin. In addition, a protein with a molecular weight of ~ 97 kDa also co-immunoprecipitated with CFTR. This 97 kDa protein is not Grp94 and represents a novel interaction with nascent CFTR. Further experiments will be needed to identify this protein.

1. INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive, single gene disorder with a complex phenotype. This disease is remarkably common in Caucasians, affecting about one in 2500, with one person in 25 being a heterozygote carrier (for a comprehensive review of CF see: Welsh et al., 1995). The hallmarks of the disease include chronic pulmonary obstruction, bacterial colonization in the airways, pancreatic enzyme insufficiency due to blockage of secretory ducts, elevated sweat electrolytes, and reduced fertility in males.

CF is a severe medical problem. It is the most prevalent cause of severe, progressive lung disease in children and has become a major cause of lung-related morbidity and mortality in young adults. The disease was first described in the 1930s, and is caused by a defect in chloride transport in CF secretory epithelial cells. However, this primary defect could only be confirmed through the identification of the CF gene and the cloning of its product, the Cystic Fibrosis Transmembrane Conductance Regulator, in 1989 (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). Since that time, there has been an explosion of scientific interest to explore the array of cellular processes on which CFTR touches. CFTR has became one of the most intensively studied proteins in biomedical science. Many of its structural and functional properties have been described, a large number of mutations have been detected in this gene, and striking progress has been made toward the understanding of related epithelial cell pathophysiology. The growing understanding of the role that CFTR plays in the pathophysiology of CF serves as a clinical basis for more effective treatment of the disease.

1.1 Clinical Features of Classic CF

CF involves a generalized dysfunction of the exocrine glands which presents in many different ways and mimics a number of clinical entities, predominantly affecting the pancreas, intestine, lung, sweat duct, and liver. Exocrine glands discharge their secretions through ducts into body cavities or directly to various body surfaces, whereas endocrine glands secrete into the bloodstream. In the pancreas of CF patients, thick, semi-solid secretions obstruct the duct system from an early age so that the delivery of enzymes to the duodenum is considerably reduced. Deficiency of these enzymes impairs digestion and absorption, resulting in nutritional deficiencies. The glands lining the bowel wall secrete excessive amounts of viscid mucus which contribute to the malabsorption. Back-pressure in the pancreas from obstruction of the main ducts produces cysts and fibrosis in the body of the gland in older CF patients. The endocrine portion of the pancreas (islets of Langerhans) is usually unaffected by these structural changes in the rest of the gland, but the production of insulin is eventually reduced in some older patients.

Approximately 10% of CF patients are born with an intestinal obstruction known as meconium ileus (Di Sant'Agnese and Powell, 1984). In these infants, the small bowel was blocked with a thick, dehydrated, rubbery, tarry, tenacious mucoid plug. The loss of pancreatic enzymes in utero gives rise to accumulations of undigested proteins which when mixed with intestinal mucus produced the hyperviscid meconium substance. These infants fail to pass meconium in the first day or two of life, develop abdominal distention and proceed to bilious emesis.

In the liver, similar obstruction of the bile ductules may result in cirrhosis and lead to back-pressure in the portal vein (portal hypertension). Total obstruction of the cystic duct may occur and this is associated with hypoplasia of the gall-bladder and the formation of gallstones.

The main life-limiting aspect of CF is a loss of pulmonary function as a result of recurrent respiratory infection and inflammation. The lungs are normal at birth but are prone to infection, which stimulates the secretion of profuse amounts of mucus. This causes obstruction, predisposing to further infection and secondary lung damage which in due course becomes irreversible. *Streptococcus* and *hemophilus* bacteria are the predominant lung pathogens in the first two years of life. Subsequent colonization of airways by *Pseudonmonas aeruginosa* provides the major clinical challenge (Rubio, 1986). Like the pancreas, the lungs deteriorate over months or years. Data indicates that, in addition to bacterial infections, an exaggerated inflammatory response also contributes to the deterioration of pulmonary tissue in CF. An elevated level of inflammatory mediators exists in the airways of CF infants prior to the development of bacterial infections (Khan et al., 1995).

The sweat glands are structurally normal. However, a failure to reabsorb chloride along the sweat gland duct causes a disturbance of the sweat electrolyte levels. This results in abnormally high concentrations of sodium and chloride, the measurement of which forms the basis for the diagnostic sweat test. In the salivary glands the changes are mainly functional, with abnormal concentrations of electrolytes, enzymes and other proteins, especially in the parotid glands. The testes are normal but abnormalities of the vas deferens and epididymis, probably due to obstruction, render the vast majority of

males sterile (Taussig, 1984). Underlying this pathology of multiple organs is a reduction in a regulated chloride conductance across the apical membrane of affected epithelia.

1.2 Diagnosis and Treatment of CF

CF is a disease that varies in severity. Some patients have the disease in a mild form which is difficult to recognize until much later, even well into adult life. The diagnosis of CF in children is based on the clinical features of chronic obstructive lung disease, persistant pulmonary infection, meconium ileus, pancreatic insufficiency, or a positive family history, and the analysis of sweat chloride levels. Approximately 15 percent of affected babies present immediately after birth with meconium ileus, but in the majority of cases, the diagnosis is made later in the first year. Any one of the major clinical features of CF, if accompanied by a sweat chloride concentration greater than 60 meq/liter or by the presence of CF mutations on both chromosomes is sufficient to make the diagnosis (Welsh et al., 1995). Newborn screening is possible using dried blood specimens for quantitation of immunoreactive trypsin in combination with mutation analysis.

The primary objectives of CF treatment are to control infection, promote mucus clearance, and improve nutrition. Treatment involves a comprehensive approach to provide postural drainage with chest percussion, administration of antimicrobials, a nutritional regimen including pancreatic enzyme replacement and fat-soluble vitamins, and psychosocial support.

1.3 Heterogeneity of CF

CF runs a highly variable course, ranging from death in the first days of life caused by complications of meconium ileus, or death in the first few months from severe respiratory tract problems, to essentially asymptomatic existence for 10 to 20 years and protracted survival. Although symptoms and presentations are highly variable, CF patients can be generally classified as either 'pancreatic insufficient' (PI) or 'pancreatic sufficient' (PS). Pedigree studies have shown that pancreatic sufficiency and insufficiency are highly concordant among affected siblings (Corey et al., 1989). Prior to the cloning of the CF gene, pancreatic status was found to be associated with certain haplotypes of closely linked markers, suggesting that the PI/PS phenotype is determined by specific CF mutations (Deoto et al., 1988; Kerem et al., 1989). Indeed, specific mutations, when presented in the homozygous state, were associated with PI 98% of the time, leading to the classification of CF mutations as mild (M) or severe (S). Based on this hypothesis, CF patients inheriting two severe mutations (S/S) would be PI, in contrast to those inheriting at least one copy of mild mutation (S/M or M/M) who are predicted to be PS. Support for this hypothesis was provided by the identification of a large number of mutations that were strongly associated with either PI or PS disease (Dean et al., 1990; Kerem et al., 1990). The link between genotype and pancreatic phenotype has been confirmed using direct measurements of pancreatic function (Durie et al., 1992).

With proper early diagnosis, medical care and treatment, the prognosis of CF has improved greatly, with a median survival of 28.2 years for females and 30.6 for males (Welsh et al., 1995). Individuals with CF do live into the sixth and seventh decades of

life (Sanders et al., 1980). More than 33 percent of the individuals in the 1992 United States CF patient registry were 18 years of age or older.

1.4 Gene Mapping and Cloning

CF was first described in the late 1930s as a distinct clinical entity. Since then a progressively more refined description of the CF syndrome and its complications have emerged. Two sets of observations in the early 1980s set the stage for rapid progress toward understanding the molecular defect of this disease. Knowles and coworkers described altered electrical properties of CF respiratory epithelium, associated with abnormalities of both sodium and chloride transport (Knowles et al., 1981). Soon thereafter, Quinton and colleagues demonstrated chloride impermeability in CF sweat gland ducts (Quinton et al., 1983). These observations focused attention on a pathogenetic role for electrolyte and water movement across CF epithelia and for the first time offered a plausible explanation for the previously noted water deficits in mucous secretions, and for the dysfunction of multiple organs. Subsequent work with epithelial cells and their membranes identified dysfunction of cAMP-dependent chloride channels as the primary pathophysiologic lesion. Subsequently, in 1989 the gene responsible for the manifestation of CF was finally identified (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). The CF gene was mapped to chromosome 7q 31.2 and was cloned using a positional cloning strategy by walking and jumping along the chromosome. The gene spans approximately 230 kb and consists of 27 exons, in sizes ranging from 38 to 724 bp (Zielenski et al., 1991). It encodes a 6.5-kb mRNA detectable by northern blot in a variety of tissues, especially those affected in CF: lung, pancreas,

sweat glands, liver, nasal polyps, salivary gland, and colon (Riordan et al., 1989). The encoded polypeptide was named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Riordan et al., 1989). Since the isolation of the gene, tremendous work has been done to characterize the function of CFTR.

1.5 Structure of CFTR

CFTR is an integral membrane protein with a single polypeptide chain of 1480 amino acids. From the amino acid sequence a theoretical model of the molecular structure was deduced (Figure 1). The protein has five distinct regions, the majority of which are found on the cytoplasmic side of the membrane. Four of the five domains form two symmetrical halves of the CFTR molecule, each being composed of a transmembrane region (TMD) made of a set of six membrane spanning segments and a nucleotide binding domain (NBD). Each of the NBDs contains consensus sequences for ATP-binding and hydrolysis (ie. Walker A and B sequences). Between the two 'halves' is situated a larger unique domain (R), that contains a high proportion (~30%) of polar amino acids and multiple consensus sequences for phosphorylation (Riordan et al., 1989). Two consensus N-linked glycosylation sites are found in the loop between transmembrane helices 7 and 8. Subsequent studies have revealed that these sites are indeed glycosylated.

Based on its primary sequence, CFTR resembles members of the traffic ATPases or ABC (ATP-binding cassette) superfamily of transporters (Doige and Ames, 1993; Higgins, 1992; Hyde et al., 1990). This family includes periplasmic permeases systems, STE6, involved in the secretion of A mating factor in yeast, and P-glycoprotein,

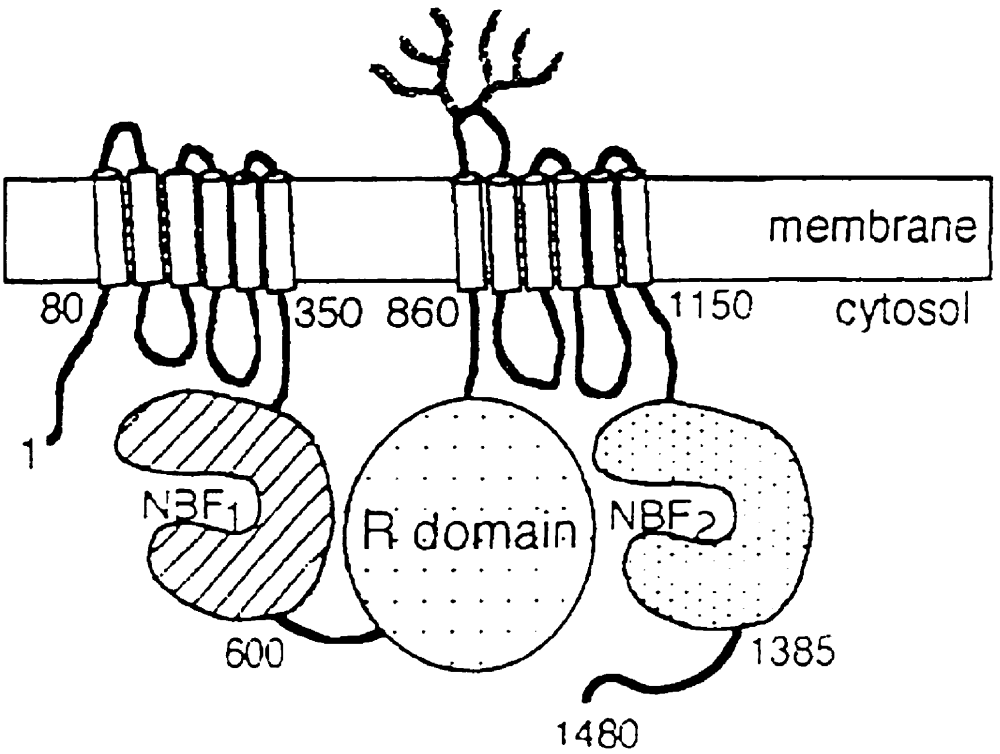


Figure 1. A schematic diagram of the CFTR. The protein is shown inserted into a lipid bilayer. The positions of selected amino acid residues are shown. Branched structures on the 4th extracellular loop represent N-linked carbohydrates.

responsible for multi-drug resistance. The R domain, with its many potential phosphorylation sites and multiple charged amino acids, is a unique feature of CFTR, not shared by other members of the family.

1.6 Function and Regulation of CFTR

Studies during the 1980s showed that the apical membrane of several epithelia have a chloride ion (Cl^-) conductance activated by cAMP agonists and that this Cl^- conductance was defective in CF patients. Years later, cloning of the CF gene and subsequent studies showed that CFTR is indeed a chloride channel (Anderson et al., 1991; Bear et al., 1992) with a linear I/V relationship and a small, single-channel conductance (6-10 pS). The channel is selective for anions over cations with an anion permeability sequence of $Br^- \geq Cl^- > I^-$ (Anderson et al., 1991). It is situated primarily in the apical membrane, where it provides a pathway for Cl^- movement across epithelia and regulates the rate of Cl^- flow. The channel activity is regulated by cAMP-dependent phosphorylation and by intracellular nucleotides. These features are conferred on CFTR by the function of its TMDs, the NBDs and the R domain. Knowledge of the contribution that these domains make to the overall function of CFTR has emerged from studies of wild type CFTR, variants containing site-directed mutations, and many agents that alter function. The TMDs contribute to the formation of the Cl^- selective pore (Cheung et al., 1996; Anderson et al., 1991; McDonough et al., 1994); the NBDs hydrolyze ATP to regulate channel gating (Anderson et al., 1991; Anderson et al., 1992; Baukrowitz et al., 1994; Carson et al., 1995); and R domain phosphorylation controls channel activity (Cheng et al., 1991; Rich et al., 1991; Winter and Welsh, 1997).

The opening and closing of the CFTR Cl^- channel is tightly controlled by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels. Activation of the cAMP-dependent protein kinase (PKA) causes the phosphorylation of multiple serine residues within the R domain. Once the R domain is phosphorylated, channel gating is regulated by a cycle of ATP hydrolysis at the NBDs. Finally, protein phosphatases dephosphorylate the R domain and return the channel to its quiescent state.

CFTR is the only ABC transporter thus far established to function as an ion channel. In addition to its role in anion translocation, the molecule has also been implicated in the regulation of epithelial amiloride-sensitive sodium channels, as the outwardly rectifying chloride channel (Jovov et al., 1995a, b).

1.7 Molecular Mechanisms of CFTR Dysfunction

To date, more than 800 gene alterations have been observed in CF patients (CF Mutation Data Base, Cystic Fibrosis Genetic Analysis Consortium, www.genet.sickkids.on.ca/cftr). Welsh and Smith (1993) have categorized CF mutations into four groups based on their influence on CFTR processing and function. Class I mutations in the CF gene are predicted to prevent synthesis of the correct full-length protein. These include nonsense mutations, splice site abnormalities, and frameshifts caused by insertion or deletion mutations. These mutations. In some cases, the mutation generates an unstable mRNA that results in no detectable protein. In other cases, a truncated protein missing part of the normal sequence or containing novel amino acids may be produced. Such proteins are often unstable and would usually either be degraded rapidly or have little or no function. Mutations found in class II affect the maturation and

transport of CFTR through the exocytic pathway. Since the N-linked oligosacchaarides on these proteins were not modified past their immature, high-mannose form, investigators concluded that they were retained in the endoplasmic reticulum (ER). This class includes the most commonly found mutant, ΔF508 with a three base pair deletion causing an in-frame loss of the phenylalanine residue in the NBD1. ΔF508 accounts for ~ 66% of all CF chromosomes identified worldwide; it is found on at least one chromosome in ~ 90% of all affected individuals (Sferra and Collins, 1993). Class III and Class IV mutants encode proteins that are fully glycosylated and that are targeted to the plasma membrane, but are defective either in channel regulation (Class III) or in ion conduction (Class IV). The Class III regulatory mutants generally exhibit single amino acid substitutions in one of the NBDs that lead to reduced channel activation by ATP. The conduction mutants (Class IV) represent a small group of rare mutations in the membrane-spanning domains. These channels exhibit reduced single channel conductance. For example, when open they conduct fewer ions per second than wild-type CFTR at the same electrochemical driving force. As expected, disease severity within these classes depends on how individual mutations affect the amount of CFTR that gets to the plasma membrane and how active it is once there.

1.8 CFTR Biosynthesis and Intracellular Transport

Intracellular targeting and processing of the CFTR protein has received much attention (for a recent review see: Riordan, 1999). The reason for this focus is the high prevalence of the ΔF508 mutant allele, and the proposal that this mutation results in local misfolding and prevents the protein from attaining its native conformation. Cells detect

such improperly folded or assembled proteins and target them for degradation through a set of processes known as biosynthetic quality control. Since CFTR processing is also the focus of this thesis, the following two sections outline the current understanding of the biogenesis, trafficking and degradation of CFTR regarding its participation in epithelial function.

1.8.1 Mislocalization of ΔF508 CFTR

Like other integral membrane glycoproteins, CFTR synthesis occurs in the endoplasmic reticulum (ER) where early events of folding and assembly take place. It is then transported by vesicular carriers through the various compartments of the Golgi appartus prior to insertion into the plasmalemma. Newly synthesized CFTR possesses two N-linked oligosaccharide chains and migrates on SDS-PAGE as a series of bands of approximately 135-140 kDa following analysis by SDS-PAGE. After transit through the Golgi apparatus, the wild type protein migrates at ~175 kDa. Pulse-chase analysis indicates that the core-glycosylated, 135-140 kDa form of CFTR is sensitive to endoglycosidase H (endoH). This immature form of CFTR, commonly referred to as form B, is a precursor to the mature 175 kDa CFTR that contains complex, endoH-resistant oligosaccharide chains (form C) (Cheng et al., 1990; Lukacs et al., 1994; Ward and Kopito, 1994 and Pind et al., 1994).

The initial suggestion that CFTR mislocalization contributes to the pathogenesis of CF was provided by Cheng et al. (1990), who observed that form C is totally absent in ΔF508 transfected cells. They also observed that wild type CFTR underwent two stages of glycosylation, a core-glycosylation (endoglycosidase H sensitive) characteristic of

processing in the ER, and more extensive glycosylation characteristic of processing in the Golgi complex. In contrast, ΔF508 CFTR only underwent core-glycosylation, this suggests that the mutant protein was retained in the ER. Pulse-chase studies have revealed that both wild type and ΔF508 CFTR were synthesized as initially indistinguishable 140 kDa immature core-glycosylated precursors. However, only wild type CFTR was chased to a 175 kDa, endoH-resistant form (Cheng et al., 1990; Lukacs et al., 1994; Ward and Kopito, 1994 and Pind et al., 1994)). These data suggest that deletion of Phe-580 interferes with a step in the maturation of immature CFTR prior to reaching the Golgi complex. In addition, immunohistochemical analysis of transiently transfected Cos cells revealed differences between wild type and ΔF508 CFTR. The immunoreactivity was restricted to a perinuclear ER-like distribution in cells expressing ΔF508 whereas the wild type CFTR signal was widespread, including the plasma membrane. Kartner et al. (1992) used monoclonal anti-CFTR antibodies to examine CFTR expression in frozen sections of human sweat glands from normal and CF skin biopsies. They observed a strong signal in the apical plasma membrane region of sweat ducts from normal donors, consistent with physiological studies of transepithelial Cl^- transport. In contrast, only weak signals were detected in duct cells from ΔF508 homozygotes. These signals were restricted to an apparent cytoplasmic and perinuclear location and were absent from the plasma membrane region. Yang et al. (1993) used electron microscopy coupled with immunoperoxidase staining to localize CFTR in cell lines transformed with recombinant adenovirus vectors, confirming a peripheral distribution for wild type CFTR, with ΔF508 CFTR being localized to the ER region.

The initial demonstration that the ΔF508 mutation may alter the folding of CFTR came from studies of synthetic polypeptide models of NBD1. A sixty-seven amino acid fragment of NBD1 (P67) containing the Walker A consensus and a region of homology around F508 was shown to specifically bind adenine nucleotides (Thomas et al., 1991). Deletion of the F508 residue (P66) had no significant effect on the ability of the polypeptide to bind nucleotide, indicating that this portion of the ΔF508 CFTR was not critical for functioning. *In vitro*, equilibrium thermodynamic studies of this domain revealed that the ΔF508 mutation does not have a large effect upon the stability of the native state (Ko et al., 1990), but dramatically destabilized the 67 amino acid peptide model of a folding intermediate. Studies of the refolding of denatured, and isolated bacterial-expressed NBD1 in vitro suggest that the F508 stabilizes a folding intermediate that is prone to self-aggregation (Qu et al., 1996; Qu and Thomas, 1997). Although the yield of NBD1 refolding is reduced by deletion of Phe-508, neither the kinetics of refolding nor the stability of the refolded protein is affected by this mutation. Moreover, the refolding yield is increased both by reduced temperature and by the presence of osmolytes like glycerol (Qu et al., 1996).

To gain insight into the structural consequences of the ΔF508 mutation on intact CFTR in a native environment, Zhang et al. (1998) employed comparative limited proteolysis in conjunction with immunoblotting and immunoprecipitation with domain-specific monoclonal antibody probes. Distinct protease susceptibilities suggest that cytosolic domain conformations of wild type and ΔF508 CFTR differ, not only near Phe508, but globally. Furthermore, ΔF508 CFTR proteolytic cleavage patterns were

indistinguishable from those of the early folding intermediate of wild type CFTR, indicating that wild type and ΔF508 CFTR share the same early conformation.

Although deletion of Phe508 (ΔF508) from CFTR results in a protein that is unable to leave the ER, this mutant may nevertheless be functional. Cl^- like channel activity has been observed in insect cells (Li et al., 1993), *Xenopus oocytes* (Drumm et al., 1991) or ER membranes (Pasyk and Foskett, 1995) containing ΔF508 CFTR. In addition, reconstitution of purified ΔF508 CFTR into planar bilayers produced wild-type like Cl^- channel activity. Vast over expression of ΔF508 cDNA in mammalian cells leads to the appearance of very low levels of CFTR protein and functional CFTR Cl^- channels in the plasma membrane (Dalemans et al., 1991), again suggesting that the ΔF508 mutation does not impair function. On the basis of these observations, it has been proposed that pharmacologic maneuvers which release ΔF508 protein from the ER and allow it to be targeted to the apical plasma membrane could be beneficial therapeutically. In cell culture systems several treatments have shown some success for this approach, such as exposure to reduced temperatures (Denning et al., 1992) or application of osmolytes, including glycerol (Sato et al., 1996; Brown et al., 1996). Following up the observation that cAMP-activated Cl^- channels appeared on the surfaces of ΔF508 CFTR-expressing SF9 insect cells and *Xenopus oocytes* (Drumm et al., 1991) cultured at reduced temperature, Denning et al. (1992) showed that a small amount of ΔF508 protein can escape the ER when mammalian fibroblasts are cultured at 23^0C – 26^0C for 2 or 3 days. The maturation of ΔF508 protein at reduced temperature was evidenced both as the appearance of the fully glycosylated form of CFTR by SDS-PAGE analysis and by the appearance of PKA-activated Cl^- channels at the surfaces of ΔF508-expressing

fibroblasts. Sato et al. (1996) and Brown et al. (1996) have shown that a small amount of ΔF508 can mature, be delivered to the cell surface, and generate cAMP-activated Cl^- currents when ΔF508-expressing cells are grown in 10% glycerol. Glycerol treatment retarded the degradation of both immature ΔF508 and wild-type CFTR protein. Other osmolytes that may promote CFTR processing include deuterated water, dimethylsulfoxide, and trimethylamine N-oxide (Brown et al., 1996). Thus, both temperature and osmolyte treatments may stabilize protein structure and allow a misfolded protein to adopt a wild type conformation, thereby resulting in its release from the ER (Brown et al., 1996). These methods promote ΔF508 CFTR movement to the cell surface at levels which are sub-wild-type. Such concentrations may be sufficient since expression as low as 10% of normal has been reported to provide normal chloride currents (Johnson et al., 1992) in patients. While these maneuvers are intriguing, they are not applicable in a clinical setting. More recently, Chang and colleagues (1999) have evaluated signaling motifs expressed on nascent ΔF508 CFTR that may act as retention or retrieval sequence signals. These sequence signals include a RXR tripeptide responsible for the ER retention or retrieval of the individual subunits of the K_{ATP} channel (Zerangue et al., 1999). Chang et al. have found four such RXR tripeptide mofits. Substitution of lysines for one of the arginines in each of these four triplets simultaneously permits nacent ΔF508 CFTR to mature about one-third as efficiently as wild-type CFTR and generates functional chloride channels at the cell surface (Chang et al., 1999). Thus, interference with recognition of these signals may be helpful in the management of CF.

1.8.2 Inefficient Processing of the Core-Glycosylated CFTR

Some insight into the molecular details underlying CFTR misprocessing was provided from biosynthetic labeling studies using transfected cells expressing recombinant CFTR (Cheng et al., 1990). One of the most striking features of CFTR biosynthesis is that wild type CFTR protein is also inefficiently processed within the ER. As was initially noted by Cheng et al. (1990), the immature, core-glycosylated forms of wild type and ΔF508 CFTR turn over rapidly ($T_{1/2}$ ~ 30 min), and conversion of the wild type protein to the mature form occurs quite inefficiently. Depending on the cell type, less than 20 – 30% of newly synthesized wild-type CFTR molecules escape from the ER to become fully glycosylated within the Golgi System and localized in the apical membrane (Ward and Kopito, 1994; Lukacs et al., 1994). Instead, the majority of immature wild-type CFTR protein and virtually all ΔF508 protein are degraded with relatively rapid kinetics ($T_{1/2}$ of 15 – 40 min depending on cell type). The 175 kDa mature species is considerably more stable and turns over much more slowly. The $T_{1/2}$ of the complex-glycosylated species was estimated to be ~16 h (Lukacs et al., 1994). This inefficient processing of immature wild type CFTR occurs both for native protein in epithelial cells and for recombinant CFTR protein when heterologously expressed in nonepithelial cells (Ward and Kopito, 1994). Degradation rates and maturation efficiency were estimated in the epithelial lines T84 and Calu-3, which express CFTR endogenously. Approximately 50% of the labeled wild type CFTR failed to mature in these epithelial lines, and disappeared with a calculated $T_{1/2}$ of 30 – 40 min (Ward and Kopito, 1994). Multiple pulse-chase experiments indicated that the $T_{1/2}$ for ΔF508 CFTR was only slightly shorter than that of the ER form of the wild type protein in CHO and

BHK cells. These observations suggest that the ΔF508 mutation does not greatly alter the susceptibility of the newly synthesized protein to degradation. It is rather remarkable that the rate of degradation of ΔF508 CFTR is virtually identical to the rate of disappearance of the wild type CFTR, implying that the wild type and mutant proteins share an early conformation that is equally susceptible to proteolysis.

Protein transport between the ER and Golgi can be inhibited by incubating at reduced temperatures, and when pulse-labeled cells were chased at 16^0C, ~50% of the initially labeled core-glycosylated wild type CFTR and all of the ΔF508 CFTR were still degraded (Lukacs et al., 1994). Continued CFTR degradation at a reduced temperature, together with its insensitivity to lysosomal protease inhibitors and to lysosomotropic agents suggested that breakdown occurs in a pre-Golgi compartment. In addition, proteolysis of wild type and ΔF508 CFTR proceeded after the disruption of vesicular transport between ER and Golgi by exposure to brefeldin A (BFA). Both ΔF508 CFTR and its breakdown products are detected in the ER after cellular fractionation, but are absent from the Golgi and plasmalemmal fractions (Lukacs et al., 1994). The sum total of all these experiments point to the ER as the primary site for degradation of the immature wild type and ΔF508 CFTR.

Lukacs et al. (1994) described two distinct core-glycosylated forms that exist along the post-translational folding pathway of CFTR: a newly synthesized, protease-susceptible intermediate that is presumably incompletely folded, and a second fraction that is converted with low efficiency to the protease-resistant and transport competent form. The fraction of wild type CFTR that is properly processed must be present in a modified state and the transition from the protease-susceptible to protease-resistant form

is energy dependent, requiring ATP. When transport from the ER to the Golgi is inhibited by brefeldin A, the fraction of wild type CFTR that does not get degraded is comparable to the amount of fully mature, complex-glycosylated CFTR generated in the absence of BFA. Therefore, it is reasonable to deduce that some essential ATP-dependent event occurring in the ER is a pre-requisite for stabilization that allows CFTR to escape endogenous proteases and to exit from this compartment.

It was proposed that the two species of core-glycosylated CFTR (maturation competent and maturation retarded) may differ in their tertiary conformation and in the state of association with other components of the ER (Lukacs et al., 1994). This hypothesis was further developed by Ward and Kopito (1994) who suggested that the inefficiency of maturation of wild type CFTR reflects the kinetics of its folding, which generally occurs in the context of polypeptide chain binding proteins, or molecular chaperones (Gething and Sambrook, 1992; Helenius et al., 1992).

Defective maturation and misprocessing of ΔF508 CFTR has been proposed to be the consequence of impaired folding, which has mostly been elucidated from cell culture systems where CFTR is heterologously expressed in nonepithelial cells. A recent report by Kälin et al. (1999) offers surprising evidence that the degree to which ΔF508 CFTR processing is impaired is tissue specific. Using several antibodies, each recognizing different sites in CFTR, they have found that ΔF508 is present in the plasma membrane in airway and intestinal cells, but not in sweat gland cells. The sweat gland findings are consistent with the findings of Kartner et al. (1992) and with those in heterologous systems, but the airway findings are surprising, contradicting a previous report showing that high level expression in the bronchial submucosal glands is undetectable in ΔF508

airways (Engelhardt et al., 1992). As the recent results remain controversial, their significance remains to be determined.

1.8.3 Transmembrane Topogenic Determinants in Influencing CFTR Folding

CFTR assembly and maturation requires coordinated folding events in the cytosol, ER lumen, and lipid bilayer. The transmembrane domains of CFTR must also fold properly, involving acquisition of correct topology and association with membrane lipids, here referred to as topogenesis. Transmembrane topology of most eukaryotic polytopic proteins is established cotranslationally at the ER membrane through the action of alternating signal and stop transfer sequences. Charged residues within TM segments have been implicated in influencing intracellular trafficking, degradation, and oligomerization of polytopic proteins (Bonifacino et al., 1990). Remarkably however, CFTR contains charged residues in predicted TM segments no. 1, 2, 6, 7, 8, 9, 10 and 11 that likely facilitate interactions between TM helices and contribute to the structure of the chloride channel pore (Anderson et al., 1991; Akabas et al., 1994). Lu and colleagues (1998) demonstrated that CFTR achieves its N terminus topology through a mechanism involving both co- and posttranslational translocation events. Using a series of defined chimeric and truncated proteins expressed in a reticulocyte lysate system, Lu et al. (1998) have identified two topogenic determinants encoded within the first (TM1) and second (TM2) membrane-spanning segments of CFTR. These two TM segments of CFTR have two distinct but independent mechanisms to ensure the correct membrane folding of the amino terminal end of CFTR. Signal sequence activity of TM1, was inefficient due to

the presence of two charged residues, Glu^{92} and Lys^{95}, located within its hydrophobic core. As a result, TM1 was able to direct correct topology for less than half of nascent CFTR chains. In contrast to TM1, TM2 was able to direct CFTR amino-terminus topology through a ribosome-dependent posttranslational mechanism. Mutating charged residues Glu^{92} and Lys^{95} to alanine improved TM1 signal sequence activity as well as the ability of TM1 to independently direct CFTR amino terminus topology. Chen and Zhang (1999) followed up on this observation and examined the topogenesis of TM3 and TM4 of CFTR in a similar cell-free expression system involving *in vitro* transcription and translation using the rabbit reticulocyte lysate system. They found that the correct membrane insertion of TM3 and TM4 of CFTR was ensured by the flanking amino acid sequences and controlled by the correct membrane insertion of their preceding TM1 and TM2. Thus, the correct membrane insertion and folding of TM1 and TM2 play an essential role in the membrane insertion and folding of the subsequent TM segments of CFTR. Therefore, mutations that affect either the cytosolic, transmembrane, or lumenal domains of a polytopic membrane protein such as CFTR, can cause misfolding and thereby recruit the ER quality control system (Kopito, 1997).

1.9 ER Quality Control

Eukaryotic membrane and soluble proteins that are destined for transit through the secretory pathway are subject to a surveillance system known as 'ER quality control' that retains aberrant proteins in the ER (Bonifacino and Lippincott-Schwartz, 1991; Hammond and Helenius, 1995). ER quality control ensures that correctly folded proteins exit the ER and proceed to their final destination. Misfolded or unassembled proteins are

retained in the ER and have been shown to be transiently bound to chaperones and subsequently degraded, which often involves the ubiquitin-proteasome system (Brodsky and McCracker, 1997; Kopito, 1997). ΔF508 CFTR is an example of a membrane protein whose trafficking is monitored by the ER quality control system.

1.9.1 Protein – Assisted Protein Folding (Molecular Chaperones)

During, or immediately after translation in the endoplasmic reticulum, peptides undergo a series of modifications. Housekeeping proteins termed 'molecular chaperones' have been identified with this process. Molecular chaperones are a class of proteins that bind transiently to newly synthesized polypeptides and promote productive folding by preventing aggregation of folding intermediates. Proteins that are unable to fold correctly often exhibit prolonged association with chaperones, which results in the retention of the misfolded proteins in ER. Aside from being the key mediators of protein folding in the cell under normal growth conditions as well as under stress, molecular chaperones are involved in protein translocation and degradation. Nascent CFTR is also recognized by components of the overall quality control apparatus and is accessible to chaperones on both sides of the ER membrane. Calnexin, a Ca^{2+} binding transmembrane protein located in the ER interacts with the oligosaccharide chains found on the extracellular loop 4 (EL4) of CFTR (Pind et al., 1994 and unpublished results). On the cytoplasmic side, Hsp70 (Yang et al. 1993) binds to immature CFTR. Both of these molecular chaperones have well documented roles in the promotion of protein folding. They have been observed to transiently interact with immature CFTR. Hsp70 was recently shown to promote the *in vitro* folding of a CFTR fragment that corresponds to NBD1 (Strickland et

al., 1997). In another recent report, Hsp70 acts in conjunction with its co-chaperone, Human DnaJ2 (Hdj-2) in the promotion of CFTR folding. It appears that Hdj-2 is localized to the cytoplasmic face of the ER and directs cytosolic Hsp70 to facilitate the folding of CFTRs cytosolic sub-domains. Meachem et al. (1999) have shown that immature ER forms of wild type and ΔF508 CFTR can be isolated in complexes with Hdj-2 and Hsp70. In experiments with purified components, Hdj-2 and Hsp70 acted synergistically to suppress NBD1 aggregation, suggesting that Hdj-2 and Hsp70 facilitate early steps in CFTR assembly.

Another heat shock protein, Hsp90, was recently identified to interact with nascent CFTR (Loo et al. 1999). Hsp90 is one of the most abundant cytosolic proteins in eukaryotes, amounting to ~1% of the total soluble proteins, even in the absence of stress. Loo et al. have demonstrated that only immature CFTR interacts with Hsp90. This interaction was found to have a major impact on the fate of the nascent CFTR polypeptide as it is being processed at the ER membrane. Disruption of interaction of wild type CFTR and Hsp90 by the benzoquinone ansamycin drugs such as geldanmycin, greatly accelerated its rate of degradation and nearly completely abrogated its maturation.

1.9.2 Degradation of CFTR by Ubiquitin-Proteasome Pathway

The degradation of immature CFTR protein within the ER appears to involve in part the proteasome, a 26S cytosolic complex of multiple peptidases. Most short-lived cytosolic proteins are degraded by the 26S proteasome complex, which contains the multi-catalytic 19S proteasome as its catalytic core and requires prior covalent attachment of multiple ubiquitin chains to the substrate. Two groups have shown that the

ER degradation of immature wild-type and ΔF508 CFTR protein is slowed substantially by cell permeant inhibitors of the proteasome such as lactacystin and N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) (Jensen et al., 1995; Ward et al., 1995). Inhibition of the ubiquitin-proteasome pathway leads to a several fold increase in steady-state levels of the immature core-glycosylated form for both wild type and ΔF508 CFTR. However, the increased level of immature CFTR is not accompanied by an increase in the mature complex-glycosylated speices. Polyubiquitinated forms of CFTR accumulated coincident with the lactocystin-induced inhibition of degradation within the ER, which can be detected as a characteristic 7 kDa 'ladder'. Furthermore, the degradation of ΔF508 CFTR within the ER is inhibited when this protein is coexpressed with a dominant-negative ubiquitin mutant (K48R) in HEK cells or when wild-type protein or ΔF508 is expressed in a cell line that contains a temperature-sensitive mutant of the ubiquitin-activating enzyme E1 (Ward et al., 1995). These results provide strong evidence for an important role for ubiquitination in the degradation of CFTR within the ER

Recently, Sato et al. (1998) have employed *in vitro* translation using the rabbit reticulocyte lysate system to show that nascent CFTR polypeptides are multiubiquitinated co-translationally before released from the ribosome. In synchronized, proteasome-inhibited lysates, translation of full-length CFTR chains was completed in approximately 30 min, whereas modification of CFTR with [^{125}I] ubiquitin was evident by 20 min, indicating that ubiquitination precedes the completion of full-length polypeptide chains. Moreover, ubiquitin was also found to be transferred to nascent CFTR chains while attached to ribosomes. Together, these data establish that ubiquitination, which is widely

assumed to be a post-translational event, can occur cotranslationally. It is possible that cotranslational ubiquition could occur because of early recognition of a misfolding/ubiquitination signal by the cellular quality control apparatus (Sato et al., 1998).

No matter how nascent CFTR distinguishes itself as competent or incompetent for ER export, it is essential to identify the molecules involved in its differential recognition. Aside from the chaperone proteins mentioned above, other chaperones may also be present in the large multimolecular complexes with which nascent CFTR is associated (Pind et al., 1994). In order to understand the biochemical basis for the intracellular retention of ΔF508 CFTR, it is also significant to determine what proteins are found in these complexes and how these associations regulate the biogenesis of CFTR. Knowing what proteins are critical for the retention of mutant CFTR in the ER may provide targets to manipulate pharmacologically.

2.0 RATIONALE AND APPROACH

An understanding of the steps involved in the biosynthesis and maturation of CFTR is of critical importance. The above sections demonstrated that the maturation of CFTR is an inefficient process. Most of the newly synthesized wild type and all of the ΔF508 mutants are retained in the ER and rapidly degraded. Previous studies have demonstrated that protein-protein interactions involving molecular chaperones are likely to play a significant role in the biogenesis of CFTR and in the overall folding of CFTR. These chaperone proteins associate with CFTR during its biosynthesis in a manner that may determine whether or not it escapes ER and matures into a functional plasma membrane channel.

Previous studies documenting the interaction of calnexin with CFTR showed that the immature form of CFTR sedimented further in glycerol gradients than did the mature form, which suggested that the immature form was in a complex with other proteins, one of which is calnexin (Pind et al., 1994). With the additional data that has accumulated since this report, it is also reasonable to suspect that Hsp70, Hdj2, and Hsp90 are present in these complexes (Yang et al., 1993; Meachem et al., 1999; Loo et al., 1998). However, the exact composition of these complexes has not been analyzed.

Prior attempts to identify proteins associated with immature CFTR have been done by co-immunoprecipitation experiments in which antibodies to known proteins of interest were used to see if they would co-immunoprecipitate newly synthesized CFTR. We propose to employ a less biased approach, to precipitate CFTR directly and determine the complement of other proteins that are specifically co-precipitated. There are three antibodies available to us to immunoprecipitate CFTR, two of which were raised against

the NBDs (M_3A_7 and $L_{12}B_4$, from J. Riordan), and the other is specific to the last four amino acids of the carboxyl terminus (M1-4, Genzyme). Although these antibodies all recognize CFTR on immunoblots and are able to immunoprecipitate CFTR, they have certain limitations for our studies. These antibodies appear to have a relatively low affinity for CFTR in that they immunoprecipitate only a fraction of the CFTR protein present in cell lysates. As a result, it becomes difficult to identify the 'signal' from proteins co-precipitating specifically over the background 'noise' of non-specific precipitation. In order to obtain a greater yield of CFTR in precipitates and increase the signal from any co-precipitating proteins, we will employ a tagging approach and add short polypeptide affinity tags onto the carboxyl terminus of CFTR. The tags chosen will be epitopes for well characterized antibodies or peptide ligands for affinity matrices (eg. His6-tag). Previous studies have shown that the addition of an epitope to the carboxyl terminus of CFTR is well tolerated (Howard et al., 1995). With the use of a PCR-based technique, a number of tag sequences will be inserted at the carboxyl terminus and tested for their ability to precipitate CFTR. The modified CFTR with the most quantitative and 'clean' precipitates will be incorporated into a human cell line and stable clones will be generated for maximal protein expression. These cells will be used to precipitate CFTR and determine which other proteins are co-precipitated. The specificity of these interactions will be checked by ensuring that the other proteins are not precipitated from control lysates by cells which do not express CFTR constructs. At least three proteins (calnexin, Hsp70 and Hsp90) are expected to be present in the co-precipitates with CFTR. Hence, the objective of this research project is to develop a 'tagged' version of

CFTR that can be used to characterize the proteins that interact with this protein during its biosynthesis and maturation.

3.0 Materials and Methods

3.1 General Molecular Biology Methods

Common molecular biology experiments were performed according to established procedures found in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) and *Current Protocols in Molecular Biology* (Ausubel et al., 1994). Transformation and propagation of plasmid constructs were done in the bacterial strains XL1-blue (Strategene, La Jolla, CA) and DH5α (Life Technologies, Burlington, ON) with 20μg/ml of carbenicillin in LB medium. DNA bands from agarose gels were purified using the QiaexII Gel Extraction Kit (Qiagen Inc., Valencia, CA). DNA used for transfection into mammalian cells were isolated and purified using the QIAfilter Plasmid Maxi Kit (Qiagen). The restriction and modifying enzymes used for DNA manipulation were purchased from New England Biolabs (Beverly, MA).

3.2 Plasmid Constructions

3.2.1 Initial Cloning Procedures

To study the biosynthesis of CFTR, the cDNA sequence needs to be incorporated into a expression vector for the production of recombinant proteins in mammalian cells. We had available to us the cDNA of CFTR in the cloning vector pBluescript (pBQ 4.7 from Dr. J. Riordon). To incorporate the CFTR sequence in the expression vector pcDNA 3.1 (-) (Invitrogen, Carlsbad, CA), the CFTR coding sequence was isolated from the pBQ 4.7 vector with the restriction enzymes *SpeI* and *KpnI*. This fragment, containing the CFTR sequence from nucleotide position -72 to 4721 was inserted into

NheI and *KpnI* restriction sites in the multiple cloning region of the pcDNA 3.1(-) expression vector. This insertion was made possible since the restriction enzymes *NheI* and *SpeI* have compatible cohesive ends. The resulting plamid is approximately 10 kb, and is designated pcDNA CFTR (Figure 2).

3.2.2 Generation of the CFTR-'Tag'

Tags were inserted at the carboxyl terminus of the CFTR sequence using a PCR based technique. A list of the tags tested, their amino acid sequences and the corresponding reverse primers bearing the tag nucleotide sequences are outlined in Table 1. A detailed diagram of the strategy used in constructing these constructs is presented in Figure 3. We wanted to sequence the PCR products in their entirety to ensure that no unwanted changes were introduced into the CFTR sequence. To reduce the amount of sequencing required to a minimum, only the C-terminal regions of CFTR was amplified and subcloned. The PCR fragments were initially cloned into the *NcoI* and *KpnI* sites of pBQ 4.7. After verifying the sequence was correct, the *BstXI-KpnI* fragment was subcloned into pcDNA 3.1 CFTR. This two-step strategy was needed as there are multiple *NcoI* sites in the pcDNA 3.1 vector. The coding sequence of the CFTR in pBQ 4.7 CFTR was linearized with the restriction enzyme *SpeI* at position 683 and used as the template for PCR amplification. A forward primer complementary to position 3923-3933 of CFTR and a reverse primer containing a *KpnI* restriction site, codons for the amino acid sequence of each epitope tag, and overlapping regions to CFTR was used to amplify the carboxyl terminus of CFTR (Table 2). The PCR reactions (50µl reactions) were carried out using 10ng of pBQ 4.7 as template, 1.0 µl of *Pfu* DNA

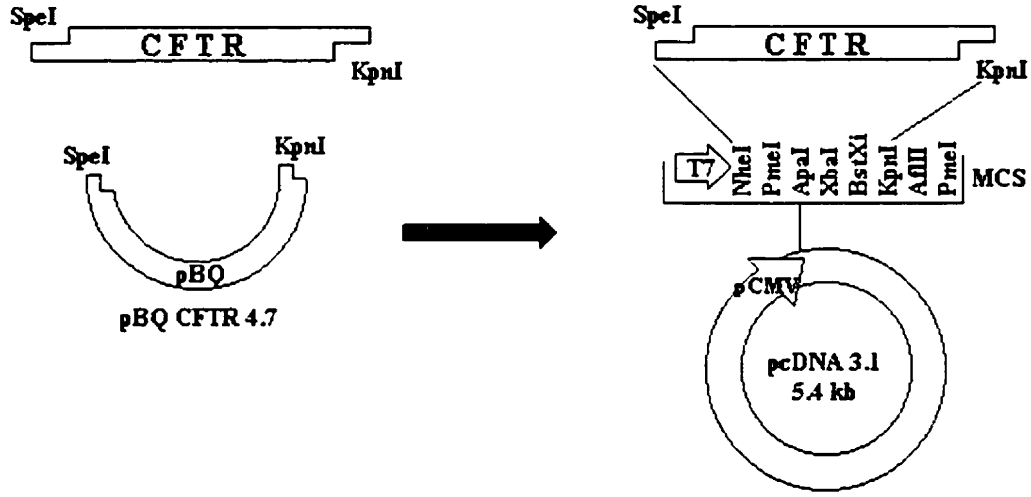


Figure 2. Initial cloning of the CFTR coding sequence from pBluescript (pBQ4.7) to the expression vector pcDNA3.1(-).

Table 1. 'Epitope' tags incorporated at the carboxyl terminus of CFTR with their corresponding amino acid sequences and the primer nucleotide sequences used in PCR

CFTR forward primer 3923-3944

Sequence (5') AAG GAG AAA TCC AGA TCG ATG G (3')

Tag	Amino Acid Sequence	CFTR reverse primer sequences
1) CFTR-Strep	AWRHPQFGG	5'-GAT GGT ACC CTA TCC TCC GAA CTG AGG GTG CCT CCA TGC AAG CCT TGT ATC TTG CAC CTC- 3'
2) CFTR-StrepII	SNWSHPQFEKAS	5'-GGA TGG TAC CCT AAG AAG CTT TTT CGA ACT GTC CGT GAG ACC AAT TAG AAA GCC TTG TAT CTT GCA CC
3) CFTR-His6	GGGHHHHHH	5'-AGA TGG TAC CCT AGT GAT GGT GAT GGT GAT GTC CAC CTC CAA GCC TTG TAT CTT GCA CC- 3'
4) CFTR-His10*	GGGHHHHHHHHHH	5'-AGA TGG TAC CCT AAT GGT GGT GAT GGT GAT GGT GAT GGT GAT GTC C- 3'
5) CFTR-Myc	EQKLISEEDL	5'-GAT GGT ACC CTA TAA ATC TTC TTC TGA AAT TAA TTT CTG TTC AAG CCT TGT ATC TTG CAC CT- 3'
6) CFTR-MycN*	EQKLISEEDLN	5'-GGA TGG TAC CCT AGT TTA AAT CTT CTT CTG AAA TTA ATT TCT GTT CAA GC- 3'
7) CFTR-Flag	DYKDDDDK	5'-GGA TGG TAC CCT ATT TGT CAT CGT CAT CTT TGT AAT CAA GCC TTG TAT CTT GCA CC- 3'

* Note: the CFTR-His10 construct was generated using pBQ4.7 CFTR-His6 as the template for PCR amplification. The CFTR-MycN construct was generated using pBQ4.7 CFTR-Myc as the template.

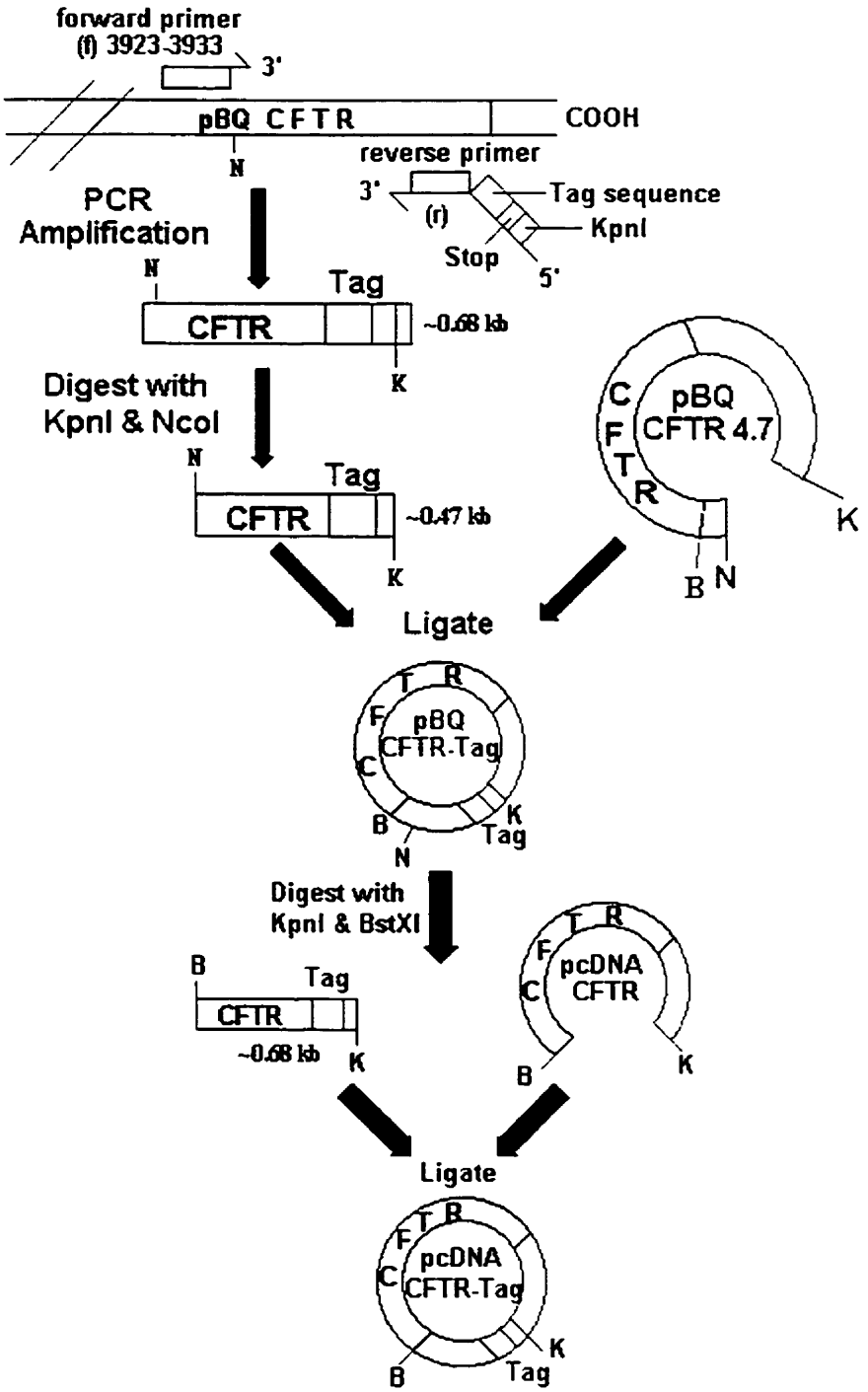


Figure 3. Constructing tags onto the carboxyl terminus of CFTR. N represents a *NcoI* site: K represents a *KpnI* site: B respresents a *BstXI* site.

polymerase (Strategene, La Jolla, CA), 75 pmol of each primer and an annealing temperature of 55 °C.

The fragments produced in these reactions were approximately 680 basepairs in length, depending upon the tag incorporated. These products were purified from agarose gels and digested with the restriction enzymes *Kpn*I and *Nco*I to give an approximately 470 bp fragment, again depending upon the sequence of the tag incorporated. This piece was then cloned back into pBQ4.7 CFTR which had been cut with the same enzymes. The resulting plasmid is pBQ CFTR-'Tag', where in each case 'Tag' is replaced by the name of the specific tag incorporated. The inserted region at the CFTR carboxyl-terminus was sequenced using the T_7 Sequencing Kit (Pharmacia, Montreal, PQ). pBQ CFTR-Tag was then digested with the *Bst*XI and *Kpn*I restriction enzymes to give an approximately 680 bp piece. The digested piece was subcloned into the pcDNA CFTR expression vector.

3.2.3 Subcloning CFTR-Flag sequences into the pIRESneo expression vector

The pIRESneo expression vector (Clontech, Palo Alto, CA) was used for the generation of CFTR-Flag stable transfectants. Unfortunately, few restriction sites were available in the multiple cloning region of this vector. The CFTR sequence was inserted into the vector in two steps and in two pieces (Figure 4). pcDNA CFTR-Flag was first digested with restriction enzymes *Sma*I and *Eco*RI, and a 2.2 kb band, the 5' half of the CFTR cDNA was isolated. The pIRESneo expression vector was cut with *Eco*RV and *Eco*RI restriction enzymes and was ligated with the 2.2 band previously isolated. A positive clone was obtained and linearlized with *Bst*XI, following which

the ends were made blunt using *Klenow* polymerase (New England Biolabs). This product was then digested with *Bsp*EI, located in the middle of the 2.2 kb fragment cloned above and a 6.2 kb band was isolated. This fragment is used as the vector for the final ligation. The insert for this ligation contained ~3/4 of the 3' end of the CFTR cDNA and was obtained by digesting pcDNA CFTR-Flag with *Bsp*EI and *Pme*I to give a 3.6 kb fragment. The 6.2 kb vector and 3.6 insert were ligated together to yield a 9.8 kb pIRES CFTR-Flag construct.

For the generation of pIRES ΔF508 CFTR-Flag, the pcDNA ΔF508 CFTR-Flag vector and the pIRESneo CFTR-Flag vector were digested with enzyme *Afl*II and the 3.6 kb ΔF508 CFTR-Flag insert was ligated to the 6.2 kb pIRES fragment using, *T4 DNA ligase* (Roche Molecular Biochemicals, Laval, PQ).

3.3 Cell Culture

CHO, BHK and HeLa cells were maintained in 5% CO_2, 95% air at 37 °C in α Minimal Eagles Medium (αMEM) supplemented with 10% fetal bovine serum (FBS) 100 units/ml penicillin and (100μg/ml) streptomycin (Life Technologies). Cells were routinely passaged at 80-90% confluence. HeLa cells transfected with pIRES CFTR-Flag and pIRES ΔF508 CFTR-Flag were maintained in the above media supplemented with 800μg/ml G418 (Life Technologies).

3.3.1 Transient Transfections

Transfections were carried out using the calcium phosphate procedure of Jordan et al (Nucleic Acid Research, 1996). Cells (4×10^5) were plated in a 60 mm culture dish

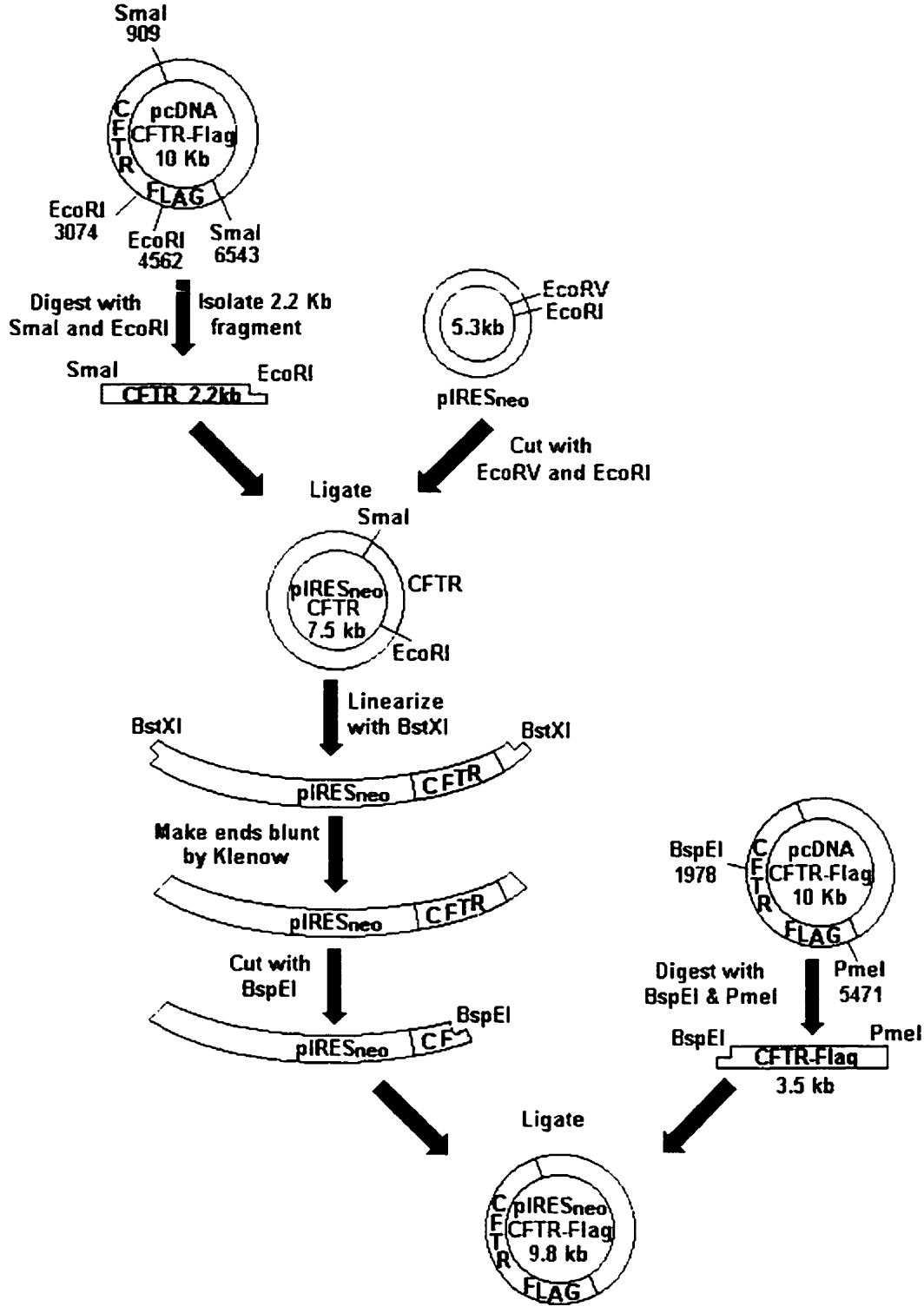


Figure 4. Subcloning CFTR-Flag sequence into expression vector pIRESneo.

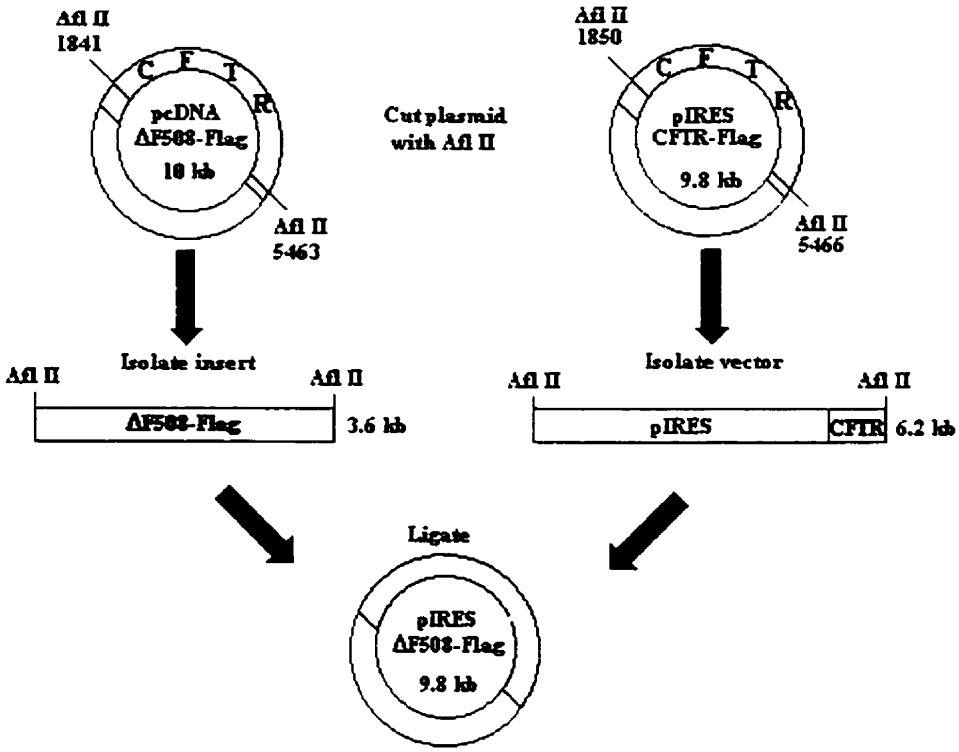


Figure 5. Cloning ΔF508 CFTR-Flag sequence into pIRESneo vector using pIRES CFTR-Flag construct.

16-20 hours prior to transfection. One hour before the precipitate was added, the medium was replaced with 3ml of fresh αMEM without serum or antibiotics. Transfection precipitates were formed by combining 7.5 μg of DNA with 15μl of 2.5 M $CaCl_2$, and bring the total volume to 150μl with 0.1 × TE buffer (1mM Tris HCl, 0.1 mM EDTA, pH 7.6). This solution was added to 150 μl of 2× HBS (140mM NaCl, 50 mM Hepes, pH 7.05 and 1.5 mM Na_2HPO_4). This mixture was incubated at room temperature for 1 min before adding to the cells. Cells were returned to the incubator and precipitates were left on for 4 hours. The medium was then removed and replaced with 4 ml of αMEM containing serum and antibiotics. The cells were returned to the incubator for 24-30 hours before they were harvested and analyzed.

3.3.2 Stable Transfection in HeLa cells

DNA plasmids containing the CFTR-Flag constructs were linearlized with the *AdhI* restriction enzyme. Ca_2PO_4 precipitates were prepared as described in section 3.3.1. Twenty-four hours following transfection, the cells were trypsinized and seeded at various dilutions in 100 mm dishes. The cells were then placed under selective pressure one day later using 800 μg/ml G418. Cell growth and death were monitored closely, and the selecting medium was changed every 3 days. After growing in G418 for approximately 21 days, individual clones of HeLa cells were isolated and continuously cultured in the presence of 800 μg/ml G418. A sample of each clone was harvested and tested for the stable expression of CFTR protein by immunoblotting.

3.4 Metabolic Labeling and Immunoprecipitation

3.4.1 Short-term pulse studies

HeLa cells stably expressing CFTR were seeded at a density of 8×10^5 cells in 60 mm dishes on the day before labeling (so they were about 80% - 90% confluent on the day of the pulse). Monolayers were washed in pre-warmed Met-free αMEM medium, then incubated in 3 ml of same medium for 30 minutes at 37 °C. Cells were pulse labeled in 0.75 ml of Met-free αMEM and 100 μCi of L-[^{35}S]methionine, (>1000 Ci/mmol, NEG-009A) (NEN, Boston, MA) for 15 minutes.

3.4.2 Pulse-chase studies

After the short-term pulse label, the labeling medium was then replaced with 4 ml of αMEM containing 10% FBS and 1mM unlabeled Met, and the cells were returned to the 37 °C incubator. Individual dishes were harvested immediately after the pulse and at increasing times of the chase.

3.4.3 Long-term labeling experiment

Cells were seeded at a density of 8×10^5 a day prior to labeling. The growth medium was aspirated and replaced with 1 ml of labeling medium containing 80% of Met-free αMEM plus 20% complete αMEM supplemented with 5% FBS and 50 μCi of L-[^{35}S]methionine. Cells were returned to the 37 °C incubator and grown under these conditions for 5 hours.

3.4.4 Cell lysis and immunoprecipitations

Metabolically labeled CFTR was isolated by immunoprecipitation. After washing the monolayers with ice-cold PBS, cells were solubilized in 1 ml of lysis buffer (for short-term and pulse-chase studies, RIPA buffer was used; for long-term labeling, buffer containing 0.2% digitonin, 25mM Hepes, pH 7.5, 150mM NaCl, 2mM CaCl2, 20mM Molybdate, 2mM ADP-MgCl2, 10mM glucose and 25units/ml hexokinase) for 30 min at 4°C. All lysis buffers contained 0.25mM AEBSF and 5 mg/ml of each of pepstatin, leupeptin, ALLN and E-64. Insoluble material was sedimented by centrifugation at 15,000 × g for 15 min at 4°C. The supernatant was incubated overnight at 4°C. The antibody of interest and immune complexes were isolated by incubation with protein G-agarose beads for 1 h at 4°C followed by six washes with lysis buffer. Immunoprecipitated proteins were eluted and denatured with Laemmli sample buffer (Laemmli, 1970) and analyzed by electrophoresis.

3.5 Electrophoresis and Fluorography

Protein samples were analyzed after separation by SDS-PAGE through gels containing 7% or a 6%-12% gradient of acrylamide. Gels were fixed in 10% acetic acid and 30% methanol for 30 min, followed by 2 washes in water. For fluorography, gels were then soaked in 1 M sodium salicylate, 3% glycerol, and 10% methanol for 1 h. The gels were dried onto a filter paper backing. Labeled proteins were visualized by exposing the gels to Kodak X-OMAT AR film at 70 °C for 1-3 days.

3.6 Protein Assay and Immunoblot Analysis

Transfected cells in 60 mm dishes were washed in PBS and harvested in 1 ml of 1% SDS. The lysates were sonicated briefly and the protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce Chemicals, Rockford, IL). Bovine serum albumin (BSA) at a concentration of 2.0 mg/ml was used to generate the standard curve. After the determination of protein concentration, equal amounts of total cellular proteins were separated by one-dimensional SDS-PAGE on a 7% acrylamide gel according to the method of Laemmli (1970). Immediately after electrophoresis, proteins were transferred electrophoretically onto a sheet of 0.2 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in the presence of a modified Towbin transfer buffer (250mM Tris, 192mM Glycine and 15% Methanol) (Towbin, 1984). The protein transfer took 2 hours at a constant voltage of 35 V at 4°C. After transfer, the nitrocellulose was incubated for 1 hour in a blocking solution containing 5% skim milk powder and 0.1% Tween-20 in PBS. The primary antibody was diluted into 5 ml of fresh blocking solution and incubated overnight at 4 °C. Following six washes in PBS containing 0.1% Tween-20, a horse-radish peroxidase linked secondary antibody was incubated with the membrane for 1 hour at room temperature (For a list of primary and secondary antibodies used in experiments, see table 2). Immune complexes were detected with an enhanced chemiluminescence detection system (Pierce Chemicals).

Table 2. Antibodies used in immunoprecipation and immunoblotting experiments

Primary Antibodies	Specificity	Supplier
1) anti-c-myc	mouse monoclonal (clone 9E10)	Roche
2) anti-6xHis	mouse monoclonal	Clontech
3) anti-flag	rabbit polyclonal (D-8) (SC-807)	Santa Cruz
4) anti-flag	mouse monoclonal (M2)	Sigma
5) anti-CFTR(M_3A_7)	mouse monoclonal	Dr. J. Riordan
6) anti-CFTR($L_{12}B_4$)	mouse monoclonal	Dr. J. Riordan
7) anti-Hsp90	rat monoclonal (SPA-840, 9D2)	StressGen
8) anti-Grp94 (CT)	rabbit polyclonal (SPA-851)	StressGen
9) anti-Hsc70	rat monoclonal (SPA-815)	StressGen
10) anti-Calnexin	rabbit polyclonal	Dr. D. William
11) anti-KDEL	mouse monoclonal (SPA-827)	StressGen

Secondary Antibodies	Manufacturer
1) Peroxidase Conjugated Goat anti-rabbit IgG	Jackson ImmunoResearch Lab
2) Peroxidase Conjugated Goat anti-mouse IgG	Jackson ImmunoResearch Lab
3) Peroxidase Conjugated Rabbit anti-rat IgG	Sigma
4) Streptavidin HRP	Jackson ImmunoResearch Lab

4.0 RESULTS

4.1 CFTR-tag Fusion Proteins

The initial goal of this project was to construct a 'tagged' version of CFTR that could be used to efficiently and specifically precipitate CFTR and its associated proteins. We initially tried the 'myc-tag', an eight amino acid epitope tag that is recognized by 9E10 monoclonal antibody (Evans et al., 1985) and the 'strep-tags' (strep and strepII), two short peptides that bind to streptavidin (Schmidt and Skerra, 1993; Schmidt and Skerra, 1994; Schmidt et al., 1996). The myc tag was chosen because the 9E10 hybridoma cells are available from the American Type Culture Collection and can be grown in culture to provide a source for large quantities of antibody. The strep-tags were also an attractive choice as they bind directly to streptavidin, yielding an antibody-independent detection and purification system. As none of these tags provided the sensitivity or specificity necessary for our experiments (see below), other alternatives were explored. In total, seven different tag sequences were successfully added onto the carboxyl terminus of CFTR using a PCR-based technique. For each construct, the region that was amplified by PCR was sequenced to be sure that it was free of PCR-induced mutations. Each construct was then transiently transfected into CHO cells and examined for CFTR protein expression by immunoblotting analysis. Cell lysates were prepared, subjected to SDS-PAGE gel electrophoresis and subsequently transferred to nitrocellulose. The samples were first immunoblotted with an anti-CFTR antibody (M_3A_7) that specifically recognizes the NBD2 of CFTR (Kartner et al., 1992). Both the mature and immature forms of CFTR can be detected in cells transfected with wild-type CFTR and with the constructs containing the inserted epitopes (Fig. 6). The protein band

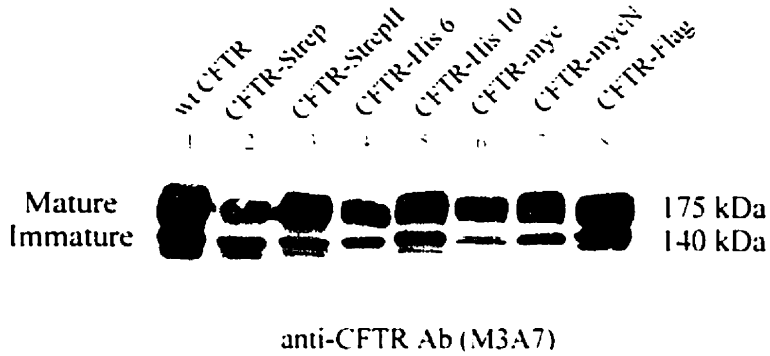


Figure 6. Immunoblot showing the CFTR-tag fusion proteins identified using the anti-CFTR (M_3A_7) antibody. CFTR constructs bearing the Strep, StrepII, His6, His10, Myc, MycN or Flag tags, along with wild-type CFTR were individually transfected into CHO cells and analyzed by SDS-PAGE for CFTR protein production.

migrating at ~175 kDa is identified as the mature form of CFTR and the lower band running at ~140 kDa is the immature form. Their mobility in SDS-PAGE is similar to previously reported results (Cheng et al., 1990; Pind et al., 1994; Lukacs et al., 1994) and results from differences in glycosylation between newly synthesized CFTR in the ER (140 kDa) and CFTR that has been modified during their transit through the Golgi apparatus (175 kDa). As both forms of CFTR are present in each of the lanes, it suggests that the tags do not prevent the maturation of CFTR, although the strep-tag did appear to significantly reduce the maturation (lane 2) . When individual tag-specific reagents were tested for their ability to detect CFTR constructs, most of the reagents could not detect the tagged fusion protein (Fig. 7). A summary of these results is presented in Table 3. For example, streptavidin-HRP could not detect Strep-tag or StrepII-tag CFTR fusion protein on an immunoblot from transfected cell lysates (Fig. 7A, lanes 2 and 3). These constructs were also unable to be precipitated from [^{35}S]-met labeled cell lysates by streptavidin-agarose (not shown). Similar negative results were seen with the His6 or His10 tagged constructs; the anti-His6 antibody was unable to recognize the His-tag CFTR fusion proteins on an immunoblot (Fig. 7B, lanes 2 and 3). In addition, the His-tagged constructs could not be precipitated from cell lysates using either the anti-His6 antibodies or immobilized metal affinity columns (not shown).

In contrast to the above results, the constructs bearing the FLAG epitope and the Myc epitopes were successfully detected on immunoblots (Fig. 7C and D). The anti-myc antibody identified both the mature and immature forms of CFTR in lysates from CHO cells transfected with the CFTR-myc and the CFTR-mycN plasmids (Fig. 7C). The antibody appears to be specific for the myc epitope because it did not recognize wild-type

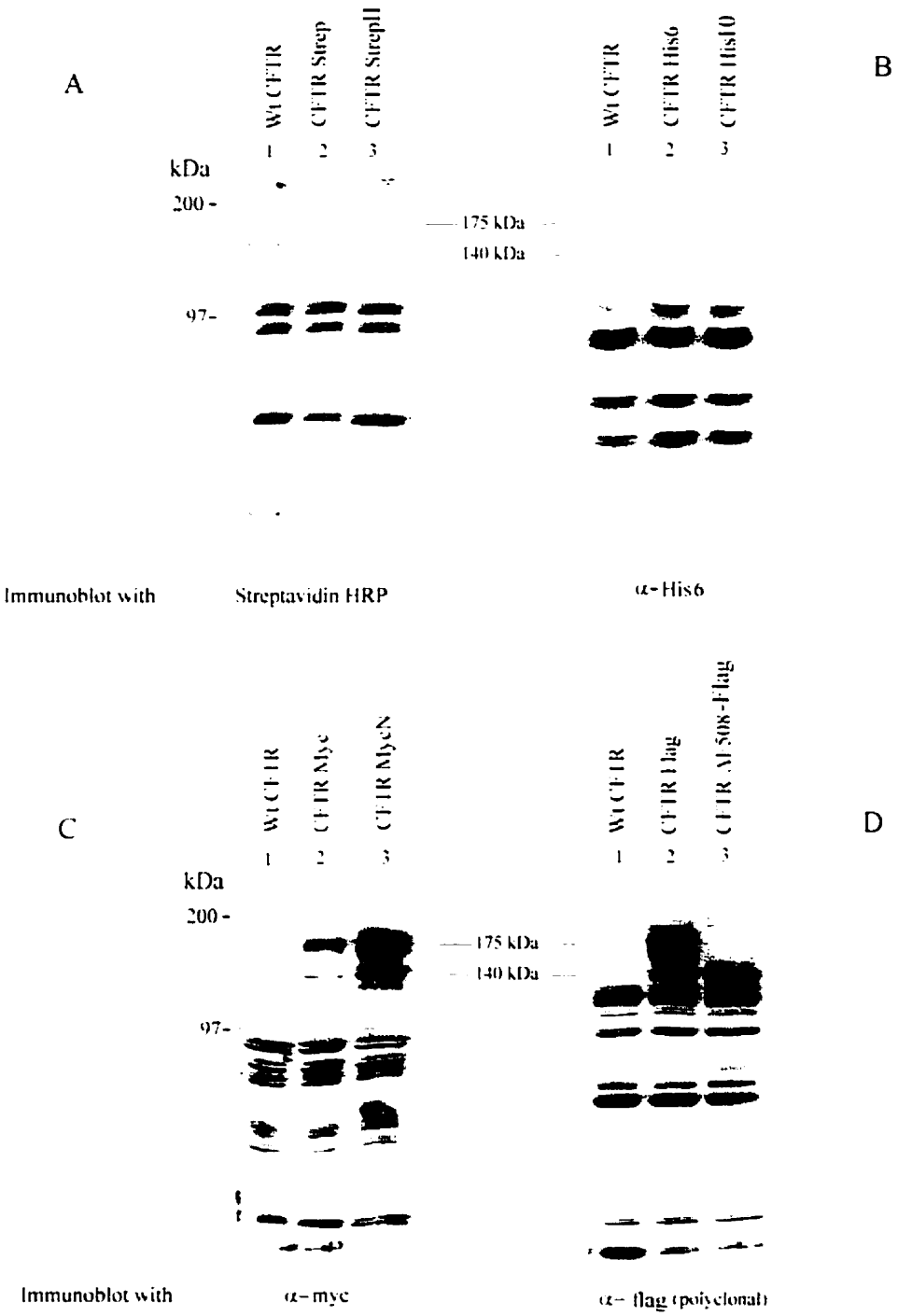


Figure 7. Immunoblots of CFTR-tag fusion proteins with tag-specific reagents. CHO cells transfected with wt CFTR or CFTR-tag constructs were harvested in 1% SDS and subjected to SDS-PAGE on 7% acrylamide gels. A. membrane containing wt CFTR, CFTR-Strep and CFTR-StrepII was probed with streptavidin-HRP; B. membrane containing wt CFTR, CFTR-His6 and CFTR-His10 was decorated by α-his antibody and goat anti-mouse HRP; C. immunoblot of CFTR-myc and CFTR-mycN proteins detected by α-myc antibody and goat anti-mouse HRP; D. wt CFTR, CFTR-flag and ΔF508 CFTR fusion proteins were detected by α-flag antibody and goat anti-rabbit HRP.

CFTR in the control cell lysate (lane 1). The CFTR-mycN construct was detected with a greater sensitivity than the CFTR-myc construct on this immunoblot (compare Fig. 7C, lanes 2 and 3). The reason for this difference may be due to different transfection efficiencies for the two constructs in this experiment. However, we consistently observed that the CFTR-myc construct yielded only very faint signals with the anti-myc antibody. It is also possible that the c-myc epitope with the extra asparagine residue at the carboxyl terminus has a higher affinity for the anti-myc antibody, thereby producing a stronger signal. While we do not understand the reason for this difference, it should be noted that in several instances in the literature, when a myc-tag was added onto carboxyl terminus of a protein, the extra asparagine residue was added (Yang and Storrie, 1997; Varughese et al., 1998; Yang et al., 1996).

The CFTR-Flag fusion protein was also recognized by its tag-specific antibody (Fig. 7D). Once again, the antibody recognized the epitope and not CFTR because it did not recognize wild-type CFTR in the control cell lysate (lane 1). Surprisingly, this recognition only occurred when using a rabbit polyclonal anti-flag antibody (Santa Cruz Biotechnology, Inc.). A monoclonal antibody that is supposed to recognize carboxyl-terminal Flag epitopes (anti-Flag M2, Sigma) failed to detect either the wild-type or the ΔF508 CFTR-flag constructs on immunoblots (not shown). As seen in Fig. 7D, the polyclonal antibody recognized several proteins in addition to CFTR, including one that migrates only slightly faster than the immature CFTR. These bands were derminated to be cross reacting proteins since they were also present in lysate prepared from untransfected cells (not shown) and cells transfected with wild-type CFTR (lane 1). This cross reaction poses a potential problem for us if these proteins also cross-react during

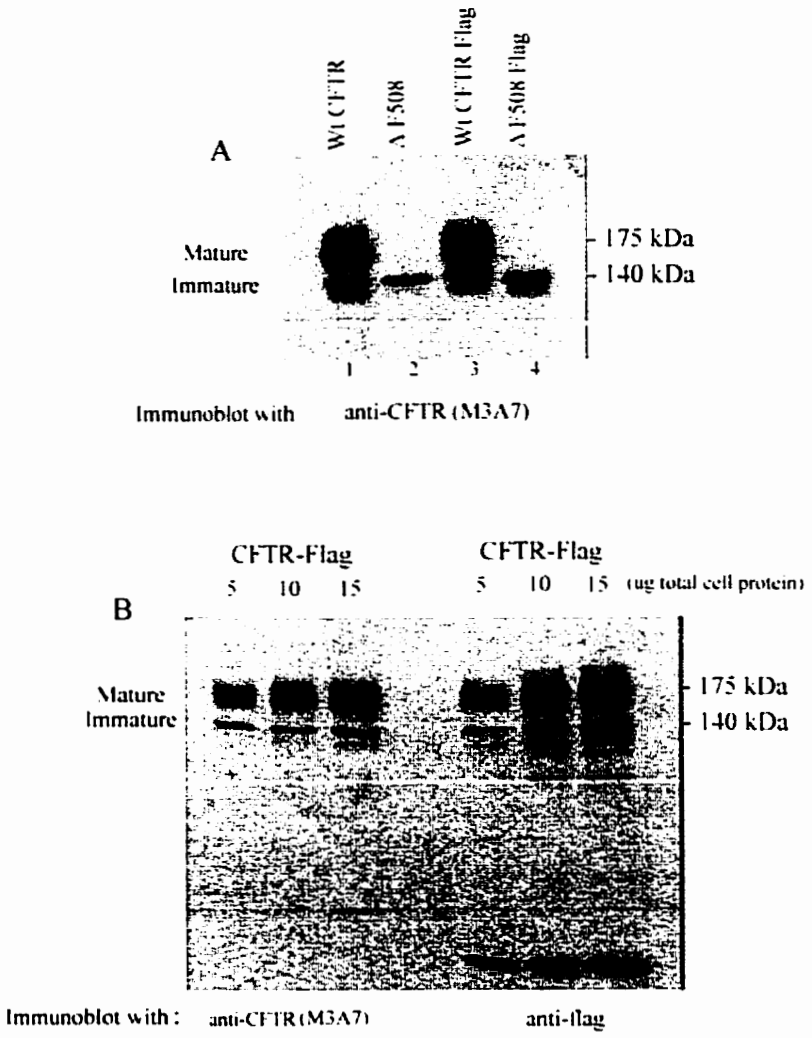


Figure 8. Immunoblot analysis of CFTR expression in transiently transfected CHO cells. A. comparison of wt CFTR, ΔF508 CFTR, CFTR-Flag and ΔF508-Flag proteins detected with the M_3A_7 anti-CFTR antibody. B. CHO cells were transfected with CFTR-flag and a lysate was prepared in 1% SDS. 5, 10, 15 μg of total cellular protein was separated by SDS-PAGE and transferred to nitrocellulose. One half of the blot was probed with the anti-CFTR (M_3A_7) antibody and the other half was probed with the anti-flag antibody.

immunoprecipitation experiments. Fortunately, preliminary experiments indicated that the cross-reaction was more pronounced on immunoblots than in immunoprecipitates.

The above results indicate that the mycN and Flag epitopes are recognized by their respective antibodies when added onto the carboxyl terminus of CFTR. Two types of experiments led us to concentrate on the CFTR-flag constructs for the rest of our experiments. First of all, the Flag-tagged CFTR produced a stronger signal on immunoblots. In comparison with the anti-myc immunoblots, the anti-flag blots required less primary antibody and the signal developed with a much shorter exposure of the blot to film. As shown in Figure 8B, the anti-flag antibody detected CFTR-flag with a sensitivity equal to, or slightly greater than, the anti-CFTR (M_3A_7) antibody. Secondly, trial immunoprecipitation experiments indicated that the flag system produced strong CFTR signals (see below), whereas the signals obtained with the mycN system were barely detectable (not shown). Since the major objective of this project was to immunoprecipitate CFTR and its associated proteins, the CFTR-flag constructs were the best suited for this purpose. Fig. 8A shows a comparison between wild-type and ΔF508 CFTR and their flag-tagged counterparts. This immunoblot was probed with the anti-CFTR antibody. The mobility of the proteins and the ratio of immature to mature CFTR is comparable between the native and the flag-tagged proteins (compare lane 1 to lane 3 and lane 2 to lane 4). This result indicates that the flag epitope has no observable effect on the overall conformation or maturation of CFTR in these cells.

4.2 Inability to Create Stable Transfectants of CFTR-FLAG Using pcDNA 3.1

The expression studies done in the previous section were all completed using transient transfections. In order to achieve constitutive expression of recombinant CFTR in heterologous cells, and eliminate the need to do a transfection prior to every experiment, it was necessary to generate stable transfectants expressing the construct of interest. For our initial attempts, the expression vector pcDNA3.1 was transfected into HeLa cells using calcium phosphate (Jordon et al., 1996). The pcDNA3.1 vector contains a neomycin (G418) resistance gene for selection of stable transfectants in mammalian cells. Forty-eight hours after transfection, medium containing 800 μg/ml G418 drug was added to the cells. Fresh G418 medium was added to the cells every three days. After approximately three weeks growing under selective pressure, individual colonies of cells were readily apparent on the plates. These clones of cells were isolated, propogated and analyzed for CFTR protein production by immunoblotting. The amount of CFTR protein detected in these stable transfectants was minimal, and certainly not useful for subsequent immunoprecipitation studies. After many trials, no clones could be isolated that expressed significant amounts of CFTR. It was concluded that the pcDNA 3.1 expression vector may be inefficient at producing high levels of recombinant proteins in HeLa cells.

4.3 Stable Expression of pIRESneo CFTR-Flag in HeLa

Since the pcDNA 3.1 vector was unable to produce high levels of CFTR expression in HeLa cells, stable expression of CFTR-flag was attempted using the

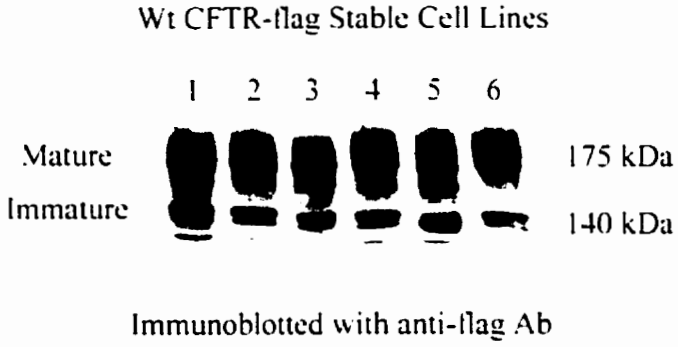


Figure 9. Different stable cell lines all express wild-type CFTR-flag fusion proteins. HeLa cells were transfected with CFTR-flag cDNAs cloned into pIRESneo expression vector by calcium phosphate. After three week of selection and propagation in G418, CFTR protein expression were examined by immunoblotting with anti-flag antibody.

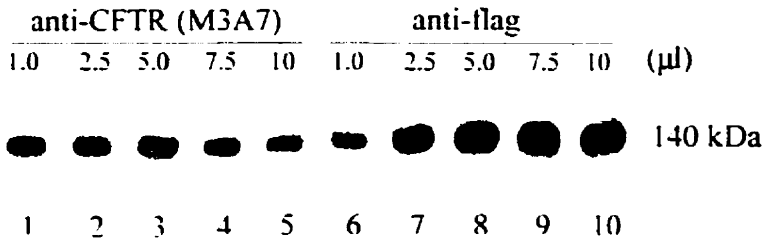
pIRESneo vector (Clontech). pIRESneo is a bicistronic vector originally described as pcIN4 by Rees et al. (1996), which is designed for the efficient production of stable cell lines expressing recombinant proteins in mammalian cells. This vector contains the internal ribosomal entry site (IRES) of the encephalomyocarditis virus in an arrangement that permits both the gene of interest and the neomycin resistance gene to be translated from a single mRNA. After selection with G418, nearly all surviving colonies should stably express the recombinant protein, thus decreasing the need to screen large numbers of clones. The vector also contains a synthetic intron between the gene of interest and the IRES, a feature that has been shown to enhance the accumulation of polyadenylated mRNA in the cytoplasm (Huang et al., 1990).

The CFTR-flag and ΔF508-Flag cDNAs were cloned into the pIRESneo expression vector and transfected into HeLa cells using calcium phosphate (Jordon et al., 1996). G418 was used to select for resistant cells as described in section 4.2. After three weeks of selection in G418, individual colonies of cells were isolated and propagated. CFTR protein expression in selected stable transfectants was examined by immunoblotting. All stable transfectants tested yielded strong CFTR protein signals, as recognized by both the anti-CFTR and anti-flag antibodies (Fig. 9).

4.4 Characterization of Immunoprecipitation Conditions Using the Stable Cell Lines

A series of experiments was done in order to characterize and optimize the immunoprecipitation conditions for the flag-tagged CFTR constructs stably expressed in HeLa cells. To determine the working concentration of the anti-flag antibody that will give the maximal recovery of CFTR, short-term labeling and immunoprecipitation

A



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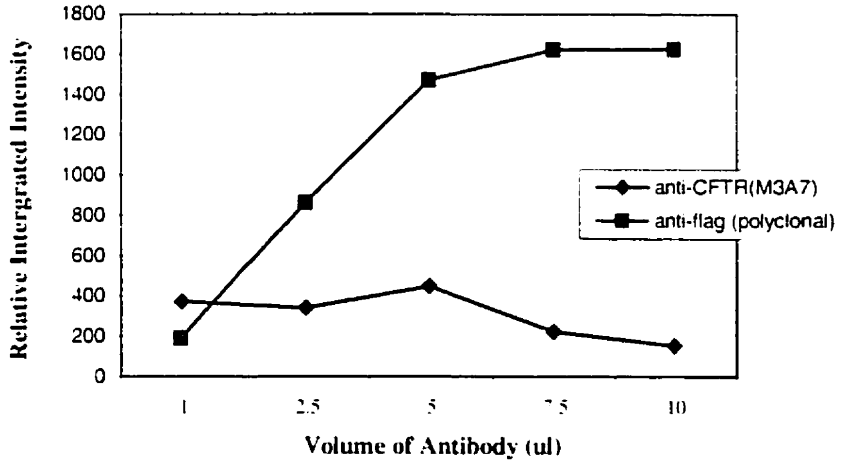

Figure 10. Comparison of the ability of anti-CFTR and anti-flag antibodies to immunoprecipitate CFTR (I). A. HeLa cells stably expressing CFTR-Flag were pulse labeled for 15 min with [^{35}S]-met. Lysates were prepared in 1.0 ml of RIPA buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS). Supernatant from 10 dishes of cells were pooled and equally divided among 10 samples. Varying volumes of M_3A_7 or anti-flag antibodies were incubated with the lysate overnight. Complexes were collected with protein-G sepharose, washed 5 times with RIPA buffer and eluted with Laemmli sample buffer. CFTR was analyzed by SDS-PAGE and fluorography. B. Quantitation of the above fluorogram. A non-saturated exposure of this gel was scanned using a flat-bed scanner with a transparency adapter and quantited using commercial program (Quanity One, BioRad). Values of the relative integrated intensities of [^{35}S] signal obtained by densitometry were plotted on the Y-axis and volumes of antibodies used in immunoprecipitation were plotted on the X-axis.

studies were performed. After a brief pulse in [^{35}S]-met, cell lysates were prepared and immature CFTR was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography. For this experiment, the lysates from several dishes of cells were pooled and equal aliquots were then immunoprecipitated with vary amounts of M_3A_7 or anti-flag antibodies (Fig. 10A). The relative amount of CFTR in the lanes was estimated by densitometry and the results are plotted in Fig. 10B. It can be seen that the recovery of CFTR is greater with the anti-flag antibody than with M_3A_7. The maximal amount of CFTR recovered with M_3A_7 (lane 3) was 3-4 fold lower than the maximal amount recovered with the anti-flag antibody (lanes 8-10). We also observed that adding more M_3A_7 eventually resulted in a lower recovery of CFTR (compare lanes 4 and 5 with lane 3). This was a repeatable result. Since the M_3A_7 antibody was supplied as an ascites fluid, we do not know the exact concentration of M_3A_7 used in this experiment. The anti-flag antibody was affinity purified by manufacturer and supplied at a concentration of 100 μg/ml. Therefore, 0.5-1.0 μg of this antibody yielded a maximal recovery of CFTR.

A further comparison between anti-CFTR and anti-flag antibodies is shown in Fig. 11A. Once again, the anti-flag antibody yielded a greater recovery of CFTR than did M_3A_7 (compare lanes 1 and 3). In fact, the anti-flag antibody recovered more CFTR than did a combination of two anti-CFTR monoclonal antibodies (M_3A_7 + $L_{12}B_4$ (Kartner et al., 1992); compare lanes 2 and 3), the previous 'gold standard' for achieving maximal recoveries of CFTR in immunoprecipitates (Pind et al., 1994).

The immunoprecipitations shown in Fig. 10 and 11A were performed following an overnight incubation of the antibody with the cell lysates. In order to study protein-protein interaction that may be labile following cell lysis, it was of interest to determine if

overnight incubations were required in order for the antibody and antigen to reach equilibrium. Fig. 11B shows that a 5 h incubation of the anti-flag antibody with the cell lysates recovered almost as much CFTR as an overnight incubation (compare lanes 5 and 6). In contrast, a 2 h incubation resulted in a lower recovery (lane 4). For the M_3A_7 antibody, the amount of CFTR was maximal at the 5 h time point (lanes 1-3).

4.5 Biosynthesis of CFTR-Flag and ΔF508-Flag in HeLa cells

To further characterize the cell lines that were developed in this study, pulse-chase experiments of the CFTR-flag and ΔF508-flag stable transfectants were performed (Figure 12). In agreement with earlier studies using heterologous expression systems (Cheng et al., 1990; Denning et al., 1992; Marshall et al., 1994; Pind et al., 1994; Lukacs et al., 1994; Ward and Kopito, 1994), newly synthesized CFTR (0 min of chase) migrates as a band of ~140 kDa in both tranfectants. This has been identified as the endoglycosidase H-sensitive, core-glycoslyated form of CFTR, also known as the B form. During the subsequent chase of wild-type CFTR-flag, label incorporated into the 140 kDa precursor band decreased, with the appearance of a slower, more diffusely migrating species of ~175 kDa. This has been identified as the mature complex-glycosylated, endoglycosidase H-resistant, or C form of CFTR. None of the pulse-labeled, core-glycosylated ΔF508-flag was processed to the complex-glycosylated species during the chase. Instead, the intensity of this band decreased during the chase and was almost undetectable by 3.5 h of chase. Previous studies have shown that the newly synthesized CFTR that does not 'mature'and transit through the Golgi apparatus to the plasma membrane is degraded in the ER, in a process that, at least in part, involves

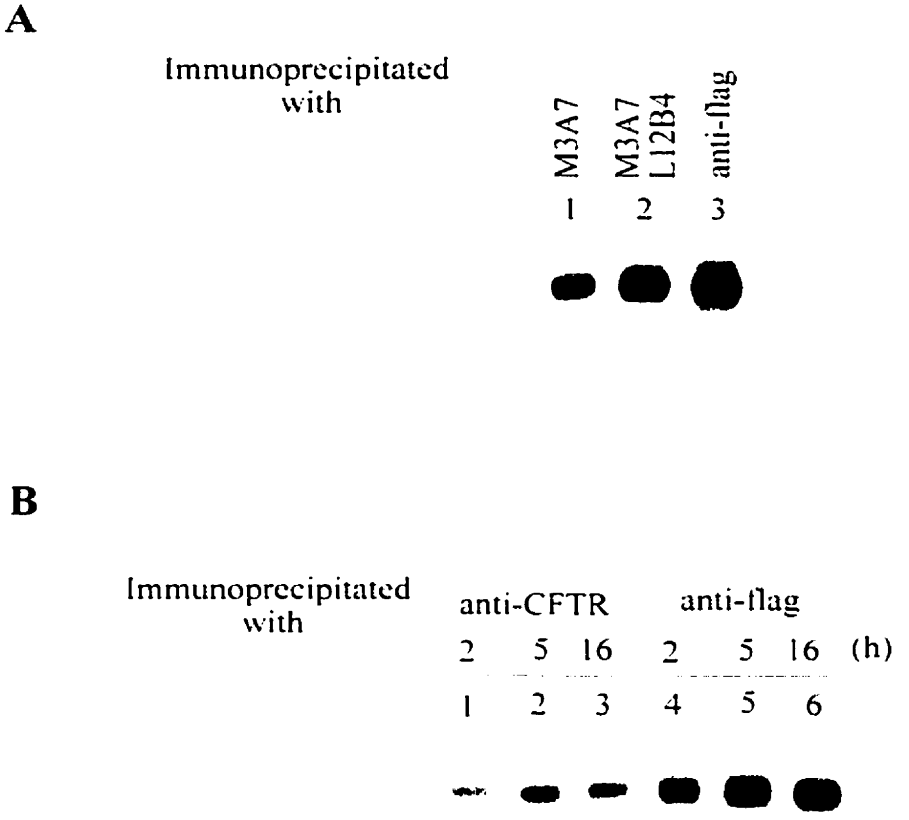

Figure 11. Comparison of the ability of anti-CFTR and anti-flag antibodies to immunoprecipitate CFTR-Flag. HeLa cells expressing wild-type CFTR-Flag were pulse-chased with 100 µCi/ml [^{35}S]-met for 15 min. Lysates were prepared in RIPA lysis buffer, and equal aliquots were immunoprecipitated with the antibodies shown in the Figure. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. A.lysates were immunoprecipitated with 5.0 µl of M_3A_7, 2.0 µl M_3A_7 and 1.0 µl $L_{12}B_4$, and 5.0 µl of anti-flag antibodies. B. Time course experiment in which CFTR was immunoprecipitated with 5.0 µl of M_3A_7 or 5.0 µl of anti-flag antibodies for 2, 5, 16 h (overnight) respectively.

ubiquitination and the proteasome (Lukacs et al., 1994; Jensen et al., 1995; Ward et al., 1995). Our results with the flag-tagged CFTR constructs in HeLa cells are consistent with this model.

As illustrated in Figure 12, the mature CFTR-flag (175 kDa) first became detectable at 30 min of chase, reaching a plateau by 2.5 h. Labeling of this band showed only a slight decline during chases of up to 6 h, suggesting that the mature CFTR has a relatively long half life. This is consistent with previous reports in which the $t_{1/2}$ for the mature, plasma membrane localized CFTR has been estimated to be 7-8 h in HEK293 cells (Ward and Kopito, 1994) and >24 h in CHO cells (Lukacs et al., 1993). Fig. 12 also shows that only a portion of the CFTR-flag that is present at 0 min of chase is converted into the mature, 175 kDa band. This result is in agreement with previous estimates that only 20-40% of newly synthesized wild-type CFTR reaches the plasma membrane in cells expressing CFTR endogenously and in heterologous expression systems (Marshall et al., 1994; Lukacs et al., 1994; Ward and Kopito, 1994). In summary of the results presented thus far in this thesis, we have created HeLa cell lines stably expressing epitope-tagged versions of wild-type and ΔF508 CFTR. These cell lines reproduce the phenotypes observed for CFTR in other cells, with the advantage that the epitope provides a means for rapid and efficient immunoprecipitation of the CFTR fusion proteins. The remainder of this thesis describes the use of these cell lines to investigate the proteins that co-precipitate with CFTR.

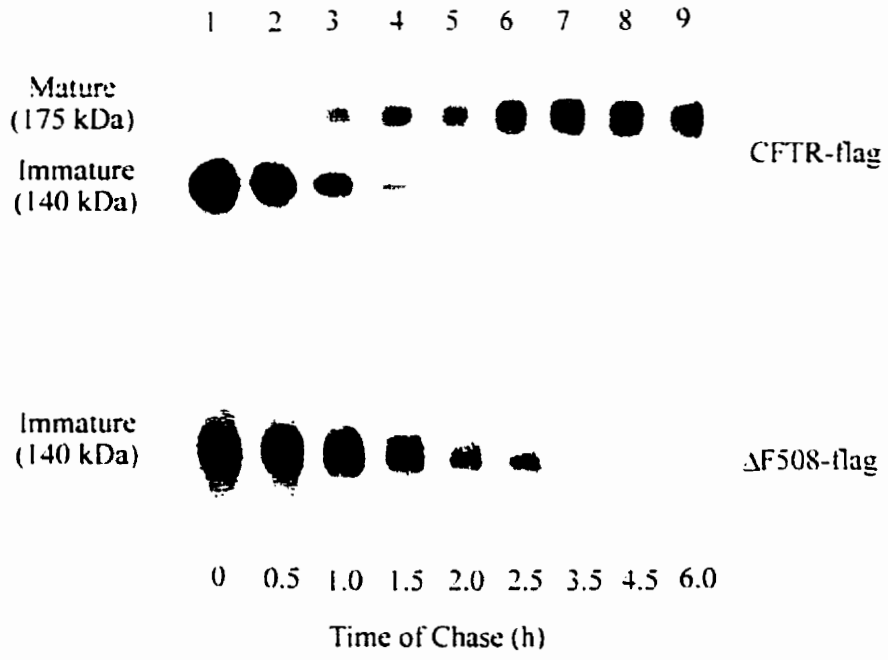

Figure 12. Biosynthesis and maturation of CFTR-Flag and ΔF508-Flag in HeLa cells. HeLa cells expressing CFTR-Flag or ΔF508-Flag were pulse labeled with [^{35}S]-met for 15 min and chased for the times indicated. Anti-flag immunoprecipitates were analyzed by SDS-PAGE and flourography.

4.6 Identifying Proteins Associated with Nascent CFTR

To study the proteins associated with CFTR during its biosynthesis in ER, the cells should be lysed in detergents that efficiently solubilize the CFTR but do not dissociate the interacting proteins. Since the protein-protein interactions we are expecting to find are non-covalent in nature, it is necessary to solubilize the cells under conditions that preserve these interactions. We set out to establish optimal lysis conditions that would allow for the co-precipitation of as many CFTR-associated proteins as possible. HeLa cells were initially solubilized in non-denaturing buffers that contain 'mild' detergent such as digitonin or Triton X-100. Immunoprecipitates recovered from these lysis buffers were compared with those solubilized by RIPA buffer, a 'harsher' detergent system containing 0.1% SDS, plus 1% Triton X-100 and 1% deoxycholate. Inspection of the resulting immunoprecipitates by SDS-PAGE and fluorography showed that digitonin, Triton X-100 and RIPA all solubilized similar amounts of CFTR. Even low concentrations of 0.2% digitonin or 0.1% Triton X-100 released CFTR from the cell (Fig. 13). This result showed that we can use Triton X-100 or digitonin to solubilize the CFTR expressed in HeLa cells.

A series of experiments done to examine the effect of solubilization conditions on the recovery of proteins that co-precipitate with CFTR are presented in Figures 13-16. For these experiments, HeLa cells stably expressing the tagged CFTR constructs were incubated in the presence of [^{35}S]-met for 5 h to label the total cellular pool of proteins. Anti-flag antibodies were then used to immunoprecipitate CFTR and any interacting proteins. From the review of the literature presented in section 1, we expect that, under the appropriate conditions, hsc70, calnexin and hsp90 should be present in these

immunoprecipitates. Our goal is to confirm these interactions in our system and to examine our results for any other protein bands that specifically co-immunoprecipitate with CFTR.

Fig. 13 compares the effects of different detergents and additives to the lysis buffer on the recovery of bands representing proteins that co-precipitated with CFTR. For this, and all the following experiments the buffer used for immunoprecipitation contained 25 mM Hepes, pH 7.5, and 150 mM NaCl. For cells solubilized in this buffer containing 0.1% Triton X-100 and no further additives, the major protein bands found in the precipitates include CFTR (only the 140 kDa band in this experiment as these cells expressed ΔF508-flag) and bands migrating at ~73 kDa and ~70 kDa (lane 1). A similar result was observed for cells solubilized in 0.2% digitonin (lane 4). In addition to the ~73 and ~70 kDa bands, the bands of ~97, ~95 and ~90 kDa present in some of the lanes of this gel were observed consistently and will be discussed below. Other faint bands that were observed on this gel were either not reproducibly present or were deemed to be non-specific as they were also present in control immunoprecipitates from untransfected HeLa cells (eg. See Figs. 14 and 16). They will not be discussed further.

The band migrating at ~73 kDa (Figs. 13-16) varied in intensity depending upon the additives present in the cell lysates. As seen in Fig. 13, the intensity of this band decreased when apyrase was added to deplete ATP concentrations in both the Triton X-100 (compare lane 3 to lanes 1 and 2) and digitonin (compare lane 6 to lanes 4 and 5) lysates. A similar result for the addition of apyrase can be seen in Fig. 15 (compare lane 4 with lanes 2 and 3) and Fig. 14 (compare lanes 4-6 with lanes 1-3). Adding glucose and hexokinase to deplete ATP had a similar effect (Fig. 14, compare lanes 7-9 with

lanes 1-3). In addition, adding MgATP or MgADP to the lysate decreased the recovery of this band (Fig. 15, lanes 5 and 6). While these results are interesting, we have concluded that the ~73 kDa band represents a non-specific contaminant of the immunoprecipitations. Figs. 14 and 16 (lane 1 in each figure) show that this band was also found in control immunoprecipitations from untransfected HeLa cells. Furthermore, Fig. 15 (lane 1) shows that this protein is likely 'sticking' to the protein–G beads as it was present in a control experiment in which the antibody was omitted.

The band at ~70 kDa in Figs. 13-15 also varied in intensity following manipulations to alter the ATP/ADP content of the lysates. The intensity increased or remained constant when apyrase (Fig. 13, compare lane 3 to lanes 1 and 2 and compare lane 6 to lanes 4 and 5; Fig. 14, compare lanes 5 and 6 with lanes 2 and 3; Fig. 15, compare lane 4 to lanes 2 and 3) or glucose/hexokinase (Fig. 14, compare lanes 8 and 9 with lanes 2 and 3) were added to deplete the lysate of ATP. In contrast, the intensity of this band decreased when the lysate was supplemented with MgATP (Fig. 15, compare lane 5 to lanes 2 and 3) and to a lesser extent with MgADP (Fig. 15, compare lane 6 to lanes 2 and 3). The mobility of this band on the gels (~70 kDa) and its dissociation by ATP indicates that this band may be Hsc70. Further evidence to support this hypothesis will be presented in Fig. 17. It has been shown that the ADP-bound form of Hsc70 binds substrates with higher affinity and has a slower rate of substrate release than the ATP-bound form (Palleros et al., 1994).

Figure 13 also shows that a band migrating at ~90 kDa was present in the immunoprecipitations when 20 mM sodium molybdate was included in the lysis buffer (compare lanes 2 and 3 with lanes 1 and lanes 5 and 6 with lane 4). This band was also

observed in Fig. 15, when molybdate was in the lysis buffer, and is much weaker in intensity in Fig. 14 when molybdate was not added. The mobility of this band and its increased intensity in the presence of molybdate promotes this band as a likely candidate for Hsp90. Molybdate has been documented to stabilize the complex formed between Hsp90 and steroid receptors (Hutchison et al., 1992). It was proposed that molybdate stabilizes substrate-Hsp90 complexes by binding to an ATP-site on Hsp90, thus stabilizing a conformation of Hsp90 with increased affinity for the substrate (Pratt and Toft, 1997). The intensity of this band also appeared to increase when MgATP or MgADP was added to the lysis buffer in addition to molybdate (Fig. 15, lanes 5 and 6). Evidence to show that this band co-migrates with Hsp90 will be shown in Fig. 17.

A faint doublet of bands migrating at ~95 kDa and ~97 kDa in Fig. 13 are also of interest. The lower band of this doublet is more readily observed when digitonin was used as the detergent than when Triton X-100 was the detergent (compare lanes 4-6 with lanes 1-3). A preservation of interactions in the presence of digitonin, but not Triton X-100, has been observed before for interactions involving calnexin and class II MHC molecules (Anderson and Cresswell, 1994) and CFTR (S. Pind and D.B. Williams, unpublished results). The possibility that this band is calnexin will be strengthened by showing that they co-migrate in Fig. 17.

The faint band migrating at ~97 kDa in Figs. 13-15 was observed in many experiments. Figure 16 provides evidence to suggest that this protein is co-immunoprecipitating specifically with CFTR. This figure represents a summary of the results presented in Figs. 13-15. Control, untransfected cells and cells expressing CFTR-flag and ΔF508-flag were labeled with [^{35}S]-met and lysed using the conditions

established in Figs. 13-15 to maintain protein-protein interactions. Once again, proteins migrating at ~70, 90, 95 and 97 kDa are present on this fluorograph. Their intensity is greatly increased in the samples expressing either CFTR-flag or ΔF508-flag (compare lanes 2 and 3 with lane 1), indicating that they are co-precipitating specifically with CFTR. We can also infer that they are interacting with the immature form of CFTR as they are present in the sample that only expresses immature CFTR (ΔF508-flag, lane 3). In fact, the intensity of the four interacting bands parallels the amount of immature form of CFTR present in the samples. The co-precipitating bands are stronger in the ΔF508-flag sample (lane 3), which has more immature CFTR than the CFTR-flag sample (lane 2). The identity of these bands will be the focus of the remainder of this thesis.

The bands migrating at ~70, 90 and 95 kDa have been previously mentioned as being candidates for the molecular chaperones, Hsc70, Hsp90 and calnexin, respectively. This speculation is based upon previous reports demonstrating that these chaperones interact with CFTR (Yang et al., 1993; Pind et al., 1994; Loo et al., 1998), the mobilities of the bands during SDS-PAGE, and how the individual bands react to detergents or the presence of additives in the lysis buffer. Fig. 17 provides additional evidence to strengthen this speculation. In this experiment, cells expressing ΔF508-flag were labeled with [^{35}S]-met and immunoprecipitated with antibodies to the flag epitope and to each of Hsc70, Hsp90 and calnexin. The anti-chaperone immunoprecipitation were run side by side with the anti-flag immunoprecipitates so that the mobilities of the individual chaperones could be compared to the prospective bands in the CFTR immunoprecipitates. As can be seen in this figure, the 95 kDa band co-migrates with calnexin, the 90 kDa band co-migrates with Hsp90 and the 70 kDa band co-migrates with Hsc70. Although

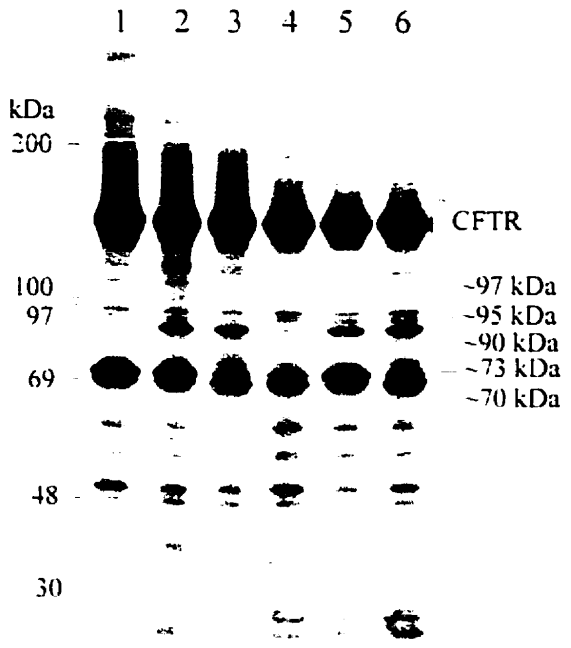

Figure 13. Effect of lysis conditions on the immunoprecipitation of CFTR and other proteins. HeLa cells expressing ΔF508-Flag were metabolically labeled with 50 μCi/ml of [^{35}S]-met for 5 h and solubilized in lysis buffer containing 25 mM Hepes, pH 7.5, and 150 mM NaCl. Detergents and other additions to the lysates are indicated below. 5.0 μl of the anti-flag antibody was used to immunoprecipitate CFTR and the precipitates were analyzed by SDS-PAGE and flourography (5-10% gradient gel).
Lanes 1-3, 0.1% Triton X-100 was used as the detergent; lanes 4-6, 0.2% digitonin was used as the detergent; lanes 2 and 5, 20 mM molybdate was added to the lysates; lanes 3 and 6, 20 mM molybdate and 25 units/ml apyrase were added to the lysates.

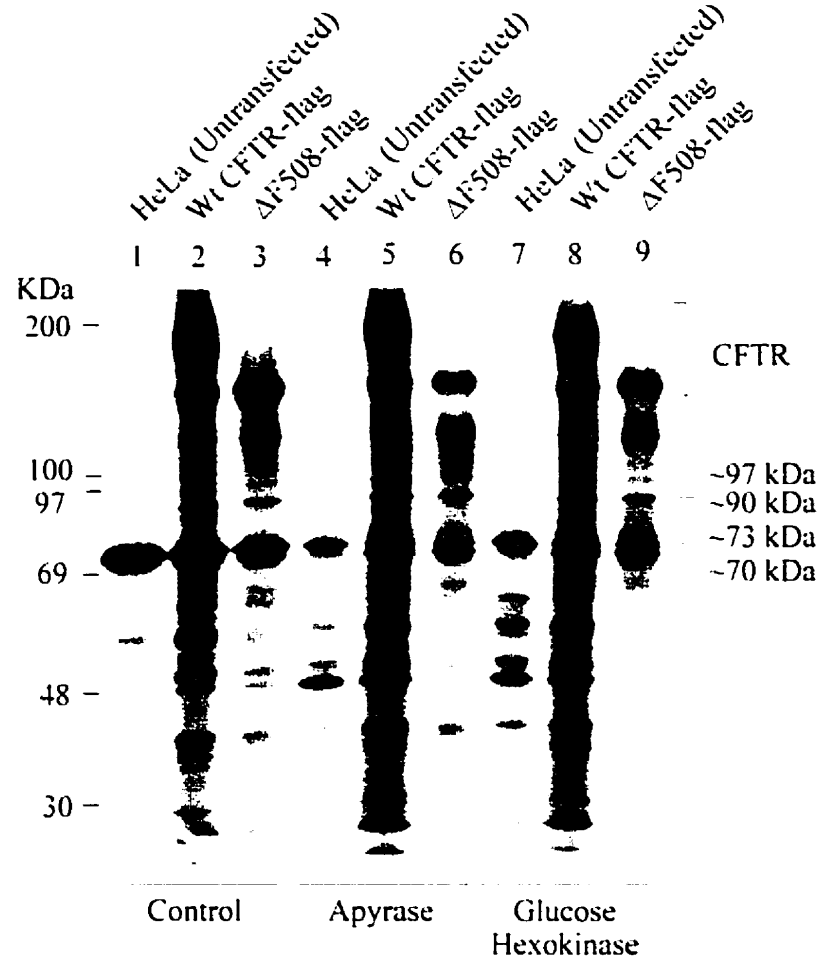

Figure 14. The effect of ATP depletion on the proteins recovered in the immunoprecipitates. Lanes 1-3, cells were labeled for 5 h and solubilized in control lysis buffer containing 0.1% Triton X-100, 25 mM Hepes, pH 7.5, 150 mM NaCl. CFTR was immunoprecipitated with 5 ml of anti-flag antibody; lanes 4-6 cells were lysed in above control buffer plus 25 units/ml apyrase to deplete ATP; lanes 7-9, cells were lysed in control buffer plus 25 units/ml hexokinase and 10 mM glucose to deplete ATP.

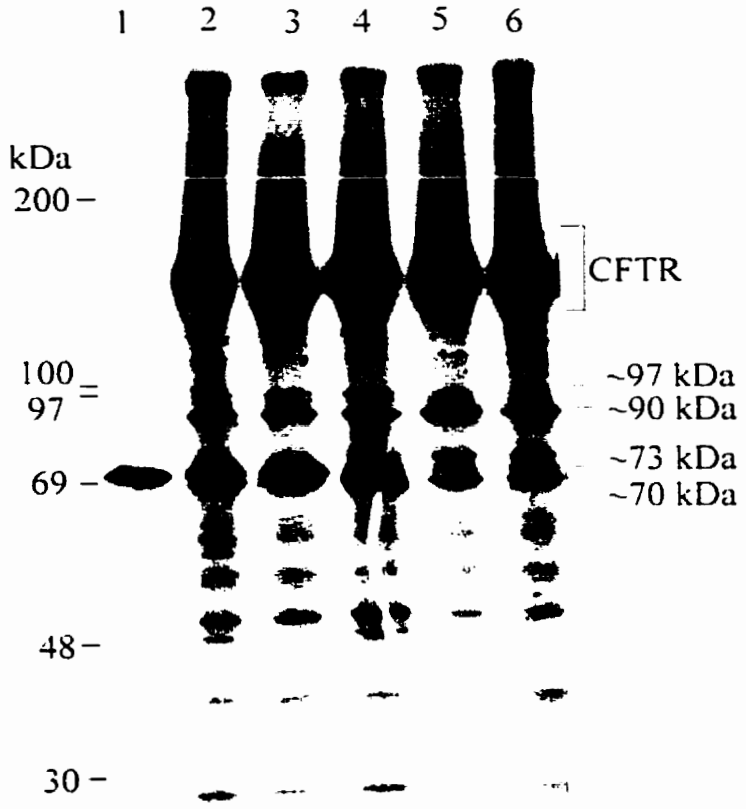

Figure 15. Manipulating the lysis conditions alters the intensity of protein bands in the immunoprecipitates. HeLa cells expressing ΔF508-Flag were radiolabeled with [^{35}S]-met for 5 h and lysed in buffer containing the common components of 0.1% Triton X-100, 25 mM Hepes, pH 7.5, 150 mM NaCl and 20 mM molybdate. Proteins were immunoprecipitated in 5.0 µl anti-flag antibody and analyzed by SDS-PAGE (5-10% gradient gel). Lane 1, no antibody was added; lane 3, cell lysates were precleared with 20 µl of protein-G sepharose before the addition of anti-flag antibody; lane 4, 25 units/ml apyrase was added to the lysis buffer; lane 5, 2 mM ATP and 2 mM $MgCl_2$ were added; lane 6, 2 mM ADP and 2 mM $MgCl_2$ were added.

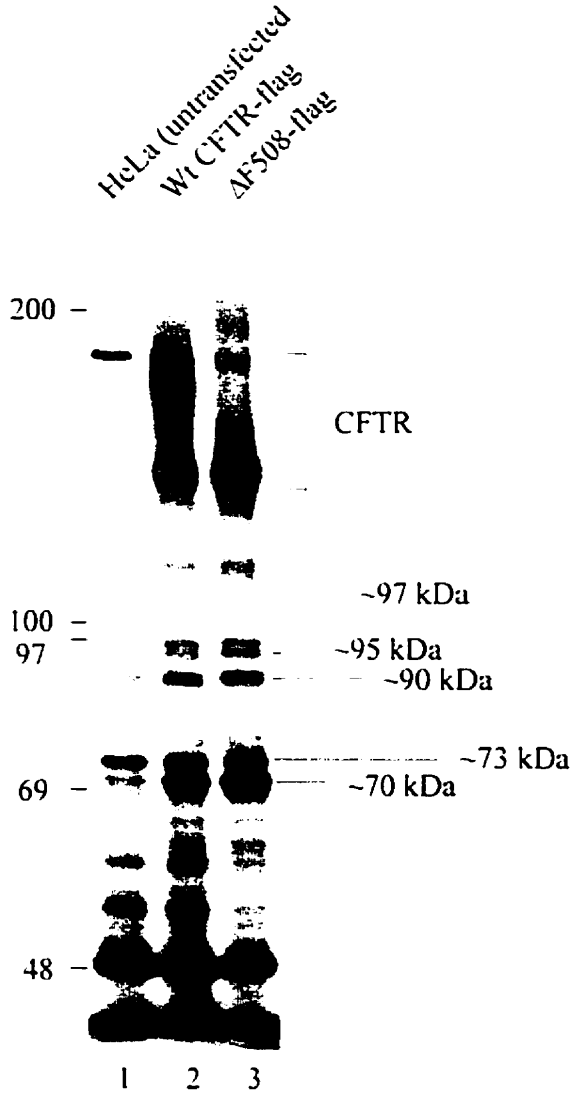

Figure 16. Immuoprecipitation of CFTR and specifically associated proteins. HeLa cells and HeLa cells expressing CFTR-Flag and ΔF508-Flag were metabolically labeled in 50 μCi/ml [^{35}S]-met for 5 h and solubilized in 1.0 ml of lysis buffer containing 0.2% digitonin, 25 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 20 mM Na molybdate, 2 mM ADP, 2 mM $MgCl_2$, 10 mM glucose and 25 units/ml hexokinase. 5.0 μl of anti-flag antibody was incubated in the lysate to precipitate CFTR and associated proteins. Proteins were analyzed by SDS-PAGE and fluorography (7% gel).

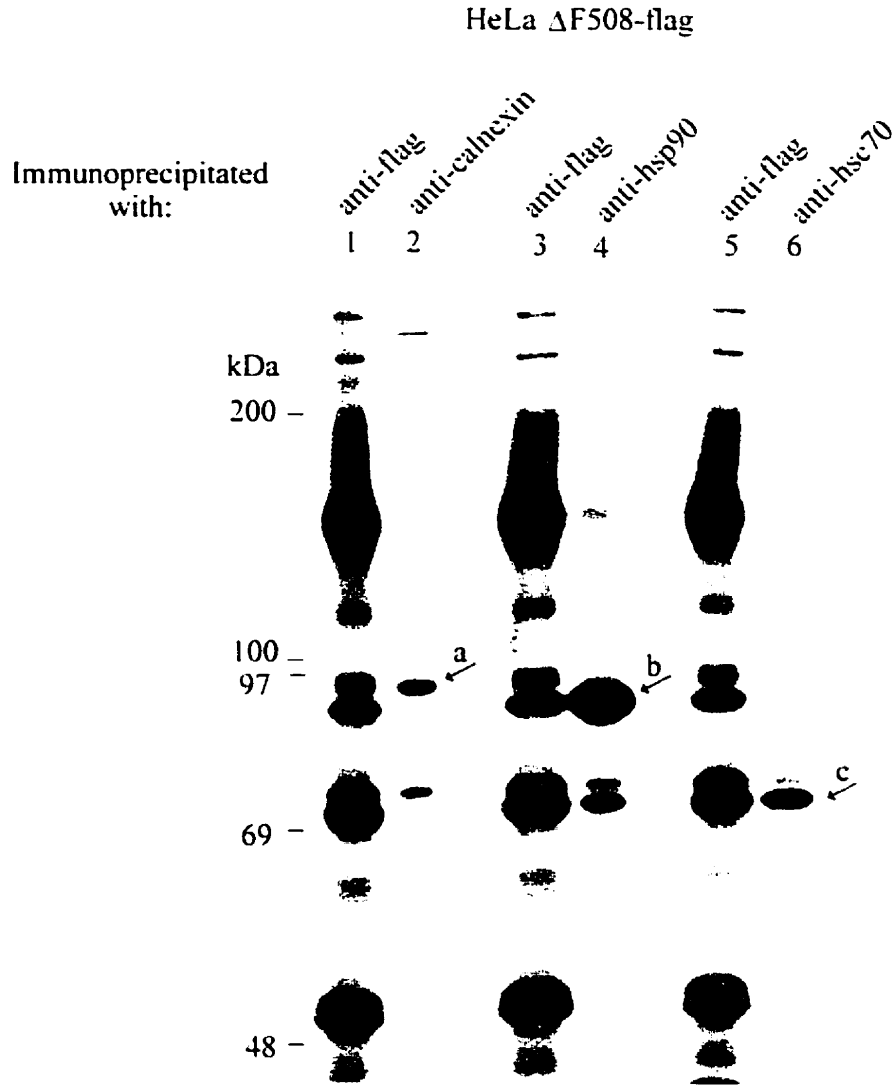

Figure 17. The identification of molecular chaperones found in the CFTR precipitates. HeLa cells expressing ΔF508-Flag were labeled for 5 h with [^{35}S]-met and solubilized in 0.2% digitonin buffer with 25 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 20 mM molybdate, 2 mM ADP and 2 mM $MgCl_2$, 25 units/ml hexokinase and 10 mM glucose. For immunoprecipitation, antibodies to CFTR (5.0 µl anti-flag), calnexin (5.0 µl R1), Hsp90 (1.0 µl), Hsc70 (2.0 µl) were added to 1.0 ml of lysate. Immunoprecipitates were washed and analyzed by SDS-PAGE (7% gel). As the calnexin, Hsp90 and Hsc70 immunoprecipitates produced very strong signals, only ¼ of their samples were loaded onto the gel. a., arrow pointing to calnexin; b., arrow pointing to Hsp90; c., arrow pointing to Hsc70.

this does not constitute a rigorous proof, the sum of all the results presented provides strong evidence that these three molecular chaperones are the bands that we observe in these co-immunopreciptations.

4.7 Is the ~97 kDa protein Grp94?

We attempted to identify the ~97 kDa protein that we observed to co-immunprecipitate with CFTR. One possible candidate for this protein is Grp94. Grp94 serves as a molecular chaperone in the ER (Melnick, 1992). It is a member of the Hsp90 family and has been shown to be a major Ca^{2+} and ATP binding component of the ER lumen (Booth, 1998; Clairmont, 1992). Grp94 has been implicated in assisting the folding and assembly of immunoglubulin apolipoprotein B and thyroglobulin (Melnick, 1992; Murasan, 1997; Linnik, 1998).

To test this possiblity, anti-Grp94 antibodies were used to immunoprecipitate Grp94 from HeLa cells stably expressing CFTR. The mobility of Grp94 following SDS-PAGE was compared to the co-immunoprecipitating with CFTR. If the mobility of Grp94 matches the unknown ~97 kDa protein, then further actions would be taken to ensure the 97 kDa protein is indeed Grp94. Stable transfectants expressing ΔF508 CFTR-Flag were metabolically labeled for 5 h. Cell extracts were prepared and proteins were isolated using anti-Flag and anti-Grp94 antibodies (Fig. 18). As illustrated in this figure, Grp94 migrated above the ~97 kDa protein (a faint band is presented at ~99 kDa). However, the anti-Grp94 antibody immunoprecipitated Grp94 poorly; a large quantity of antibody was used in this experiment, but it produced a very weak signal. To verify the above observation, a similar experiment was performed using an anti-KDEL antibody

(Fig. 19). Like many proteins localized to the lumen of the ER, Grp94 contains a carboxyl-terminal KDEL retention signal. The anti-KDEL produced much stronger signals than the anti-Grp94 antibody, with prominent bands at ~80 kDa (Grp78/Bip) and at ~99 kDa (Grp94). Once again however, the band identified as Grp94 migrated slower on the gel than the ~97 kDa band in the anti-flag immunoprecipitates. From this we conclude that the 97 kDa band is not Grp94.

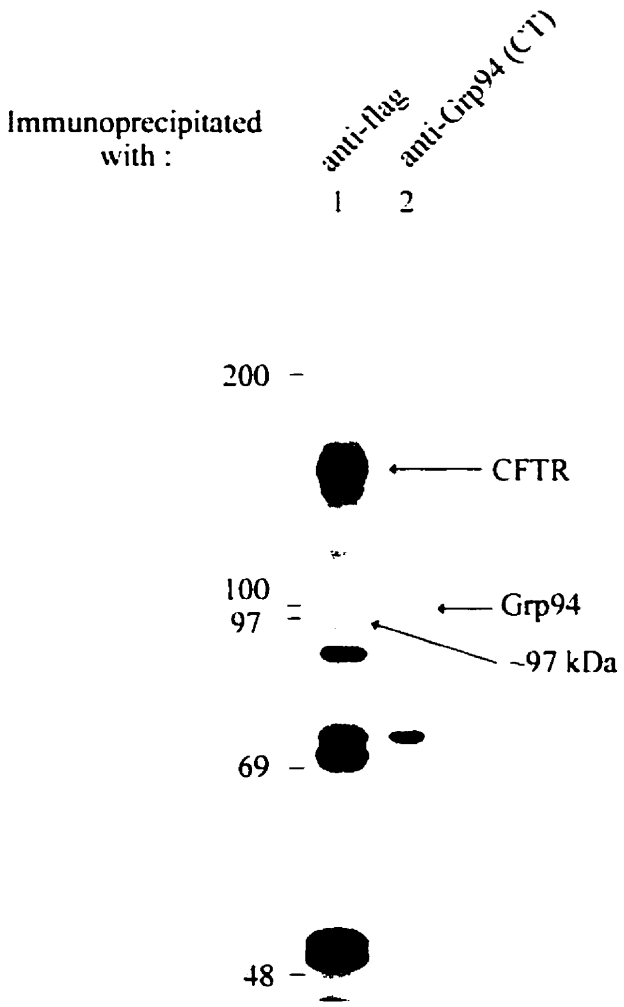

Figure 18. The ~97 kDa protein co-precipitating with CFTR does not co-migrate with Grp94 (I). HeLa ΔF508-Flag cells were labeled for 5 h and solubilized in 0.2% digitonin buffer. Anti-flag (5.0 µl) or anti-grp94 CT (20 µl) antibodies were used to immunoprecipitate CFTR and Grp94. Proteins were analyzed by SDS-PAGE and fluorography.

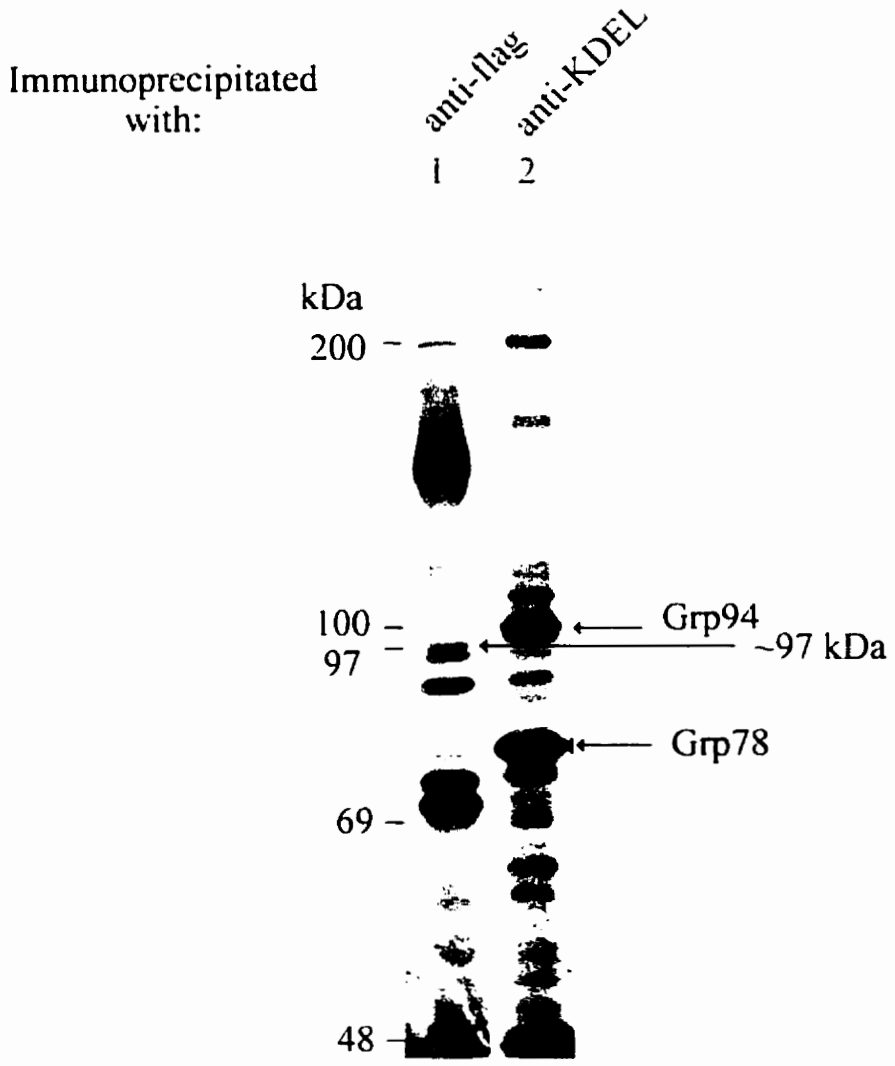

Figure 19. The ~97 kDa protein co-precipitating with CFTR does not co-migrate with Grp94 (II). [^{35}S]-labeled HeLa ΔF508-Flag cells were immunoprecipitated with anti-flag (5.0 µl) and anti-KDEL (10 µl) antibodies, and analyzed by SDS-PAGE and fluorography (7% gel).

5.0 DISCUSSION

Defective folding of nascent CFTR polypeptides and their subsequent inefficient maturation from the ER to the plasma membrane underlies the pathogenesis of most cases of CF (as reviewed in Chapter 1 of this thesis). This phenomenon is observed for many of the more than 800 mutations of CFTR that have been studied, including the common ΔF508 mutation (for review see: Riordan, 1999). The characteristics of this mutant are of prime importance to our understanding of the disease because it is present on at least one allele in ~90% of all patients with CF (Sferra and Collins, 1993). Loss of this amino acid decreases the efficiency of folding of *in vitro* models encompassing the NBD1 domain (Thomas et al., 1992; Qu and Thomas, 1996; Qu et al., 1997). It also appears to increase the probability that nascent CFTR chains become committed to degradation, by a mechanism that at least in part involves the ubiquitin/proteasome pathway (Ward and Kopito, 1994; Lukacs et al., 1994; Jensen et al., 1995; Ward et. al., 1995; Sato et al., 1998; Zhang et al., 1998). However, the F508 residue is not critically required for the Cl^- channel function of the CFTR molecule, as treatments to 'coax' small amounts of ΔF508 CFTR to the plasma membrane have shown this mutant to be functional. Such treatments have included growth of cells at reduced temperatures (Denning et at., 1992; Lukacs et al., 1993), or in the presence of 'chemical chaperones' such as glycerol (Sato et al., 1996; Brown et al., 1996). Thus, the frequency of the ΔF508 allele among CF patients and the fact that the Cl^--channel activity is not absolutely disrupted by this mutation, have made it important to determine if interventions could be identified to increase the efficiency of folding and intracellular processing of this mutant.

Clearly, the development of any potential strategies towards this goal would require a better understanding of the early steps in the folding process.

In recent years, much attention has been focused on identifying components of the quality control system that interact with newly synthesized CFTR. Logical candidates include molecular chaperones as well as mediators of ER degradation. Molecular chaperones identified to date that interact with CFTR during its biogenesis include Hsc70 and its co-chaperone Hdj-2, calnexin, and Hsp90 (Yang et al., 1993; Pind et al., 1994; Loo et al., 1998; Meacham et al., 1999). These chaperones form transient complexes with nascent, immature CFTR molecules. Dissociation of these complexes correlates temporally with the transit of CFTR to a post-ER compartment and with a large increase in the metabolic stability of the CFTR molecules. This maturation is an ATP-dependent process (Lukacs et al., 1994), producing a conformational change in the molecule that can be differentiated experimentally by distinct proteolysis patterns between the immature and the mature forms (Zhang et al., 1998). This study is the best evidence to date that there are conformational differences between the immature and mature forms of CFTR, differences that are critical determinants as to whether molecules are exported to the plasma membrane or retained in the ER and degraded. It is also the best evidence that wild-type CFTR attains a 'mature' conformation or structure that ΔF508 CFTR doesn't normally achieve.

Detailed studies of protein-protein interactions require specific and sensitive methods of detection. The CFTR-associated proteins that have been documented thus far were identified following immunoprecipitation studies with antibodies to known proteins of interest to see if they would co-precipitate nascent CFTR. Using an alternate strategy,

we wished to immunoprecipitate CFTR directly and examine for proteins that were specifically co-precipitated. An epitope-tagged version of CFTR was developed for these studies. We obtained a tool for the efficient and near-quantitative immunoprecipitation of CFTR from cell lysates by engineering a Flag-tag onto the carboxyl terminus of CFTR. A greater than 3.5-fold higher recovery of CFTR in immunoprecipitates was achieved using the anti-Flag antibody as compared to the M3A7 anti-CFTR antibody (Figure 9). The anti-Flag immunoprecipitations also yielded more CFTR than those performed using a combination of anti-CFTR monoclonals (M3A7 and L12B4), the previous 'gold standard' for maximal recoveries of CFTR (Figure 10A). While the gains achieved may seem small compared to the amount of work that was needed to develop this epitope-tagged system, these reagents were prerequisites to the results generated in the remainder of this thesis. Our goal was to identify proteins interacting with CFTR, interactions that were predicted to be transient *in vivo* and potentially labile during the cell lysis and immunoprecipitation procedure. Maximal recoveries of CFTR in the immunoprecipitates was essential in order to detect these interacting proteins.

An epitope tagging or fusion protein approach has been employed in several other studies in order to facilitate the detection of CFTR. Fusion proteins incorporating the green fluorescent protein at the amino (Moyer et al., 1998) or carboxyl (Loo et al., 1999) terminus of CFTR have been shown to function in a manner similar to wild-type CFTR. CFTR has also been tagged at the amino terminus with the influenza hemagglutinin (HA) epitope and detected at the cell surface by immunofluorescence (Haardt et al., 1999). Howard et al. (1995) tested both the Flag tag and the HA tag as epitopes added to the carboxyl terminus of CFTR. The resulting constructs were expressed in HeLa cells and

Xenopus oocytes and analyzed for Cl^--channel activity at the plasma membrane. The activity of the Flag-tagged construct could not be distinguished from wild-type CFTR in their assays, indicating that this epitope did not impair the function or intracellular transport of the protein. In contrast, the construct with the HA epitope displayed very low activity in HeLa cells and they concluded that this was due to defective targeting to the plasma membrane. A more thorough analysis of this construct was not reported.

Most of the tags that we added onto the carboxyl terminus of CFTR preserved its maturation and transport through the Golgi apparatus (as measured by modification of the carbohydrate chains) and presumably to the plasma membrane. The most dramatic exception to this was the Strep-tag, which consistently had a lower ratio of mature to immature CFTR than the other constructs (Figure 6). Although we did not assay any of our constructs for Cl^- channel activity in the plasma membrane, the above studies demonstrate that modifications to the carboxyl terminus do not impair function, as long as the protein reaches this destination. Our interest was to identify a tag that would function well in precipitation experiments of newly synthesized molecules and produce better recoveries of CFTR than the available anti-CFTR antibodies. The Flag-tag was clearly the best tag for this purpose. It did not interfere with the maturation of the protein and it produced the most sensitive immunoblots and highest recovery during precipitations. Surprisingly, these results were only found using the affinity-purified rabbit anti-Flag antibody (Santa Cruz) and not the M2 monoclonal antibody (Sigma) against this same epitope. We still do not know the reason for this difference. It is possible that the rabbit antibody has a higher affinity than the monoclonal, or that it recognizes a specific conformation of the epitope that is found on the CFTR construct. It

is also possible that the monoclonal antibody we purchased was defective, although we got the same result using two separate samples of this antibody. Nevertheless, the Flag-tagged CFTR was chosen for the rest of the studies because this eight amino acid peptide did not interfere with the maturation of CFTR and gave a strong recognition signal for immunoblots and immunoprecipitation studies using the rabbit antibody.

To obtain constitutive expression of CFTR-Flag fusion proteins, wild type and ΔF508 CFTR-Flag were subcloned into the pIRESneo expression vector and transfected into HeLa cells. This was necessary following several failed attempts at obtaining reasonable levels of stable expression using the pcDNA 3.1 expression vector. Numerous stable clones were obtained using this vector, however, they expressed low or undetectable levels of CFTR. The pIRES vectors were developed with two features to facilitate the isolation of stable clones expressing the gene of interest at high levels (Rees et al., 1996). The first of these is that the recombinant cDNA and the selection marker are transcribed from a single promoter element as a single, contiguous mRNA. Translation of the selection marker is enhanced by the presence of the encephalo-myocarditis virus internal ribosome entry site before the start codon of this sequence. This increases the probability that a clone expresses the gene of interest if it survives the selection process, as both genes are translated from the same bicistronic mRNA. The second feature of these vectors is that there is a synthetic intron incorporated into the mRNA, a feature that has been shown to enhance message stability, possibly by aiding export of the mRNA from the nucleus to the cytoplasm (Huang and Gorman, 1990). Regardless of the mechanism, we were able to successfully isolate several clones of HeLa cells expressing CFTR-Flag or the ΔF508-Flag using this expression vector and found

them all to express relatively high levels of the recombinant proteins. These cell lines reproduced the phenotypes observed for CFTR in other cells, with the advantage that the epitope provided a means for rapid and efficient immunoprecipitation of the recombinant proteins.

To enhance the protein-protein interactions between CFTR and its partners, the lysis condition and the components of lysis buffer were examined. After much manipulation to prevent the dissociation of molecules from CFTR, we could clearly distinguish radiolabelled Hsc70, Hsp90 and calnexin in the co-immunoprecipitates, in agreement with our predictions based upon previous reports (Yang et al., 1993; Pind et al., 1994; Loo et al., 1998). The depletion of ATP from cell lysates increased the recovery of Hsc70; the addition of molybdate to the lysis buffer strongly increased the recovery of Hsp90; and the presence of digitonin, but not Triton X-100, preserved the interaction of calnexin and CFTR. These interactions were only observed, or greatly increased, in the samples expressing either CFTR-Flag or ΔF508-Flag, indicating that they co-precipitated specifically with CFTR. They also interacted specifically with the immature form of CFTR, as they were present in the samples that only express immature CFTR (i.e. ΔF508 CFTR). The intensity of the three interacting proteins paralleled the amount of immature CFTR present in the samples. We conclude that our cell lines have provided an accurate representation of nascent CFTR interacting with these previously reported chaperone proteins. In fact, the intensities of the chaperone bands in our immunoprecipitates are greater and more convincing than those reported in the original papers. The novelty of our approach is that it does not require antibodies to the chaperones to detect the interactions. The interacting bands were detected as specific co-

precipitating, radiolabelled bands following labelling cells with [^{35}S]-met and immunoprecipitating CFTR. This approach provides the benefit that novel interacting proteins can be identified in the immunoprecipitates.

Along with the chaperone proteins that were found in the CFTR co-precipitates, we also consistently observed a faint band migrating at ~97 kDa, just above calnexin. This protein was co-immunoprecipitated specifically with CFTR since it was not present in control immunoprecipitates from untransfected HeLa cells. As an initial approach to identify this protein, we searched for known proteins with similar molecular weights that might interact with proteins on either side of the ER. We attempted to determine if the ~97 kDa protein was the ER chaperone Grp94, by comparing their migration following SDS-PAGE. The anti-Grp94 or anti-KDEL immunoprecipitates were run side by side with the anti-flag immunoprecipitates and the band identified as Grp94 migrated slower on the gel than the ~97 kDa band (Figs 17 and 18). Thus, Grp94 is not the ~97 kDa band that co-immunoprecipitated with CFTR.

A second potential candidate for the ~97 kDa protein was revealed following a literature search. The 26S proteasome is composed of a 20S proteolytic core (the proteasome) and a 19S regulatory complex. A protein of 97 kDa named subunit 2 is part of the regulatory complex (Rechsteiner et al., 1993; Goldberg, 1995). This protein has also been identified as TRAP2, a protein isolated from a HeLa cell library that interacts with the cytoplasmic domain of the type 1 receptor for tumor necrosis factor (Dunbar et al., 1997). Studies with the yeast equivalent to TRAP2 suggest that this component of the regulatory complex is important to ubiquitin-dependent protein proteolysis (Tsurumi et al., 1996). In addition, the observations that the yeast homolog of TRAP2, Hrd2, is

involved in the degradation of the ER protein HMG-CoA reductase (Hampton et al., 1996) and that CFTR can be degraded by the proteasome (Ward et al., 1995; Jensen et al., 1995), makes it pertinent to ask whether the p97 protein we see is TRAP2. This would be a very significant finding, showing a direct connection from CFTR to the proteasome. One way to test this possibility is to obtain an antibody to TRAP2 to examine whether it recognizes and had the same mobility as the ~97 kDa protein in our immunoprecipitates. A sample of the antibody raised against the HeLa TRAP2 protein was obtained from Dr. D. B. Donner (Dunbar et al., 1997), however this reagent arrived after the experimental section of this thesis was completed. It will be an important future direction to test whether this antibody recognizes the CFTR-associated p97. If the mobility of TRAP2 on SDS-PAGE does not match the ~97 kDa protein found in the CFTR precipitates, then an alternate approach would be advisable to identify this protein. Scaling up the anti-flag immunoprecipitation protocol would hopefully isolate enough of the p97 protein for analysis by microsequencing. Obtaining sequence data on this protein will be important. Comparison with sequence databases will reveal whether this protein has been previously identified. A possible alternative to this identification involves the preparation of monoclonal antibodies to the proteins eluted from anti-flag affinity columns. Antibodies specific for the ~97 kDa protein will be selected and used for the further characterization of the protein. In addition, antibodies to the ~97 kDa protein generated can be used to determine the kinetics and specificity of the interaction of with CFTR.

The above data suggests that at least three chaperones and the ~97 kDa protein participate in the biogenesis of CFTR. However, it remains to be established whether these chaperones act sequentially or simultaneously during this process. Another key

unresolved question is how or whether these chaperone interactions contribute to the retention or degradation of misfolded CFTR chains in the ER. Hsc70, Hsp90, calnexin and the ~97 kDa protein all interact with the immature forms of CFTR. However, all of these interactions occur with both the wild-type and the mutant CFTR — no major differences in the stoichiometry or the kinetics of the chaperone-CFTR interactions have been found. This result may be a reflection of the fact that like ΔF508 CFTR, most of the wild-type CFTR is also misfolded and degraded in the ER. It is also unresolved as to whether these chaperones are acting in an attempt to assist the folding of the immature molecules, or if they are binding specifically to the misfolded molecules and marking them for degradation. In this regard, if the ~97 kDa protein co-immunoprecipitating with CFTR is the TRAP2 protein, it may be a link for targeting misfolded molecules to the proteosome for degradation.

To account for the inefficient processing of wild-type CFTR and the virtually complete retention of ΔF508 mutants in the ER, several groups have proposed a model that is based on the relative kinetics of CFTR folding and degradation (Ward and Kopito, 1994; Lukacs et al., 1995). According to this model, the folding of CFTR occurs with relatively slow kinetics and consequently, the kinetics of folding overlap with the kinetics of sorting misfolded proteins to the degradative pathway. As a result, a large fraction of newly synthesized CFTR becomes targeted for degradation before it can fold completely. The ΔF508 mutation likely further retards the kinetics of folding, such that virtually all of these mutant molecules are sorted to the degradative pathway before they can fold. In addition, the commitment to degrade misfolded CFTR may occur early in its biosynthesis, possibly even co-translationally. *In vitro* assays have shown that nascent

CFTR polypeptides can be ubiquitinated cotranslationally, before release from the ribosome (Sato et al., 1998). Indeed, misfolded CFTR and ΔF508 molecules are degraded rapidly, without an apparent lag after synthesis (Ward and Kopito, 1994). How can the "quality control" machinery discriminate between misfolded CFTR molecules and those are in the process of folding? Are there more chaperones that participate in folding and degradation of CFTR than those that we have observed in our assays? Pulse-chase and co-immunoprecipitation studies suggest that at least two chaperones of the ER lumen, BiP and Grp94, do not interact with either ΔF508 or CFTR. However, the participation of other chaperones or interacting proteins remains a possibility. Investigation of these interactions may provide answers to some of the questions posed above. Once all the interactions have been identified, extensive kinetic analyses will be required to elucidate the sequence of events in the biogenesis of nascent CFTR. What is perhaps the most clear conclusion we can reach from this discussion is that the steps involved in this process are complex and a far way from being understood. Any further progress towards this goal will increase our general knowledge as to how membrane proteins are synthesized and how these processes contribute specifically towards cystic fibrosis.

6.0 SUMMARY

The major conclusions that can be drawn from this thesis are as follows:

1) The addition of the Flag-tag onto the carboxyl terminus of CFTR provided a valuable reagent for our studies on the biogenesis of CFTR. The rabbit antibody to this epitope detected very low levels of CFTR on immunoblots and also yielded a high recovery of CFTR following immunoprecipitation. The addition of this epitope did not interfere with the maturation or the intracellular transport of the modified CFTR.

2) Stable HeLa cell lines expressing the CFTR-flag constructs were generated using the pIRES expression vector. The isolation of stable clones expressing acceptable levels of CFTR was dramatically easier with this vector than with pcDNA.

3) We confirmed an interaction of Hsp70, calnexin and Hsp90 with the immature forms of wild-type and ΔF508 CFTR. Specific, radiolabelled bands representing each of these three chaperone proteins were observed to co-precipitate with the Flag-tagged CFTR constructs. The recovery of the chaperone bands was sensitive to the composition of the buffer used to lyse the radiolabelled HeLa cells and wash the immunoprecipitates.

4) A novel protein of ~97kDa was observed to co-precipitate with both wild-type and ΔF508 CFTR. The identity of this band remains to be determined, however it is not the ER chaperone Grp94. It will be of interest to continue the studies to identify this protein and determine its role in the biogenesis of CFTR.

7.0 REFERENCES

Akabas, M.H., C. Kaufmann, T.A. Cook, and P.Archdeacon. 1994. Amino acid residues lining the chloride channel of the cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry* 269:14865-14868

Anderson, K.S. and P. Cresswell. 1994. A role for calnexin (IP90) in the assembly of class II MHC molecules. *European Molecular Biology Organization Journal* 13:675-682

Anderson, M.P., H.A. Berger, D.P. Rich, R.J. Gregory, A.E. Smith and J.M. Welsh. 1991. Nucleotide triphosphates are required to open the CFTR chloride channel. *Cell* 67:775-784

Anderson, M.P., R.J. Gregory, S. Thompson, D.W. Souza, and S. Paul. 1991. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253:202-205

Anderson, M.P., D.P. Rich, R.J. Gregory, A.E. Smith, and J.M. Welsh. 1991. Generation of cAMP-activated chloride currents by expression of CFTR. *Science* 251: 679-682

Anderson, M.P., and J. M. Welsh. 1992. Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains. *Science* 257:1701-1704

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1994. *Current Protocols In Molecular Biology*. New York: Greene Publishing Associates and Wiley-Interscience.

Baukrowitz, T., T.C. Hwang, A.C. Nairn, and D.C. Gadsby. 1994. Coupling of CFTR Cl^- channel gating to an ATP hydrolysis cycle. *Neuron* 12:473-482

Bear, C.E., C. Li, N. Kartner, R.J. Bridges, and T.J. Jensen. 1992. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* 68:809-818

Bonifacino, J.S., and J. Lippincott-Schwartz. 1991. Degradation of proteins within the endoplasmic reticulum. *Current Opinion in Cell Biology* 3:592-600

Booth, C.L., K.R. Brouwer, and K.L. Brouwer. 1998. Effect of multidrug resistance modulators on the hepatobiliary disposition of doxorubicin in the isolated perfused rat liver. *Cancer Research* 58 (16):364-368

Brodsky, J.L., and A.A. McCracken. 1997. ER-associated and proteasome-mediated protein degradation: how two topologically restricted events came together. *Trends in Cell Biology* 7: 151-156

Brown, C.R., L.Q. Hong-Brown, J. Biwersi, A.S. Verkman, and W.J. Welsh. 1996. Chemical chaperones correct the mutant pheno-type of the delta F508 cystic fibrosis transmembrane conductance regulator protein. *Cell Stress Chaperones* 1: 117-125

Carson, M.R., S.M. Travis, and M.J. Welsh. 1995. The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions controlling channel activity. *Journal of Biological Chemistry* 270:1711-1717

Chang, X.-B., L. Cui, Y.-X. Hou, T.J. Jensen, A.A. Aleksandrov, A. Mengos, and J.R. Riordan. 1999. Removal of multiple arginine-framed trafficking signals overcomes misprocessing of ΔF508 CFTR present in most patients with cystic fibrosis. *Molecular Cell* 4: 137-142

Chen, M., and J.-T. Zhang. 1999. Topogenesis of cystic fibrosis transmembrane conductance regulator (CFTR): regulation by the amino terminal transmembrane sequences. *Biochemistry* 38: 5471-5477

Cheng, S.H., R.J. Gregory, J. Marshall, S. Paul, D.W. Souza, G.A. White, C.R. O'Riordan, and A.E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 63:827-834

Cheng, S.H., D.P. Rich, S. Marshall, R.J. Gregory, M.J. Welsh and A.E. Smith. 1991. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* 66: 1027-1036

Cheung, M., and M.H. Akabas. 1996. Identification of cystic fibrosis transmembrane conductance regulator channel-lining residues in and flanking M6 membrane-spanning segment. *Biophysical Journal* 70: 2688-2695

Clairmont C.A., A. De Maio, and C.B. Hirschberg. 1992. Translocation of ATP into the lumen of rough endoplasmic reticulum-derived vesicles and its binding to luminal proteins including BiP (GRP 78) and GRP 94. *Journal of Biological Chemistry* 267(6):3983-90

Corey, M., P. Durie, D. Moore, G. Forstner, and H. Levison. 1989. Familial concordane of pancreatic function in cystic fibrosis. *Journal of Pediatrics* 115:274-277

Dalemans, W., R. Barbry, G. Champigny, S. Jallat, K. Dott, D. Dreyer, R.G. Crystal, A. Pavirani, J.-P. Lecocq and Lazdunski, M. 1991. Altered chloride ion channel kinetics associated with the ΔF508 cystic fibrosis mutation. *Nature* 354:526-528

Dean, M., M.B. White, J. Amos, B. Gerrard, C. Stewart, K.-T. Khaw and M. Leppert. 1990. Multiple mutations in highly conserved residues are found in mildly affected cystic fibrosis patients. *Cell* 61: 863-870

Denning, G.M., M.P. Anderson, J.F. Amara, J. Marshall, A.E. Smith, and M.J. Welsh. 1992. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature* 358: 761-764

Devoto, M., L. De Benedetti, Seia, M., L. Piceni Sereni, M. Ferrari, M.L. Bonduelle, A. Malfroot, W. Lissens, A. Balassopoulou, G. Adam, D. Loukopoulos, P. Cochaux, G. Vassart, R. Szibor, J. Hein, K. Grade, W. Berger, B.Wainwright and B. Romeo. 1989. Haplotypes in cystic fibrosis patients with or without pancreatic sufficiency from four European populations. *Genomics* 5: 894-898

Di Sant'Agnese, P.A. and V. S. Hubbard. The intestinal tract. In: *Cystic Fibrosis*, edited by L.M. Taussig. New York: Thieme-Stratton, 1984. p. 212-229

Doige C.A., and G.F. Ames. 1993. ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. *Annual Review of Microbiology* 47:291-319

Drumm, M.L., D.J. Wilkinson, L. S. Smit, R.T. Worrell, T.V. Strong, R.A. Frizzell, D.C. Dawson, and F.S. Collins. 1991. Chloride conductance expressed by ΔF508 and other mutant CFTRs in *Xenopus oocytes*. *Science* 254: 1797-1799

Durie, P., Corey, M., Kristidis, P., Markiewicz, D., Zielenski, J., and L.-C. Tsui. 1992. The range of exocrine pancreatic disease and specific CF gene mutations. *Pediatric Pulmonology* 8 [Suppl]:240

Dunbar, J., H.Y. Song, D. Guo, L.-W. Wu, and D.B. Donner. 1997. Two-hybrid cloning of a gene encoding TNF receptor-associated protein 2, a protein that interacts with the intracellular domain of the type 1 TNF receptor. *Journal of Immunology* 158: 4252-4259

Engelhardt, J.F., J.R. Yankaskas, S.A. Ernst, Y.Yang, C.R. Marino, R.C. Boucher, J.A. Cohn, and J.M. Wilson. 1992. Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genetics* 2: 240-247

Evan, G.I., G.K. Lewis, G. Ramsay and J. M. Biship. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Molecular and Cellular Biology* 5:3610-3616

Fedorov, A.N., and T.O. Baldwin. 1997. Co-translational protein folding. *Journal of Biological Chemistry* 272: 32715-32718

Frissell, R.A., G. Rechkemmer, and R.L. Shoemaker. 1986. Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science* 233:558-560

Gething, M.-J. and J. Sambrook. 1992. Protein folding in the cell. *Nature* 355:33-45

Gilbert, A., M. Jadot, E. Leontieva, S. Wattiaux-De Coninck, and R. Wattiaux. 1998. Delta F508 CFTR localizes in the endoplasmic reticulum-Golgi intermediate compartment in cystic fibrosis cells. *Experimental Cell Research* 242: 144-152

Goldber, A. L. 1995. Functions of the proteasome: the lysis at the end of the tunnel. *Science* 268:522

Gregory, R.J., S.H. Cheng, D.P. Rich, J. Marshall, S. Paul, K. Hehir, L. Ostedgaard, K.W. Klinger, M. J. Welsh, and A.E. Smith. 1990. Expression and characterization of the cystic fibrosis transmembrane conductance regulator. *Nature* 347:382-386

Gregory, R.J., D.P. Rich, S.H. Cheng, D.W. Souza, G.A. White, C.R. O'Riordan, and A.E. Smith. 1991. Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Molecular Cell Biology* 11:3886-3893

Gunderson, K.L., and R.R. Kopito. 1995. Conformational states of CFTR associated with channel gating: the role of ATP binding and hydrolysis. *Cell* 82:231-239

Hammond. C., and A. Helenius. 1995. Quality control in the secretory pathway. *Current Opinions in Cell Biology* 7: 523-529

Hampton, R.Y., R.G. Gardner, and J. Rine. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Molecular Biology of the Cell* 7: 2029-2044

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbour, New York. Cold Spring Harbour Laboratory Press

Heleniun, A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Molecular Biology of the Cell* 5: 253-265

Helenius, A., T. Marquart, and I. Braakman. 1992. The endoplasmic reticulum as a protein folding compartment. *Trends of Cell Biology* 2:227-231

Higgins, C.F. 1992. ABC transporters: from microorganisms to man. *Annual Review of Cell Biology* 8:67-113

Howard, M., M.D. DuVall, D.C. Devor, J.-Y. Dong, K. Henze, and R.A. Frizzell. 1995. Eitope tagging permits cell surface detection of functional CFTR. *American Journal of Physiology* 269: C1565-C1576

Huang , T., and C.M. Gorman. 1990. Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. *Nucleic Acids Research* 18:937-946

Hutchison K.A., L.F. Stancato, R. Jove, W.B. Pratt. 1992. The protein-protein complex between pp60v-src and hsp90 is stabilized by molybdate, vanadate, tungstate, and an endogenous cytosolic metal. *Journal of Biological Chemistry* 267(20):13952-7

Hyde, S.C., P. Emsley, M.J. Hartshorn, M.M. Mimmack, and H. Gileadi. 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346:362-65

Jackson, M. R., M. F. Cohen-Doyle, P. Peterson, and D. B. Williams. 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science* 263: 384-387

Jensen, T.J., M.A. Loo, S. Pind, D.B. Williams, A.L. Goldberg, and J.R. Riordan. 1995. Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* 83:129-135

Johnson L.G., J.C. Olsen, B. Sarkadi, K.L. Moore, R. Swanstrom, R.C. Boucher. 1992. Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. *Nature Genetics* 2(1):21-5

Jordon, M., A. Schallhorn, and F. Wurm. 1996. Transfection mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Research* 24: 596-601

Jovov, B., I.I Ismailov, and D.J. Benos. 1995a. Cystic fibrosis transmembrane conductance regulator is required for protein kinase A activation of an outwardly rectified anion channel purified from bovine tracheal epithelia. *Journal of Biological Chemistry* 270: 1521-1528

Jovov, B., I.I Ismailov, B.K. Berdiev, C.M. Fuller, E.J. Sorscher, J.R, Dedman, M.A. Daetzel and D.J. Benos. 1995b. Interaction between cystic fibrosis transmembrane conductance regulator and outwardly rectified chloride channels. *Journal of Biological Chemistry*. 270:29194-29200

Kälin, N., A. Claab, M. Sommer, E. Puchelle and B. Tümmler. 1999. ΔF508 CFTR protein expression in tissues from patients with cystic fibrosis. *Journal of Clinical Investigation* 33:1379-1389

Kartner, N., O. Augustinas, T.J. Jensen, A.L. Naismith, and J.R. Riordan. 1992. Mislocalization of ΔF508 CFTR in cystic fibrosis sweat gland. *Nature Genetics* 1:321-327

Kerem, B.S., J.A. Buchanan, P. Durie, M.L. Corey, H. Levison, J.M. Rommens, M. Buchwald and L.-C. Tsui. 1989. DNA marker haplotype association with pancreatic sufficiency in cystic fibrosis. *New England Journal of Medicine* 279:65-69

Kerem, B.S., J.M. Rommens, J.A. Buchanan, D. Markiewicz, T.K. Cox, A. Chakravarti, M. Buchwald, and L.C. Tsui. 1989. Identification of the cystic fibrosis gene: genetic analysis. *Science* 245:1073-1080

Khan, T.Z., J.S. Wagerner, T. Bost, J. Martinez, F.J. Accurso, and D.W. Riches. 1995. Early pulmonary inflammation in infants with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine* 151:1075-1082

Knowles M, J. Gatzy, and R. Boucher. 1981. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *New England Journal of Medicine* 305:1489

Ko, Y.H., and P.L. Pedersen. 1995. The first nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator can function as an active ATPase. *Journal of Biological Chemistry* 270:22093-22096

Ko, Y.H., M. Delannoy, and P.L. Pedersen. 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

Kopito, R.R. 1997. ER quality control: the cytoplasmic connection. *Cell* 88:427-430

Kopito, R.R. 1999. Biosynthesis and degradation of CFTR. *Physiological Reviews* 79: S167-S173

Li, C., M. Ramjesinghm, E. Reyes, T. Jensen, X. Chang, J.M. Rommens, and C.E. Bear. 1993. The cystic fibrosis mutation (DeltaF508) does not influence the chloride channel activity of CFTR. *Nature Genetics* 3:311-316

Linnik K.M., and H. Herscovitz. 1998. Multiple molecular chaperones interact with apolipoprotein B during its maturation. The network of endoplasmic reticulum-resident chaperones (ERp72, GRP94, calreticulin, and BiP) interacts with apolipoprotein regardless of its lipidation state. *Journal of Biological Chemistry* 273(33):21368-73

Loo, M.A., T.J. Jensen, L. Cui, Y.-X. Hou, X.-B. Chang and J.R. Riordan. 1998. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *European Molecular Biology Organization Journal.* 17:6879-6887

Lu, Y., X. Xiong, A. Helm, K. Kimani, A. Bragin and W.R. Skach. 1998. Co- and posttranslational translocation mechanisms direct cystic fibrosis transmembrane conductance regulator N terminus transmembrane assembly. *Journal of Biological Chemistry* 273: 568-576

Lukacs, G.L., X.B. Chang, C. Bear, N. Kartner, A. Mohamed, J.R. Riordan and S. Grinstein. 1993. The ΔF508 mutation decreases the stability of cystic fibrosis

transmembrane conductance regulator in the plasma membrane. *Journal of Biological Chemistry* 268:21592-21598

Lukacs, G.L., A. Mohamed, N. Kartner, X.B. Chang, J.R. Riordan and S. Grinstein. 1994. Conformational maturation of CFTR but not its mutant counterpart (ΔF508) occurs in the endoplasmic reticulum and requires ATP. *European Molecular Biology Organization Journal* 13:6076-6086

Massiah, M.A., Y.-H. Ko, P.L. Pedersen and A.S. Mildvan. 1999. Cystic fibrosis transmembrane conductance regulator: solution structures of peptides based on the Phe508 region, the most common site of disease-causing ΔF508 mutation. *Biochemistry* 38:7453-7461

Marshall, J., S. Fang, L.S. Ostedgaard, C.R. O'Riordan, D. Ferrara, J.F. Amara H. Hoppe, R.K. Scheule, M.J. Welsh, A.E. Smith, and S. H. Cheng. 1994. Stoichiometry of recombinant cystic fibrosis transmembrane conductance regulator in epithelial cells and its functional reconstitution into cells in vitro. *Journal of Biological Chemistry* 269:2987-2995

Meacham, G.C., Z.Lu, S. King, E. Sorscher, A. Tousson, and D.M. Cyr. 1999. The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *European Molcular Biology Organization Journal* 18:1492-1505

Melnick J., S.Aviel, and Y. Argon. 1992. The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. *Journal of Biological Chemistry* 267(30):21303-6

McDonough, S., Davidson, N., Lester, H.A., and N.A. McCarty. 1994. Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron* 13:623-634

Murasan, Z., and P. Arvan. 1997. Thyroglobulin transport along the secretory pathway. Investigation of the role of molecular chaperone, Grp94, in protein export from the endoplasmic reticulum. *Journal of Biological Chemistry* 272(42):26095-26102

Palleros, D.R., L. Shi, K.L. Reid, and A.L. Fink. 1994. Hsp70-protein complexes. Complex stability and conformation of bound substrate protein. *Journal of Biological Chemistry* 269: 13107-13114

Pasyk, E.A., and J.K. Foskett. 1995. Mutant (ΔF508) cystic fibrosis transmembrane conductance regulator Cl^- channel is functional when retained in endoplasmic reticulum of mammalian cells. *Journal of Biological Chemistry* 270:12347-12350

Pind, S., J.R. Riordan and D.B. Williams. 1994. Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis

transmembrane conductance regulator. *Journal of Biological Chemistry* 269:12784-12788

Price, E.M. 1994. Pediatric Pulmonology Supplement 10.

Qu, B.H., and P.J. Thomas. 1996. Alteration of the cystic fibrosis transmembrane conductance regulator folding pathway. *Journal of Biological Chemistry* 271: 7261-7264

Qu, B.H., E.H. Strickland, and P.J. Thomas. 1997. Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. *Journal of Biological Chemistry* 272: 15739-15744

Quinton P.M., and J. Bijman. 1983. Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with Cystic Fibrosis. *New England Journal of Medicine* 308:1185

Rajagopalan, S., Y. Xu and M. B. Brenner. 1994. Retention of unassembled components of integral membrane proteins by calnexin. *Science* 263: 387-390

Rechsteiner, M., L. Hoffman, and W. Dubiel. 1993. The multicatalytic 26 S proteases. *Journal of Biological Chemistry* 268:6065

Rees, R., J. Coote, J. Stables, S. Goodson, S. Harris and M.G. Lee. 1996. Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. *Biotechniques* 20: 102-110

Rich, D.P., M.P. Anderson, R.J. Gregory, S.H. Cheng and S. Paul. 1991. Expression of cystic fibrosis transmembrane conductance regulator corects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature* 347: 358-363

Riordan. J.R, J. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.L. Chou, et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245: 1066-73

Riordan, J.R. 1999. Cystic fibrosis as a disease of misprocessing of the cystic fibrosis transmembrane conductance regulator glycoprotein. *American Journal of Human Genetics* 64(6):1499-1504

Rommens J.M., M.C. Iannuzzi, B. Kerem, M.L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J.L. Cole, D. Kennedy, N. Hidaka, M. Zsiga, M. Buchwald, J.R. Riordan, L.-C. Tsui, F.S. Collins. 1989. Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science* 245:1059-1066

Rosenstein, B.J., and T.S. Langbaum. Diagnosis. In: *Cystic Fibrosis*, edited by L.M. Taussig. New York: Thieme-Stratton, 1984, p. 85-114

Rubi, T.T. 1986. Infection in patients with cystic fibrosis. *American Journal of Medicine* 81:73-77

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, Second Edition. Plainview, New York: Cold Spring Harbor Laboratory Press.

Sanders J.S., T.D. Pryor, and M.K.Wedel. 1980. Prolonged survival in an adult with cystic fibrosis. *Chest* 77(2):226-7

Sato. S., C.L. Ward. M.E. Krouse. J.J. Wine. and R.R. Kopito. 1996. Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *Journal of Biological Chemistry* 271: 635-638

Sato, S., C.L. Ward, and R.R. Kopito. 1998. Cotranslational ubiquitination of the cystic fibrosis transmembrane conductance regulator in vitro. *Journal of Biological Chemistry* 273:7189-7192

Schmidt, T.G.M., and A. Skerra. 1993. The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. *Protein Engineering* 6:109-122

Schmidt, T.G.M., and A. Skerra. 1994. One-step affinity purification of bacterially produced proteins by means of the "Strep tag" and immobilized recombinant core streptavidin. *Journal of Chromatography A* 676:337-345

Schmidt, T.G.M., J. Koepke, R. Frank and A. Skerra. 1996. Molecular interaction between the *strep*-tag affinity peptide and its cognate target, streptavidin. *Journal of Molecular Biology*. 255:753-766

Seibert, F.S., Loo, T.W., Clarke, D.W., and J.R. Riordan. 1997. Cystic fibrosis: channel, catalytic, and folding properties of the CFTR protein. *Journal of Bioenergetics and Biomembranes* 29:429-442

Sferra, T.J. and F.S. Collins. 1993. The molecular biology of cystic fibrosis. *Annual Review of Medicine* 44:133-14

Strickland, E., B.-H. Qu, L. Millen and P.J. Thomas. 1997. The molecular chaperone Hsc70 assists the *in vitro* folding of the N-terminal nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry* 272: 25421-25424

Teasdale R.D., and M.R. Jackson. 1996. Lignal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annual Review of Cell Developmental Biology* 12:27-54

Thomas, P.J., P. Shenbagamurthi X, Ysern and P.L. Pedersen. 1991. Cystic fibrosis transmembrane conductance regulator: nucleotide binding to a synthetic peptide. *Science* 251: 555-557

Thomas, P.J. Y.H. Ko and P.L. Pedersen. 1992. Altered protein folding may be the molecular basis of most cases of cystic fibrosis. *FEBS Letters* 312:7-9

Thomas, P.J., P. Shenbagamurthi, J. Sondek, J.M. Hullihen, and Pedersen PL. 1992. The cystic fibrosis transmembrane conductance regulator. Effects of the most common cystic fibrosis-causing mutation on the secondary structure and stability of a synthetic peptide. *Journal of Biological Chemistry* 267: 5727-5730

Thomas, P.J. and P.L. Pedersen. 1993. Effects of the delta F508 mutation on the structure, function, and folding of the first nucleotide-binding domain of CFTR. *Journal of Bioenergetics and Biomembr*anes 25:11-19

Towbin H, and J. Gordon. 1984. Immunoblotting and dot immunobinding--current status and outlook. *Immunological Methods* 72(2):313-40

Tsurumi, C., Y. Shimizu, M. Saeki, S. Kato, G. M. Demartino, C.A. Slaughter, M. Fujimuro, H. Yokosawa, M. Yamasaki, K.B. Hendil, A. Toh-e, N. Tanahashi and K. Tanaka. 1996. cDNA cloning and functional analysis of the p97 subunit of the 26 S proteasome, a polypeptide identical to the type-1 tumor-necrosis factor receptor associated protein-2/55.11. *European Journal of Biochemistry* 239:912

Varughese, M., A. Chi, A.V. Teixeira, P.J. Nicholls, J.M. Keith and S.H. Leppla. 1998. Internalization of a Bacillus anthracis protective antigen-c-Myc fusion protein mediated by cell surface anti-c-Myc antibodies. *Molecular Medicine* 4(2):87-95

Ward, C.L. and R.R. Kopito. 1994. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *Journal of Biological Chemistry* 269: 25710-25718
Ward, C.L., Omura, S., and Kopito, R.R. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83:121-127

Welsh, M.J. and A.E. Smith. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell* 73:1251-1254

Welsh, M.J., L.-C. Tsui, F.T. Boat, and A.L. Beaudet. 1995. Vol 3, 7th Ed. *Cystic fibrosis, in The Metabolic and Molecular Bases of Inherited Disease.* C.R. Scriver, et al., editors. McGraw-Hill: New York, p. 3799-3876

Winter, M.C. and M.J. Welsh. 1997. Stimulation of CFTR activity by its phosphorylated R domain. *Nature* 389: 294-296

Yang, Y., S. Janich, J.A. Cohn and J.M. Wilson. 1993. The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. *Proceedings of the National Academic Sciences of USA*. 90: 9480-9483

Yang, W. and B. Storrie. 1997. Expression of a cytoplasmically epitope-tagged human Golgi glycosyltransferase in homologous cells results in mislocalization of multiple Golgi proteins. *Cell Biology Int* 21: 223-228

Yang, W., R. Pepperkok, P. Bender, T.E. Kreis and B. Storrie. 1996. Modification of the cytoplasmic domain affects the subcellular localization of Golgi glycosyl-transferase. *European Journal of Cell Biology* 71: 53-61

Zerangue, N., B.Schwappach, Y.N. Jan and L.Y. Jan. 1999. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K_{ATP} channels. *Neuron* 22: 537-548

Zhang, F., N. Kartner and G.L. Lukacs. 1998. Limited proteolysis as a probe for arrested conformational maturation of ΔF508 CFTR. *Nature Structural Biology* 5:180-183

Zielenski J, R. Rozmahel, D. Bozon, B. Kerem, Z. Grzelczak, J.R. Riordan, J. Rommens, and L.C. Tsui. 1991. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* 10: 214-28