Expression, Purification and Characterisation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in *Saccharomyces cerevisiae*

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Abstract

Mutations in the eukaryotic integral membrane protein Cystic Fibrosis Transmembrane conductance Regulator (CFTR) cause the hereditary disease cystic fibrosis (CF). CFTR functions as an ion channel at the surface of epithelial cells and regulates the movement of chloride ions and water across the plasma membrane. CFTR is difficult to express and purify in heterologous systems due to its propensity to form insoluble aggregates and its susceptibility to degradation. Obtaining good yields of highly purified CFTR has proven problematic and contributes to our limited understanding of the structure and function of the protein. The most prevalent disease causing mutation, F508del, results in misfolded CFTR which is particularly unstable and is quickly targeted for degradation by the host system and is prevented from being trafficked to the plasma membrane. There are limited treatment options for patients with the F508del mutation and it is therefore of significant interest within CF research. New methods and assays are required to identify potential compounds which could correct the F508del mutation. This thesis investigates the use of *Saccharomyces cerevisiae* to express and purify codon optimised recombinant CFTR. The use of a green fluorescent protein (GFP) tag enabled quick and simple detection of CFTR in whole cells and after extraction from the plasma membrane. By optimising the culture conditions for CFTR expression and detergent solubilisation conditions, relatively high yields of full-length protein were obtained. When used as a chemical chaperone at the time of inducing CFTR expression, glycerol increased yields of full-length protein. Degradation of CFTR could be limited by inducing expression at an optimal cell density and by harvesting cells within a specific time window. CFTR was extracted by solubilisation in the mild detergent dodecyl-β-D-maltopyranoside (DDM) in the presence of up to 1 M NaCl with up to ~87% efficiency in some cases.

Using a gene optimisation strategy in which additional purification tags and a yeast Kozak-like sequence were added, the human CFTR (hCFTR) protein was expressed and purified. Fluorescence microscopy revealed CFTR localisation at the periphery of yeast cells. Immunoaffinity chromatography facilitated by the GFP tag at the C terminus of CFTR produced protein of up to 95% purity. An assessment of the thermal stability of this highly purified CFTR using a fluorescent probe binding assay revealed a denaturation midpoint (T_m) of ~43 °C. The ability of this assay to determine the stability of CFTR is encouraging and there is the potential to further develop it in a high-throughput manner to identify compounds which stabilise the F508del protein and which may hold the key to developing new treatments for CF.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

List of abbreviations

~	approximately
ΔF/ΔT	first derivative plot of fluorescence change
10His	deca-histidine tag
6His	hexa-histidine-tag
aa	amino acid
ABC	ATP-binding cassette
ATP	adenosine triphosphate
BHK	baby hamster kidney
CB	CFTR buffer
cCFTR	codon-optimised chicken CFTR
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFTR	cystic fibrosis transmembrane conductance regulator gene
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cl^-	chloride ion
COPII	coat protein complex II
CPM	7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin
CTAB	cetyltrimethylammonium bromide
DDM	dodecyl-β-D-maltopyranoside
DMSO	dimethylsulphoxide
E. coli	*Escherichia coli*
ECL	extracellular loop
eGFP	enhanced green fluorescent protein
endFP	endogenous fluorescent protein
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
GFP	green fluorescent protein

hCFTR	human CFTR
HSP70	heat shock protein 70
ICL	intracellular loop
LB	Lysogeny (Luria) broth
LBA	LB agar
LDAO	lauryldimethylamine oxide
LiPFO	lithium perfluoro-octanoic acid
LPG	lysophosphatidylglycerol
LPG14	lysophosphatidylglycerol-14
LPG-14	1-tetradecanoyl-*sn*-glycero-3-phospho- (1'-*rac*-glycerol)
mCFTR	codon-optimised murine CFTR
MPC	mass population culture
MRB	membrane resuspension buffer
MSD	membrane-spanning domain
MWCO	molecular weight cut off
NaPFO	sodium perfluoro-octanoic acid
NBD	nucleotide-binding domain
NBD1	nucleotide binding domain 1
OD_{600}	optical density at 600 nm
Opti-h*cftr*	codon optimised human CFTR gene
Opti-hCFTR	codon optimised human CFTR
Opti-Pgp	codon optimised Pgp
P. pastoris	*Pichia pastoris*
PBS	phosphate-buffered saline
PDB	protein data bank
Pgp	P-glycoprotein
PIC	protease inhibitor cocktail
PKA	protein kinase A

plCFTR	codon-optimised platypus CFTR
PTM	post-translational modification
R-domain	regulatory domain
RFU	relative fluorescence units
RPL3	ribosomal protein L3
S. cerevisiae	*Saccharomyces cerevisiae*
SB	SDS solubilisation buffer
SDS	sodium dodecyl sulphate
SMT3	suppressor of mif two 3
SOC	super optimal broth with catabolite repression
SP	stationary phase
SUMO	small ubiquitin-like modifier
T	temperature
TBS	Tris-buffered saline
T_m	denaturation midpoint
-URA agar	CFTR agar -uracil
WT	wild-type
WT-h*cftr*	wild-type CFTR gene with Kozak
WT-hCFTR	wild-type CFTR with Kozak
YPD	Yeast peptone dextrose
YSB	Yeast suspension buffer

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Finally, I would like to thank my family: my mum and step-dad (Lesley and Allen), my sister Michelle, and my wonderful nephew, Alex, whose enquiring mind and enthusiasm for learning inspires me every day. Their support and understanding over the last few years has been truly invaluable and I cannot thank them enough. I would like to dedicate this thesis to them in the hope that it helps in some way show my gratitude.

Chapter 1 - Introduction

1.1 Overview

Cystic fibrosis (CF) is a life-shortening disease which affects around 1 in 2500 live births within Caucasian populations (Trzcinska-Daneluti *et al.*, 2009). It is a recessive genetic disease and approximately 1 in 26 people in the UK population are carriers of one of the mutated alleles responsible for CF (Bobadilla *et al.*, 2002). Currently treatments for the large majority of CF patients are limited, and a major factor is the lack of structural and functional data available for the protein responsible for CF, the cystic fibrosis transmembrane conductance regulator (CFTR).

1.2 Biological membranes and membrane proteins

Biological membranes are an essential component of all living organisms. They are primarily composed of phospholipids and proteins (Storch and Kleinfeld, 1985). The amphipathic nature of phospholipids enables the formation of bilayers in which hydrophobic tails are sandwiched between hydrophilic heads (Fig. 1.1). This conformation allows fluidity within the membrane and lipids and proteins are able to diffuse laterally along the membrane plane as proposed in the 'fluid mosaic model' by S. J. Singer and G. L. Nicolson in 1972 (Singer and Nicolson, 1972). Although numerous modifications have been made to the model (Israelachvili, 1977, Jacobson *et al.*, 1995) the underlying principles are still widely accepted to describe membrane structure.

The function of biological membranes varies greatly and is dependent on the types of lipids and proteins they are composed of and their location within the cell. Functions include transportation, signal transduction and protein secretion (Storch and Kleinfeld, 1985).

Membrane proteins account for ~30% of proteins coded for in the human genome (Nugent and Jones, 2009). They are found either at the surface of a biological membrane, a peripheral membrane protein, or within the lipid bilayer, an integral, or transmembrane protein (Fig. 1.1).

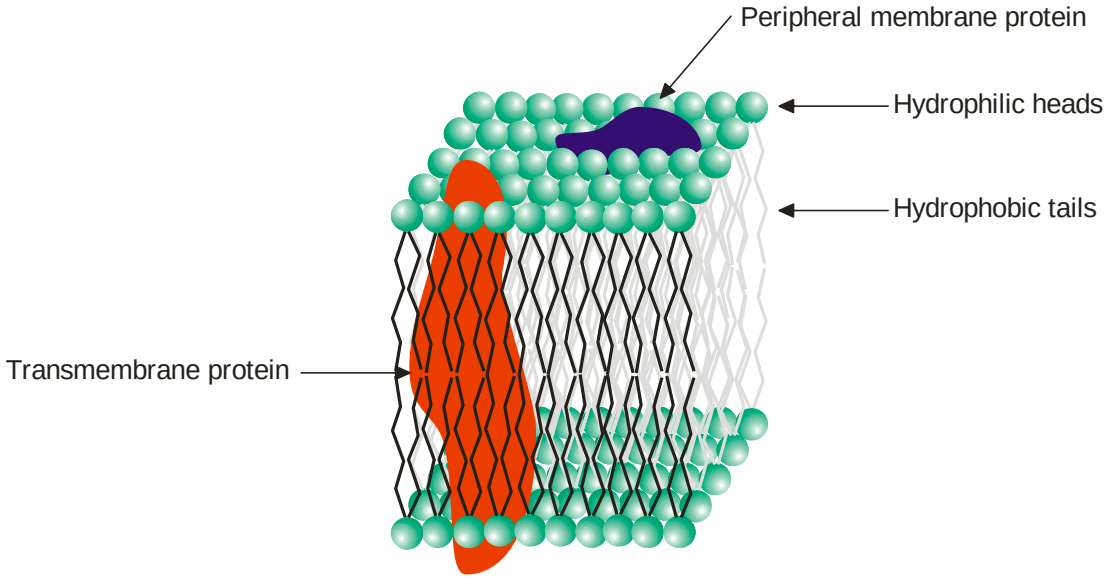

Figure. 1.1. Types of membrane protein and their association with the lipid bilayer. Transmembrane proteins are found within the bilayer and peripheral membrane proteins are found at the surface.

Integral membrane proteins can act as channels and mediate movement of molecules, including ions, through the lipid bilayer (Castle *et al.*, 2009). It is estimated that ~60% of drug targets are integral membrane proteins (Arinaminpathy *et al.*, 2009) due to their importance in fundamental cellular processes.

1.2.1 The study of membrane proteins

Membrane proteins are one of the most difficult protein classes to study and probably one of the least well understood. In order to study activity and function of membrane proteins, sufficient quantities must first be obtained from a suitable expression system (Newstead *et al.*, 2007b). This can be problematic as membrane proteins are often expressed at low levels in their native environment and as a result represent <3% of the total proteins deposited (total of 101948) in the protein data bank (PDB) (http://www.rcsb.org/pdb/results/results.do?qrid=A017AD14&tabtoshow=Current; accessed 27/07/2014, search results included alpha-helical, beta-barrel and monotopic membrane proteins).

1.2.2 Heterologous expression of membrane proteins

Membrane proteins are often present at low concentrations under normal physiological conditions and expression levels are usually insufficient to perform the desired functional and structural studies. To overcome this problem, the use of recombinant expression systems, which can be manipulated to overexpress a protein of interest, can be employed (McLuskey *et al.*, 2008). Genetic manipulation of recombinant proteins can also enhance yields of membrane proteins obtained from these systems. An array of recombinant protein expression systems are available including those derived from bacteria, yeast, insect and mammalian cells. Bacterial expression systems, such as *Escherichia coli* (*E. coli*) exhibit the advantage of being inexpensive and fast to grow in the laboratory but unfortunately, they are usually unsuitable for the production of lengthy eukaryotic membrane proteins such as CFTR. Conversely, insect and mammalian cell systems possess the necessary cellular components to produce membrane proteins such as CFTR, but are expensive and for mammalian cell cultures, yields are low. Insect cell expression using a baculovirus system offers a compromise, but for CFTR as well as several other human ATP-binding cassette (ABC) proteins, solubilisation of the protein can only be achieved with harsh detergents, implying that the system generates poor quality protein (Fitzgerald, E., Imperial College of Science, Technology, Medicine, personal communication). Recombinant protein expression in yeast systems such as *Saccharomyces cerevisiae* (*S. cerevisiae*) or *Pichia pastoris* (*P. pastoris*) is relatively inexpensive and the cells are fast to grow whilst also being capable of performing similar folding and post-translational modification processes to those found in higher eukaryotic cells. Yeast expression systems are also relatively easy to manipulate and recombinant protein expression can be tightly regulated. Cultures can be scaled up with ease and for *P. pastoris*, grown to high cell densities allowing greater yields of protein.

1.2.3 Use of tagged proteins

A protein tag is a peptide sequence that can be fused to a recombinant protein. The properties of a tag can be exploited for a variety of purposes. Fluorescent proteins are extensively used in cell biology as a reporter of expression and also in microscopy for real time expression and localisation of proteins (Phillips, 2001). Fluorescence is produced when a specific functional group of a molecule, the fluorophore, absorbs energy at a particular wavelength and emits the energy at another, equally specific but

longer wavelength. A commonly used tag, enhanced green fluorescent protein (eGFP) is ~27kDa and is the product of a point mutation (S65T) in the native green fluorescent protein (GFP) (Tsien, 1998, Zacharias and Tsien, 2006). eGFP emits fluorescence at a wavelength of ~507-509 nm when excited at a wavelength of ~488 nm (Patterson *et al.*, 1997, Ilk *et al.*, 2004, Zacharias and Tsien, 2006) and has an extra excitation peak at ~475 nm which native GFP does not have. Various derivatives of GFP are commercially available including a range of coloured fluorescent proteins produced by inducing various mutations in native GFP.

A useful tag used for recombinant protein expression in prokaryotic systems is the cleavable small ubiquitin-like modifier (SUMO) protein which has also been shown to improve expression of functional proteins in eukaryotic systems by increasing their stability and solubility during the post-translational modification. Sumoylation is involved in a variety of essential cellular processes including protein activation, stability and cell cycle progression (Panavas *et al.*, 2009). In eukaryotic systems, proteolytic cleavage of the SUMO tag occurs by naturally occurring proteases. To combat this problem, LifeSensors Inc. designed and generated a variant of SUMO. The improved tag, designated SUMOstar and based on the yeast homologue, suppressor of mif two 3 (SMT3), can be cleaved from the fusion protein post-purification, using a SUMOstar-specific protease, also developed by LifeSensor Inc. (Liu *et al.*, 2008). SUMOstar fusions exhibit improved expression, folding and solubility in eukaryotic expression systems (Liu *et al.*, 2008, Rozen-Gagnon *et al.*, 2012).

Affinity tags are widely used to purify proteins, the most common being the poly(His) tag which binds to metal matrices, including nickel and cobalt. Recombinant proteins fused to this tag can therefore be separated using affinity chromatography. His tags usually consist of at least five histidine residues but may contain up to ten, with the most commonly used being the hexa-histidine-tag (6His), with six histidine residues (Terpe, 2003). Unfortunately, purification of His-tagged proteins usually requires a further step due to binding and subsequent elution of His-containing contaminants. More specific binding, and therefore greater purity, can be achieved using epitope tags, such as the FLAG® tag. The FLAG tag is a short polypeptide sequence which comprises eight amino acid residues (DYKDDDDK) for which commercial antibodies are available including ANTI-FLAG M2 gel (*Sigma Aldrich*) in which ANTI-FLAG is covalently attached to agarose and may be used for purification or immunoprecipitation of FLAG fusion proteins. The high specificity of epitope tags can greatly increase purity of recombinant proteins.

1.2.4 Extraction of membrane proteins from heterologous expression systems

Following heterologous expression of membrane proteins, extraction from the host system is necessary. To facilitate this, cells expressing the protein of interest must first be lysed to disrupt the cell membranes. Crude membranes are then isolated by either density gradient fractionation or a series of differential centrifugation steps to isolate the cellular fractions. Proteins are then dissociated from the membranes and are solubilised in detergents to allow standard chromatographic purification methods to be applied (Duquesne and Sturgis, 2010). Solubilisation is a necessary step to prepare samples for purification and the choice of detergent for this process is critical to maintain structure, stability and functionality of membrane proteins. Upon solubilisation, proteins are separated from the lipid membrane and form protein-detergent complexes. As with lipids, detergents are amphipathic molecules, comprising a hydrophilic (polar) head group and a hydrophobic (non-polar) tail. Detergent molecules however, are much more soluble than lipids, and have the ability to form micelles in aqueous solution when their concentration exceeds the critical micelle concentration (CMC). The CMC varies for different detergents being strongly influenced by the hydrocarbon chain length, and is an important factor when solubilising membrane proteins. A range of detergents are available to facilitate solubilisation and they generally fall in to one of three groups; ionic, non-ionic and zwitterionic. Ionic detergents, such as sodium dodecyl sulphate (SDS), sodium perfluoro-octanoic acid (NaPFO), lysophosphatidylglycerol (LPG) and cetyltrimethylammonium bromide (CTAB) exhibit anionic and (CTAB) cationic head groups. They are usually highly efficient at solubilising membrane proteins but are often denaturing resulting in protein unfolding. Non-ionic detergents, including dodecyl-β-D-maltopyranoside (DDM) are generally considered non-denaturing with uncharged head groups and are much milder for proteins than their ionic counterparts. Although solubilisation efficiency is usually lower, DDM is often the preferred detergent for extraction of functional and structurally stable membrane proteins (Seddon *et al.*, 2004, Lin and Guidotti, 2009, Duquesne and Sturgis, 2010). Despite their net zero charge, zwitterionic detergents, such as lauryldimethylamine oxide (LDAO) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) are not as mild as non-ionic detergents, but are generally less denaturing than ionic detergents. The choice of detergent is protein specific and it is common to test a range of detergents and select the most suitable dependent on the intended downstream applications for the extracted membrane protein. This may require a compromise of solubilisation efficiency to obtain functional, correctly folded and structurally stable protein.

1.3 ABC transporters

The ABC transporter family of proteins is one of the largest superfamilies of membrane proteins with members found in all taxanomic phyla recognized today (Jones and George, 2004). Members of the family typically have four domains, two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs) which bind adenosine triphosphate (ATP; (Rosenberg *et al.*, 2005, Kos and Ford, 2009, Ward *et al.*, 2009). It is this binding, and subsequent hydrolysis of ATP that enables gating and transport activity of the ABC proteins (Chami *et al.*, 2002) by causing conformational changes in the MSDs (Hollenstein *et al.*, 2007). A mechanism which is not yet fully understood is the interaction and transport of drugs across the TMDs (Lee *et al.*, 2002, Lee *et al.*, 2008).

1.4 Cystic Fibrosis Transmembrane conductance Regulator

The cystic fibrosis transmembrane conductance regulator gene (*CFTR)*, also known as ABCC7, encodes the CFTR protein which is a unique member of the ABC family of proteins (Riordan and Chang, 1992). CFTR is a large, 1480 amino acid (aa) long, multidomain, transmembrane protein (Amaral, 2005) which, as with other ABC transporters, consists of four domains: two MSDs with six alpha-helical transmembrane segments each and two NBDs (Fig. 1.2) (Kos and Ford, 2009). The TMDs each have three extracellular loops (ECLs) and two intracellular loops (ICLs). Unlike other members of the ABC family, CFTR has a regulatory domain (R-domain) (Gadsby *et al.*, 2006, Zhang *et al.*, 2009, Tosoni *et al.*, 2013) and also contains a 32-residue domain known as the regulatory insertion (RI), found within NBD1 of the protein (Aleksandrov *et al.*, 2010, Zhang *et al.*, 2011). It is generally accepted that CFTR functions as an ion channel in the transport of chloride ions (Cl^-) and bicarbonate ions across epithelial cell membranes (Bear *et al.*, 1992) and can also facilitate the movement of other ions such as iodide (I^-). It's function as an ion channel is unique among ABC transporters (Gadsby *et al.*, 2006). Some researchers propose that CFTR is not actually a chloride ion channel itself (Hyde *et al.*, 1990) but may be an ATP-powered pump which regulates other chloride ion channels (Miller, 2010). There is also a proposal that CFTR is a glutathione pump that maintains a relatively high concentration of this compound in the airway surface liquid (Kogan *et al.*, 2003).

As with other ABC transporters, both NBDs can bind ATP although NBD1 has a higher binding affinity for ATP than NBD2 (Aleksandrov *et al.*, 2002, Basso *et al.*, 2003).

Distinct from most other ABC transporters, ATP hydrolysis occurs primarily at NBD2, and ATP bound to NBD1 does not readily dissociate as it does from NBD2 (Szabó *et al.*, 1999, Aleksandrov *et al.*, 2002) although it is thought that binding at both sites is required for channel gating activity (Vergani *et al.*, 2003, Vergani *et al.*, 2005, Zhou *et al.*, 2006, Rosser *et al.*, 2008). A region rich in protein kinase A (PKA) phosphorylation sites, the R-domain of CFTR consists of ~200 residues (Hegedus *et al.*, 2009). CFTR channel gating is dependent on phosphorylation of the R-region, with nine phospho-residues identified (Dulhanty and Riordan, 1994, Winter and Welsh, 1997, Awayn *et al.*, 2005, Seavilleklein *et al.*, 2008, Bozoky *et al.*, 2013).

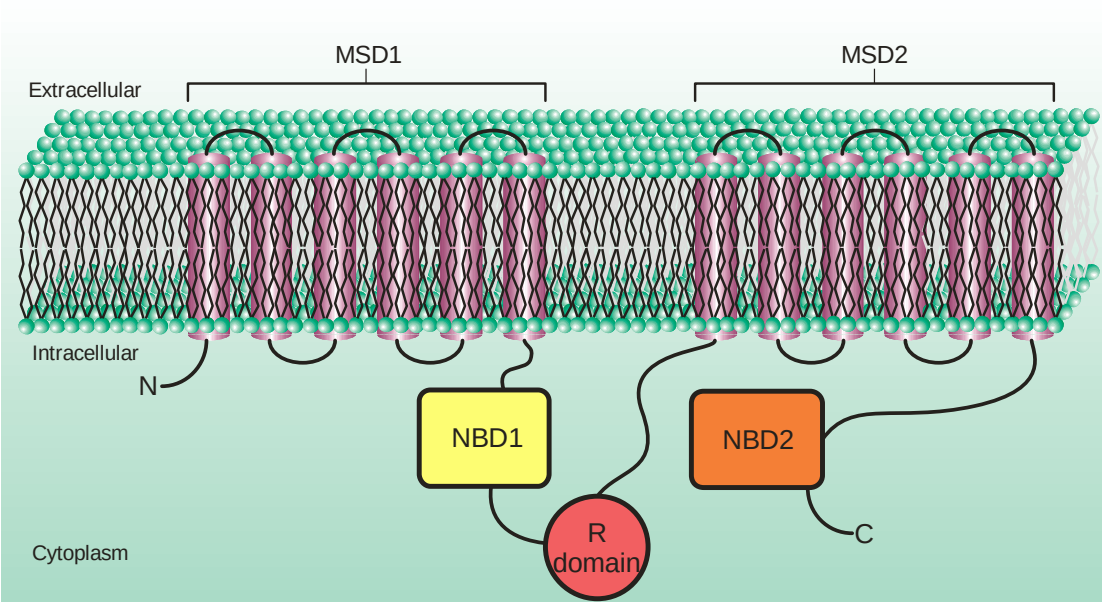

Figure 1.2. Schematic of CFTR showing two membrane-spanning domains (MSD1 and MSD2), two nucleotide-binding domains (NBD1 and NBD2) and the regulatory domain R-domain).

Our knowledge of CFTR structure is limited (Zhang *et al.*, 2009) but there is also a lack of functional data available for CFTR in its native environment. To overcome this, it is desirable to study the protein in a more native like environment such as an artificial lipid membrane. This can be achieved by insertion of the protein via membrane reconstitution to create proteoliposomes (Geertsma *et al.*, 2008). A major obstacle in obtaining structural and functional data is the difficulty in producing large enough quantities of pure, full-length CFTR due to its low expression levels in heterologous expression systems (Riordan, 2008).

There are over 1960 known mutations of the *CFTR* gene (http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html; accessed 14/02/14) causing an array of disorders with varying degrees of severity (Collawn *et al.*, 2009, Rosser *et al.*, 2009). F508del, a mutation resulting in the deletion of a phenylalanine at the 508th

residue of the protein, accounts for approximately 70% of CF cases (Bobadilla *et al.*, 2002). Patients homozygous for the F508del mutation have reduced ion channel function and water is unable to move through cell membranes, resulting in the production and accumulation of thick mucus in the epithelial cells that line the lungs, pancreas and other organs (Van Goor *et al.*, 2008). Bacteria can colonise the mucus and infections resulting in reduced lung function are the most common cause of deterioration and morbidity of CF patients (Ciofu *et al.*, 2013).

1.4.1 Classes of CFTR mutations

Five classes of CFTR mutation exist, each causing disruption of the *CFTR* gene (Guggino and Stanton, 2006). Genes which carry class I mutations are the most severe and often have premature stop codons leading to truncated or unstable proteins. The protein is usually targeted for ubiquitination by the endoplasmic reticulum (ER) (Rowntree and Harris, 2003). The results of class V mutations are similar to that of class I due to alternative or aberrant splicing (Kerem, 2005). Class II mutations, including F508del, result in protein misfolding and degradation by the endoplasmic reticulum-associated degradation (ERAD) machinery (Zeitlin, 2000). Where a CFTR mutant form evades ER degradation, reaches the plasma membrane but fails to carry out its intended function, it is termed a class III or class IV mutation. Faulty channel regulation is responsible for Class III mutations whereas a decrease in ion conductance characterises class IV mutations (Rosser *et al.*, 2009). The phenotypic consequence is still typical of CF (Kerem, 2005).

1.4.2 Trafficking of CFTR through the secretory pathway

CFTR is synthesised in the ER where it undergoes co-translational folding and core, N-linked glycosylation at residues 894 and 900 in the 4th ECL (Kornfeld and Kornfeld, 1985, Glozman *et al.*, 2009). During synthesis, membrane proteins interact with a number of chaperones (e.g. HSP70 and calnexin) which assist in assembly and folding (Swanton *et al.*, 2003). Following a quality control check for correct protein folding by the ERAD system, CFTR is trafficked to the Golgi complex where further, more complex glycosylation occurs (Amaral, 2005). Whilst N-linked glycosylation in the ER is relatively conserved in eukaryotes (Hamilton *et al.*, 2003, Wildt and Gerngross, 2005), further modifications of glycoproteins show differences between mammals and

yeast (Wildt and Gerngross, 2005). Despite these differences, complex glycoproteins are produced and released from the yeast Golgi complex (Wildt and Gerngross, 2005).

It is thought that interactions of residues from each TMD1, TMD2, NBD1 and the R-region are required for correct assembly of CFTR via co-translational folding events prior to translation of NBD2 (Rosser *et al.*, 2008). The defective protein resulting from the class II F508del mutation leads to incorrect folding and the defective protein is targeted for ubiquitin-proteasome degradation by the ERAD system (Collawn *et al.*, 2009, Venerando *et al.*, 2013). This cellular checkpoint prevents F508del CFTR from reaching the cell membrane and performing its normal ion channel function (Amaral, 2005). Although the precise mechanism of CFTR folding is not yet fully understood, it is thought that a number of protein chaperones assist in the processing pathways (Amaral, 2005, Riordan, 2008). It is therefore conceivable that small molecules may hold the key to developing drugs that target mutated CFTR and potentially aid in correct folding of the protein and trafficking through the secretory pathway to the plasma membrane.

1.4.3 Overexpression of CFTR

To obtain sufficient quantities of pure protein for both structural and functional analysis, heterologous expression systems are often used which allow proteins to be expressed at much higher levels than normal (Hammon *et al.*, 2009). The budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), has been used for many years as a model organism for expression of proteins and the study of human genes (Wells and Fridovich-Keil, 1996). Overexpression of membrane proteins is difficult and the yield is often low as membrane integrity may be compromised resulting in a toxic effect on the cell (Miroux and Walker, 1996, Carpenter *et al.*, 2008). Optimal culture conditions for *S. cerevisiae* growth are essential to obtain maximum protein yield. Yeast culture growth is dependent on the availability of a carbon source and nutrients for energy and cell growth. When *S. cerevisiae* cultures exhaust one or more essential nutrients and reach the stationary phase (SP), growth rate is reduced considerably and cells undergo several changes to prepare for this stage of their life cycle (Herman, 2002). As a result of these processes, protein degradation is initiated and hence recovery levels of full-length recombinant protein may begin to decline. It is possible to optimise conditions for overexpression by investigating several variables such as expression vectors used, growth media, purification tags, method of induction, cell harvest time and growth

temperature (McLuskey *et al.*, 2008). It is important to explore these conditions in detail to ensure expressed protein is trafficked to, and incorporated into the cell plasma membrane and does not accumulate as aggregates in inclusion bodies in the cytoplasm (Drew *et al.*, 2003).

Maintaining the structure and function of expressed membrane proteins is essential to enable assessment of native structure and function. Upon obtaining purified CFTR, there are several options available to determine its structural and functional state. The oligomeric state of the protein should be established to ensure that aggregation is minimal so as not to interfere with downstream analysis. Due to CFTR's function as an ATP-dependent Cl^- channel, it is important that purified protein maintains its ATP activity and folded state to enable characterisation and functional assays to be performed.

1.5 Scope of the thesis

The work described in this thesis focuses on the use of *S. cerevisiae* as a heterologous expression system from which full-length, functional CFTR can be obtained. The studies are presented as five separate papers which address the various stages of the project.

1.5.1 Context of the PhD within the field of CFTR research

In order to elucidate the underlying molecular mechanisms responsible for CF, in-depth functional and structural analyses of the CFTR protein are required. By expanding our knowledge and understanding of CFTR structure and function, more targeted therapies could be developed. For example, the binding sites and the bound configuration of drugs currently in development could be determined if suitable structural studies could be performed (e.g. X-ray crystallographic studies of CFTR-drug co-crystals). Such structural studies would potentially allow further optimisation of a drug's structure to improve the binding or effectiveness. Moreover, if purified and active CFTR protein could be obtained, assays could be developed to examine the mode of action of drugs or to search for compounds directly interacting with the CFTR protein.

Current CFTR therapy for patients with the G551D mutation (about 3% of patients) involves the use of the channel potentiator VX-770/ivacaftor. This drug has proven to be highly effective in clinical trials, restores lung function and reduces considerably exacerbations (lung infections). The transformative effect of this drug illustrates the power of chemotherapy to treat this genetic disease. However much work remains to be done on the most commonly found mutation in patients (F508 deletion). 'Corrector' drugs aim to correct the folding/instability problems of the CFTR protein with this mutation, however so far even the most effective compounds (in cell-based assays) have proven ineffective in patients. A combination therapy of corrector and potentiator compounds is also being trialled in patients with the F508 deletion. Recent reports show little or no improvement of lung function, but interestingly, a reduction in exacerbations. Hence there is encouraging signs that a combination therapy with better corrector and potentiator compounds may work.

To perform structural studies or to develop new assays for CF-therapeutic compounds, highly purified, functional CFTR must first be obtained, a feat which has proven difficult to achieve in the expression systems previously tested (Bear *et al.*, 1992, Huang *et al.*,

1996, Ramjeesingh *et al.*, 1997, Huang *et al.*, 1998, Kiser *et al.*, 2001, Zhang *et al.*, 2002b, Ketchum *et al.*, 2004, Sun *et al.*, 2006, Fu and Sztul, 2009). Progress has been hindered by: (i) the large size of this multi-domain transmembrane protein; (ii) the presence of large hydrophobic regions; (iii) low level expression in heterologous systems; (iv) protein which is readily degraded; (v) protein that exhibits limited solubility in detergents and (vi) is prone to aggregation (Ward *et al.*, 1995, Zhang *et al.*, 2002b). This thesis aims to provide solutions to some of these problems and to identify areas where future progress needs to be made, as summarised in the paragraphs below.

1.5.2 The CFFT CFTR 3D Structure Consortium

The CFTR 3D Structure Consortium was established by Cystic Fibrosis Foundation Therapeutics (CFFT) Inc. with the ultimate goal of obtaining structural information for CFTR to assist the development of structure-based drug discovery to develop new therapeutics for treating CF. To address these aims, the consortium brings together expertise from several laboratories worldwide that have been testing CFTR expression in several heterologous expression systems to obtain milligram quantities for functional and structural analysis. The expression systems include mammalian human embryonic kidney (HEK) cells (Kappes lab, University of Alabama at Birmingham) and baby hamster kidney (BHK) cells (Riordan lab, University of North Carolina), yeast cells *Saccharomyces cerevisiae* (Ford lab, University of Manchester) and *Pichia pastoris* (Urbatsch lab, Texas Tech University) and bacterial cells *Escherichia coli* and *Lactococcus Lactis* (Slotboom lab, University of Groningen). This collaboration also includes groups with more specific expertise in the biophysical characterisation of CFTR (Brouillette lab, University of Alabama at Birmingham; Ford lab, University of Manchester; Hunt lab, Colombia University; Riordan lab, University of North Carolina).

1.5.3 CFTR orthologues

Although expression and purification of human CFTR is of greatest medical relevance in line with the aims of the consortium, expression of twelve additional CFTR orthologues has also been attempted. The rationale for this approach is that the natural divergence in amino acid sequences of CFTR (Table 1.1) could by chance generate an orthologue that is a more stable, soluble protein and which is more amenable to expression, purification and structural and functional studies. Indeed,

previous data suggest that possible differences in protein structure affects stability of different CFTR orthologues (Price *et al.*, 1996, Ostedgaard *et al.*, 2007, Stahl *et al.*, 2012). The orthologues selected by the consortium represent a broad range of species across the chordate phylum with protein sequence homology to human CFTR ranging from 57% for the killifish orthologue to 92% for the pig, rabbit and ferret orthologues. The orthologues used to optimise protocols for this project were chicken, mouse and platypus and these optimised protocols were then used to express, purify and study the human CFTR orthologue.

Table 1.1 Protein sequence homology of CFTR orthologues relative to human CFTR. Data obtained from http://www.uniprot.org/blast/uniprot/uniprot/2014072262XN1XMLAG?offset=150&sort=identity&filter=annotated%3ayes&filter=taxonomy%3a2759 accessed 22/07/14.

Class/order	Species	Protein sequence homology
Eutherian (placental mammals)	*Homo sapien* (human)	100.0
	Sus scrofa (pig)	92.0
	Oryctolagus cuniculus (rabbit)	92.0
	Mustela putorius furo (european domestic ferret)	92.0
	Ovis aries (sheep)	91.0
	Canis familiaris (dog)	90.0
Metatherian (marsupial mammals)	*Trichosurus vulpecula* (brush-tailed possum)	86.0
Prototherian (egg-laying mammals)	*Ornithorhynchus anatinus* (duckbill platypus)	83.0
Aves (birds)	*Gallus gallus* (chicken)	80.0
Rodentian (rodents)	*Mus musculus* (mouse)	78.0
	Rattus norvegicus (rat)	78.0
Osteichthyes (bony fish)	*Salmo salar* (Atlantic salmon)	59.0
	Fundulus heteroclitus (Killifish) (Mummichog)	58.0

1.5.4 Yeast expression system

The use of *S. cerevisiae* to express and purify CFTR has previously proven to be challenging (Huang *et al.*, 1996, Huang *et al.*, 1998, Kiser *et al.*, 2001, Zhang *et al.*, 2002b, Ketchum *et al.*, 2004, Sun *et al.*, 2006, Ahner *et al.*, 2007, Fu and Sztul, 2009) and, where low levels of CFTR could be obtained, the protein was highly susceptible to

degradation and aggregation (Ward *et al.*, 1995, Kiser *et al.*, 2001, Drew *et al.*, 2008). Whilst higher levels of CFTR can be generated from some expression systems, it is often necessary to extract CFTR from the lipid membrane using harsh detergents rendering the protein in a less native-like state (Huang *et al.*, 1996, Ramjeesingh *et al.*, 1997, Huang *et al.*, 1998). Improvements to the yeast CFTR expression system and, in particular, extraction and purification using milder detergents, would undoubtedly benefit CF research as large amounts of protein could be rapidly generated for use in high-throughput assays providing a useful platform for screening drugs/small molecules to identify novel therapeutics to treat CF. Protein generated could also be used in structural and functional investigations to answer key questions in the field which still elude researchers despite the fact that research into CFTR has been ongoing for more than 25 years. The optimisation of this system, described in this thesis has enabled our group, and other members of the CFFT CFTR 3D Structure Consortium to exploit the advantages it poses over other systems to generate large amounts of functionally active CFTR. The protein has so far not been crystallised. However it has been used to investigate the mechanism of action of drugs currently used to treat CF. CFTR expressed from this system has been shared with labs worldwide in line with the need for collaborative research to develop new treatments or ultimately even cure CF.

1.6 Experimental aims

- To optimise the *S. cerevisiae* heterologous expression system for optimal recovery of full-length CFTR.
    - This aim is addressed in chapter 2: 'Optimisation of *Saccharomyces cerevisiae* culture conditions for expression of recombinant Cystic Fibrosis Transmembrane conductance Regulator (CFTR)'. This paper describes the investigation of several variables affecting expression of CFTR and explores how optimising each could improve expression of recombinant CFTR in yeast.

- To express and purify the murine CFTR protein in *S. cerevisiae*.
    - This aim is addressed in chapters 3 and 4: 'Expression and purification of the cystic fibrosis transmembrane conductance regulator protein in *Saccharomyces cerevisiae*' and 'Purification of the Cystic Fibrosis Transmembrane Conductance Regulator Protein Expressed in *Saccharomyces cerevisiae*'. These papers describe methods for expression and purification of murine CFTR using the optimised conditions established in chapter 2.

- To obtain sufficient levels of expression of the human CFTR protein in *S. cerevisiae* for subsequent purification to enable functional and structural studies.
    - This aim is addressed in chapter 5: 'A gene optimisation strategy to enhance expression of human CFTR from yeast expression systems'. This paper explores the use of codon- and gene-optimised constructs to improve hCFTR expression in yeast.

- To purify and characterise hCFTR expressed in *S. cerevisiae*.
    - This aim is addressed in chapter 6: 'Comparison of CFTR purification methods and thermal stability analysis of purified CFTR'. Various purification methods are explored and protein yield and purity from each are compared. Function and structure of hCFTR are then assessed to determine the state of the purified protein.

1.7 Alternative format

This thesis is being submitted in the alternative format in accordance with the rules and regulations from the University of Manchester. The five papers presented form a cohesive body of work which describes the various stages of the project in a logical sequence which is best represented by submission in this format. The manuscripts are detailed below along with each authors contribution.

Chapter 2: Optimisation of *Saccharomyces cerevisiae* culture conditions for expression of recombinant Cystic Fibrosis Transmembrane conductance Regulator (CFTR)
Authors: Tracy L Rimington, Natasha Cant, Liam O'Ryan, Robert C Ford
Intended journal: Protein Expression and Purification

Author contributions: The majority of the experimental work in this manuscript was carried out by myself. Dr. Natasha Cant assisted with sampling during the timecourse experiments. I designed the schedule of timepoints for sampling and performed all subsequent processing and data analysis of the timecourse samples. The CFTR microsomes used for the solubility experiments were provided by Dr. Liam O'Ryan and this work was a continuation of some initial observations of this author. However, all experiments contained in this manuscript are my own work and he did not contribute to the experimental work. Advice and guidance on the design and implementation of experiments was provided by my supervisor, Prof. Robert C Ford and further discussed with Dr. Liam O'Ryan and Dr. Natasha Cant. As first author on this manuscript, I was responsible for writing the text. My supervisor, Prof. Robert C Ford reviewed the initial draft and provided comments and suggestions which were incorporated by myself into the final version.

Chapter 3: Expression and Purification of the Cystic Fibrosis Transmembrane Conductance Regulator Protein in *Saccharomyces cerevisiae*
Authors: Liam O'Ryan, Tracy L Rimington, Natasha Cant, Robert C Ford
Journal published in: Journal of Visualized Experimentation (*J. Vis. Exp.* (61), e3860, doi:10.3791/3860 (2012).)
Author contributions: The protocols used for the experimental work in this manuscript were partly based on the data obtained in chapter 2. The protocols performed during filming of the JOVE video were carried out by myself and Dr. Natasha Cant. The protocols demonstrated are used routinely by members of the Ford research group, including myself. Generation and analysis of the representative SDS-PAGE gels for CFTR expression and purification contained within the manuscript were provided by Dr.

Liam O'Ryan but are routinely produced by members of the Ford research group, including myself. The timecourse data was provided and analysed by myself. CFTR expressing *S. cerevisiae* cells harvested for fluorescence microscopy were provided and prepared by myself and images captured and analysed by myself. Samples for DNA sequencing were prepared by myself and sequenced by the University of Manchester DNA Sequencing Facility. All sequencing results were analysed and assembled by myself. The text for the manuscript was written by Prof Robert Ford and reviewed by myself, Dr. Natasha Cant and Dr Liam O'Ryan. Preparation of DNA for distribution as a result of this publication has been carried out by myself and communication with recipients was by myself and Prof. Ford.

Chapter 4: Purification of the Cystic Fibrosis Transmembrane Conductance Regulator Protein Expressed in *Saccharomyces cerevisiae*
Authors: Naomi Pollock, Natasha Cant, Tracy Rimington, Robert C Ford
Journal published in: Journal of Visualized Experimentation (*J. Vis. Exp.* (), e51447, doi:10.3791/51447 (2014).

Author contributions: The protocols used for the DDM solubilisation were partly based on the data obtained in chapter 2. The protocols performed during filming of the JOVE video were carried out by Dr. Naomi Pollock and Dr. Natasha Cant and introductory and discussion excerpts were performed by each author, including myself. The experimental procedures demonstrated are used routinely by members of the Ford research group, including myself and I assisted with their design and optimisation. Generation and analysis of the representative SDS-PAGE gels for CFTR purification contained within the manuscript were provided by Dr. Pollock but are routinely produced by members of the Ford research group, including myself. Glycerol stocks of CFTR expressing *S. cerevisiae* cells were provided by myself. Samples for DNA sequencing were prepared by myself and sequenced by the University of Manchester DNA Sequencing Facility. All sequencing results were analysed and assembled by myself. The text for the manuscript was written by Dr. Pollock and reviewed by myself, Dr. Cant and Prof. Ford. Preparation of DNA for distribution as a result of this publication has been carried out by myself and communication with recipients was by myself, Dr. Pollock and Prof. Ford.

Chapter 5: A gene optimisation approach to enhance expression of human CFTR in *Saccharomyces cerevisiae*

Authors: Tracy Rimington, Naomi Pollock, Bala M. Puna, John Kappes, Robert C. Ford, Ina Urbatsch

Intended journal: Journal of Cystic Fibrosis

Author contributions: The majority of the experimental work in this manuscript was carried out by myself. The generation of constructs was carried out during my visit to the Urbatsch lab (Texas Tech University, Lubbock, Texas) under the supervision of Dr. Ina Urbatsch and with the help of a PhD student, Bala M Purna. FLAG-tagged hCFTR was provided by Dr. John C. Kappes (University of Alabama at Birmingham, Alabama). Dr. Naomi Pollock assisted with sampling during the timecourse experiments. I designed the schedule of timepoints for sampling and performed all subsequent processing and data analysis of the timecourse samples. Samples for DNA sequencing were prepared by myself and sequenced by the Manchester University DNA Sequencing Facility. All sequencing results were analysed and assembled by myself. Advice and guidance on the design and implementation of experiments was provided by my supervisor, Prof. Robert C Ford and Dr. Ina Urbatsch and further discussed with Dr. Naomi Pollock and Bala M Purna. As first author on this manuscript, I was responsible for writing the text. My supervisor, Prof. Robert C Ford reviewed the initial draft and provided comments and suggestions which were incorporated by myself into the final version.

Chapter 6: Comparison of CFTR purification methods and thermal stability analysis of purified CFTR

Authors: Tracy L Rimington, Naomi Pollock, Natasha Cant, Robert C Ford

Intended journal: Protein Expression and Purification

Author contributions: The majority of the experimental work in this manuscript was carried out by myself. Advice and guidance on the initial design and implementation of experiments was provided by my supervisor, Prof. Robert C Ford, Dr. Naomi Pollock and Dr. Natasha Cant. All protocol optimisation was carried out by myself as was the experimental work and all subsequent processing and data analysis. Advice on the CPM thermal stability assay was provided by Dr. Pollock and Dr. Cant. As first author on this manuscript, I was responsible for writing the text. My supervisor, Prof. Robert C Ford reviewed the initial draft and provided comments and suggestions which were incorporated by myself into the final version.

Chapter 2 - Optimisation of *Saccharomyces cerevisiae* culture conditions for expression of recombinant Cystic Fibrosis Transmembrane conductance Regulator (CFTR)

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2.1 Abstract

Mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) eukaryotic integral membrane protein are responsible for the disease cystic fibrosis (CF). As with many other membrane proteins, CFTR is difficult to express in heterologous expression systems and obtaining high yields for functional and structural studies has been a limitation in CF research. Our aim was to optimise cell culture conditions for the yeast *Saccharomyces cerevisiae* to allow overexpression of full-length CFTR. We investigated several aspects of the yeast cell culture and assessed the effect of altering these conditions on CFTR expression. Using GFP-tagged CFTR we were able to monitor CFTR expression relative to an endogenous fluorescent protein. The use of glycerol as a chemical chaperone at the time of induction of CFTR expression increased relative CFTR yields by ~6-fold and, when used in combination with DMSO, ~7-fold. Timecourse analysis revealed peak relative CFTR expression at ~14 hours following induction. Cultures in which protein expression was induced at an optical density at 600 nm (OD_{600}) of 2.0 exhibited the highest levels of CFTR expression and there was a substantial reduction in detectable CFTR from cultures induced above this. Detergent solubilisation efficiency of three CFTR orthologues was assessed. LPG was found to be the most efficient solubilising >90% of CFTR in all cases. DDM was less efficient ranging from ~27-50% and NaPFO exhibited very poor efficiency, solubilising <20% of CFTR in all cases. The DDM solubilisation efficiency could be substantially improved with addition of up to 1 M NaCl. The data highlights the importance of optimisation of these systems for expression of difficult membrane proteins and that by performing these trials, expression of previously poorly expressed proteins can be achieved.

2.2 Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR, also known as ABCC7) is an integral membrane protein in which mutations can give rise to the hereditary disease cystic fibrosis (CF). The CFTR gene was first identified in 1989 (Kerem *et al.*, 1989, Riordan *et al.*, 1989, Rommens *et al.*, 1989) and has since been studied extensively with the ultimate aim of discovering new therapeutic treatments and a cure for CF. *In-vitro* analysis of CFTR has predominantly been carried out using mammalian or insect cell expression systems. Our aim was to develop a heterologous expression system using the budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) to express and purify sufficient quantities of the CFTR protein to enable characterisation, a task which has previously proven tractable, but difficult (Huang *et al.*, 1996, Kiser *et al.*, 2001, Zhang *et al.*, 2002b).

The cellular stresses resulting from overexpression of recombinant proteins in *S. cerevisiae* have been widely studied (Mattanovich *et al.*, 2004) and the host machinery is often not able to cope with these stresses which leads to low yields of protein. One approach to compensate for this is to optimise the culture conditions for yeast strains to facilitate expression and extraction of high yields of membrane proteins such as CFTR which can then be purified for further biophysical and biochemical analysis. Using GFP-tagged, codon-optimised genes from three previously cloned CFTR orthologues (chicken, mouse and platypus, referred to as cCFTR, mCFTR and plCFTR respectively), several factors were investigated to assess their effect on expression of full-length CFTR. The protocols used for cloning of the constructs were based on (Drew *et al.*, 2008). Where appropriate, CFTR expression levels in the cell were estimated using the ratio of GFP relative fluorescence units (RFU) relative to a yeast endogenous fluorescent protein (endFP). Once culture conditions had been optimised, we explored the use of detergents to solubilise the full-length CFTR protein in preparation for downstream purification.

The use of chemical chaperones has been widely used to improve heterologous expression of membrane proteins (Figler *et al.*, 2000) and has also been reported to rescue the folding defect of the most prevalent mutant form of CFTR, the F508del mutation (Brown *et al.*, 1996, Sato *et al.*, 1996). We investigated the effects of two of these compounds, glycerol and dimethylsulphoxide (DMSO), which are believed to stabilise misfolded proteins and promote refolding (Diamant *et al.*, 2001). The effect of trehalose, a naturally occurring disaccharide produced by yeast cells in response to high or low temperatures (Kandror *et al.*, 2004), was also tested. In addition, the effect

of galactose was explored, which is used to induce protein expression with the vector used. Data from (Newstead *et al.*, 2007a) showed that the addition of 10% glycerol at the time of induction had a negative effect on overexpression of some membrane proteins in *S. cerevisiae*. We tested up to 8% glycerol and observed a considerable increase in expression of full-length CFTR. Improvements in expression were also observed with the other compounds tested, although to a lesser extent. Based on the findings of this first optimisation trial, glycerol was used in the induction media for all subsequent experiments.

Due to CFTR's susceptibility to degradation (Ward *et al.*, 1995, Zhang *et al.*, 2002b), the time at which cells are harvested following induction of recombinant protein expression is critical for optimal extraction of CFTR. The use of a galactose inducible promoter, which is heavily repressed in the presence of glucose (Johnston *et al.*, 1994, Drew *et al.*, 2008) allows strict control over CFTR expression and post induction cell harvest time can be accurately tuned for optimal recovery of the full-length protein. CFTR expression was monitored over time following induction and using the ratio of CFTR to endFP as described above. We identified the post-induction timepoint at which the highest levels of full-length CFTR was observed for the mCFTR orthologue and used this for subsequent experiments.

To obtain optimal yields, it is important to induce recombinant protein expression in *S. cerevisiae* before cells reach the diauxic shift and enter the late-log phase (5×10^7 - 2×10^8 cells/mL, an OD_{600} of 1.0 is ~5×10^7 cells/mL (Bergman, 2001)). After this point growth slows down as cell growth moves toward the stationary phase (SP). During SP, metabolic activity is reduced along with protein expression (Martinez *et al.*, 2004) and expression of stress response proteins is initiated. During this phase the cell wall thickens making cell lysis and therefore protein extraction more difficult (Galdieri *et al.*, 2010). To evaluate the effect of inducing expression at various stages of the yeast growth cycle, we analysed CFTR expression using in-gel fluorescence from mCFTR expressing cultures induced at various cell densities. The aim was to establish the optimum cell density at induction before substantial degradation of CFTR occurred.

Detergent solubilisation is required to extract membrane proteins from crude cell lysates and to facilitate purification by conventional liquid chromatography. Detergents provide an environment which emulates that of the lipid bilayer (Seddon *et al.*, 2004) by binding to hydrophobic regions of proteins and enabling solubilisation (Garavito and Ferguson-Miller, 2001). The choice of detergent fundamentally depends on the downstream application intended for the protein of interest (Lin and Guidotti, 2009).

Previously, CFTR has been successfully solubilised in the negatively charged, relatively denaturing detergents lysophosphatidylglycerol (LPG) (Huang *et al.*, 1998, Ketchum *et al.*, 2004) and sodium perfluoro-octanoic acid (NaPFO) (Ramjeesingh *et al.*, 1997). Previous studies have shown that solubilisation in these detergents can affect protein tertiary interactions (Therien and Deber, 2002) and it is preferable to use milder, non-denaturing detergents, such as n-Dodecyl-β-D-maltopyranoside (DDM). To investigate detergent solubility of CFTR, crude membranes were incubated with a detergent:protein ratio >5:1 (w/w) of DDM, LPG-14, LPG-16 or NaPFO in CFTR buffer (CB) containing Tris- and phosphate-buffered saline (TBS & PBS). Following centrifugation, soluble and insoluble proteins were resolved by SDS-PAGE and CFTR solubilisation was calculated using in gel-fluorescence. We were able to considerably improve solubilisation using DDM in PBS at a lower pH compared to TBS. To investigate this further, the effect of pH was first tested. As little difference was observed across a suitable pH range, we repeated the DDM solubilisation this time at a range of NaCl concentrations. There appeared to be a correlation between increasing salt concentration and CFTR solubility.

This study explores some of the factors which affect expression of CFTR in *S. cerevisiae* and how optimising culture conditions can maximise protein yield in this system. We also investigate some commonly used detergents for CFTR solubilisation and again how optimising this method can increase the amount of protein which can be extracted for further applications.

2.3 Materials and methods

2.3.1 Culture media and agar

Unless otherwise stated, media was prepared according to protocols detailed in (Sambrook & Russell, 2001). Glucose-free media and agar was sterilised by autoclaving at 121 °C for a minimum of 15 minutes prior to. Media containing glucose was sterilised by autoclaving at 110 °C for a minimum of 15 minutes.

2.3.2 Yeast culture media and agar

Yeast peptone dextrose (YPD) *(10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose)* was used throughout yeast transformations.

CFTR culture media (0.15% or 2% glucose) - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 0.75 g/L D-glucose (for 0.1%) or 20 g/L glucose (for 2%))*

CFTR induction media - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L D-galactose, 8% glycerol (v/v))*

CFTR chaperone media - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L D-galactose)*

2.3.3 Buffers and stock solutions

Yeast suspension buffer (YSB) - *(50 mM Tris-HCl pH 7.6, 5 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol)*

Membrane resuspension buffer (MRB) - *(20 mM Tris-HCl pH 7.6, 300 mM sucrose, 0.1 mM $CaCl_2$)*

Phosphate buffered saline (PBS) - *(10 mM $NaHPO_4$ pH 7.5, 150 mM, 500 mM or 1 M NaCl)*

CFTR buffer (CB) - *(50 mM Tris-HCl pH 8.0, 1 M NaCl, 20% glycerol, 1mM dithiothreitol (DTT))*

100X Protease inhibitor cocktail (PIC) - *(20 mM AEBSF, 600 µM bestatin, 400 mM chymostatin, 700 µM E-64, 2 mM leupeptin, 1.5 mM pepstatin A, 100 mM PMSF in dry DMSO plus 300 mM benzamidine in dH_2O)*

2X SDS solubilisation buffer (SB) - *(50 mM Tris-HCl pH 7.6, 5% glycerol (v/v), 5 mM EDTA, 0.02% bromophenol blue in 700 µl aliquots. 200 µl of 20% (w/v) sodium dodecyl sulphate (SDS) and 100 µl of 0.5 M DTT added just prior to use)*
10X SDS running buffer - *(30 g/L Tris-HCl, 144 g/L glycine and 10 g/L SDS in dH_2O)*
Detergent solutions - (10% (w/v) detergent in MRB or PBS)

2.3.4 CFTR expression

CFTR culture medium (2% glucose) was inoculated with 1 colony per 10 ml from fresh cCFTR, mCFTR or plCFTR glycerol stock streaked agar plates in either 50 ml-Falcon tubes or 250 ml-baffled Erlenmeyer flasks. Cultures were incubated overnight in an orbital shaker at 225 rpm at 30 °C. Unless otherwise stated, cultures were diluted to an OD_{600} of 0.1 in fresh CFTR culture medium (0.1% glucose) in either 250 ml-baffled Erlenmeyer flasks or 2 L-baffled Erlenmeyer flasks and incubated in an orbital shaker at 225 rpm at 30 °C for ~8 hours. Unless otherwise stated, cultures were induced at an OD_{600} of 1.0 by the addition of 2% galactose and 8% glycerol and were incubated for 14 hours in an orbital shaker at 225 rpm at 25 °C.

2.3.5 Harvesting and cell lysis

All steps were carried out at 4 °C or on ice. Cells were harvested 15 hours post induction by centrifugation at 4,500 x *g* for 5 min at 4 °C. The supernatants were discarded and cells were resuspended in 500 µl of YSB + PI. Cell suspensions were transferred to 1.5 ml screw-top tubes containing ~400 µl of acid washed glass beads. Cells were lysed by 4 x 1 minute at 3450 oscillations/min in a BioSpec Mini-Beadbeater-16 (Bartlesville, OK, USA) with 1 minute on ice between each round of homogenisation. Following lysis, tubes were centrifuged at 4,500 x *g* for 5 minutes at 4 °C to pellet glass beads and unbroken cells and large cell debris. 450 µl of the supernatants were transferred to sterile 1.5 ml microfuge tubes. Cell lysates were centrifuged at 21,000 x *g* for 2 hours at 4 °C. The supernatants were discarded and crude membrane pellets were resuspended in 50 µl of YSB + PIC.

2.3.6 SDS-PAGE analysis

Unless otherwise stated, 25 µl of each sample was added to an equal volume of 2X SB. Samples were loaded onto NuSep nUView 10% Tris-glycine gels and electrophoresed at 150 V for ~45 minutes in 1X SDS running buffer.

2.3.7 In-gel fluorescence of CFTR

GFP-tagged CFTR was analysed following SDS-PAGE using either a Typhoon Trio™ Variable Mode Imager (GE Healthcare Life Sciences) using a blue argon ion laser with an excitation wavelength of 488 nm and using an emission wavelength of 520 nm with images captured and saved for further analysis using ImageQuant™ TL (GE Healthcare Life Sciences) or using a ChemiDoc™ MP Imaging System (Bio-Rad) fitted with a Blue LED Module Kit with an excitation wavelength of 488 nm and using an emission wavelength of 520 nm with images captured using a Supercooled CCD camera and saved for further analysis using Image Lab™ Software (Bio-Rad) or ImageJ (http://rsb.info.nih.gov/ij). GFP fluorescence was measured using RFU following densitometry analysis.

2.3.8 Chemical chaperones trial

mCFTR expressing cultures were prepared as previously described. To compare the effect of additives on CFTR expression, the mCFTR cultures were divided into 45 ml sub-cultures in 250 ml-baffled Erlenmeyer flasks and expression was induced by the addition of 2% galactose, plus one of the following: 50 mM Trehalose, 100 mM Trehalose, an additional 2% galactose, 2.5% DMSO, 4% glycerol, 8% glycerol or 4% glycerol + 2.5% DMSO. Stock solutions of the chaperone compounds were prepared in 5 ml of CFTR chaperone media to give the final concentrations described.

2.3.9 Timecourse of hCFTR expression

A 600 ml mCFTR expressing culture was prepared as previously described. Following induction, 25 ml sample aliquots were taken at 8, 10, 12, 13, 14, 16, 18, 20 and 24 hours. Cells were harvested as previously described and stored at -80 °C until required for analysis. Crude membranes were obtained and analysed as previously described.

2.3.10 Optimising cell density at induction for CFTR expression

A 250 ml mCFTR expressing culture was prepared as previously described. Following overnight incubation, the culture was diluted into 6 x 30 ml culture in 250 ml- baffled Erlenmeyer flasks to OD_{600} values of 0.05, 0.1, 0.15, 0.2, 0.3 and 0.4 in CFTR culture medium (2% glucose). Cultures were incubated in an orbital shaker at 225 rpm at 30 °C for ~8 hours to OD_{600} values of 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0. Excess glucose was removed by centrifuging the cultures at 4,500 x *g* for 5 min at 4 °C and washing cell pellets with 30 ml of sterile dH_2O. CFTR expression was induced by resuspending cells in 30 ml of CFTR induction media. Cells were harvested, lysed and crude membranes obtained as previously described.

2.3.11 Optimising detergent solubility of CFTR

cCFTR, mCFTR and plCFTR crude membranes obtained from 2 x 0.5 L cultures each were resuspended in 500 µl each of MRB and PBS (500 mM NaCl) + PIC. 100 µl of resuspended crude membranes (total protein concentration of ~5 mg/ml) were added to 100 µl of 10% detergent solutions (5% final volume). Detergents used were: DDM, LPG14, LPG16 and NaPFO. Samples were incubated with end-over-end rotation for 1 hour at 4 °C. Following centrifugation at 21,000 x *g* for 1 hour, soluble material was transferred to fresh tubes and insoluble pellets were resuspended in the corresponding buffers. Samples were analysed by SDS-PAGE as previously described.

2.3.12 DDM solubilisation of CFTR - pH

mCFTR crude membranes (total protein concentration of ~5 mg/ml) obtained from 1 L of culture were resuspended in 100 µl of PBS + PIC. 10 µl of resuspended crude membranes were diluted in final volumes of 100 µl of 2% DDM in PBS + PIC at pH 7.0, 7.8, 8.5 and 9.0. Samples were incubated with end-over-end rotation for 1 hour at 4 °C. Following centrifugation at 100,000 x *g* for 1 hour, soluble material was transferred to fresh tubes and insoluble pellets were resuspended in the corresponding buffers. Samples were analysed by SDS-PAGE as previously described.

2.3.13 DDM solubilisation of CFTR - NaCl

plCFTR crude membranes (total protein concentration of ~5 mg/ml) obtained from 2 x 0.5 L cultures were resuspended in 500 µl each of CB + PIC and PBS + PIC. 100 µl of resuspended crude membranes were added to 100 µl of 4% DDM solution (2% final volume) in CB + PIC (1 sample) or PBS + PIC (3 samples). The NaCl concentration was adjusted in two of the PBS samples to a final concentration of 500 mM and 1 M. Samples were incubated with end-over-end rotation for 1 hour at 4 °C. Following centrifugation at 100,000 x *g* for 1 hour, soluble material was transferred to fresh tubes and insoluble pellets were resuspended in the corresponding buffers. Samples were analysed by SDS-PAGE as previously described.

2.4 Results

2.4.1 CFTR expression is enhanced by the addition of chemical chaperones

In an aim to increase CFTR expression a range of small molecule chemical chaperones were used as additives to CFTR induction media to test their effect on expression of the full-length protein. In the first study, CFTR expression was increased, to some extent, by each of the compounds tested relative to the control culture which was induced with 2% galactose only (Fig. 2.1a). Trehalose had the least effect on yield, with a 1.1-fold increase at a concentration of 50 mM. This increased slightly to a 1.2-fold increase with 100 mM trehalose. The addition of 2.5% DMSO had a greater effect, increasing yield 2.2-fold but the greatest effect was observed by adding 4% glycerol at the time of induction. This produced a 3.0-fold increase in yield compared to the control sample of 2% galactose alone (Fig. 2.1c).

Based on the results of the experiment, further studies were performed to optimise the use of glycerol as an induction media additive. As in the previous study, the expression of CFTR was tested relative to the control culture which was induced with 2% galactose only. DMSO and glycerol were tested again at the same concentrations as previously and additional cultures were prepared, one with the addition of 8% glycerol and another with 4% glycerol + 2.5% DMSO. A further culture was also set up using double the standard concentration of galactose at induction (4% instead of 2%). Once again, an increase in CFTR expression was observed with all additives tested (Fig. 2.1b). 2.5% DMSO and 4% glycerol showed similar results as the previous study, increasing expression 2.4-fold and 4.0-fold respectively. A 1.9-fold increase was observed with an additional 2% galactose (4% total). As anticipated, the addition of glycerol produced the greatest increase in CFTR expression with a 6.2-fold increase observed in the 8% glycerol sample. When combined with 2.5% DMSO, this was further improved to a 7.2-fold increase (Fig. 2.1c).

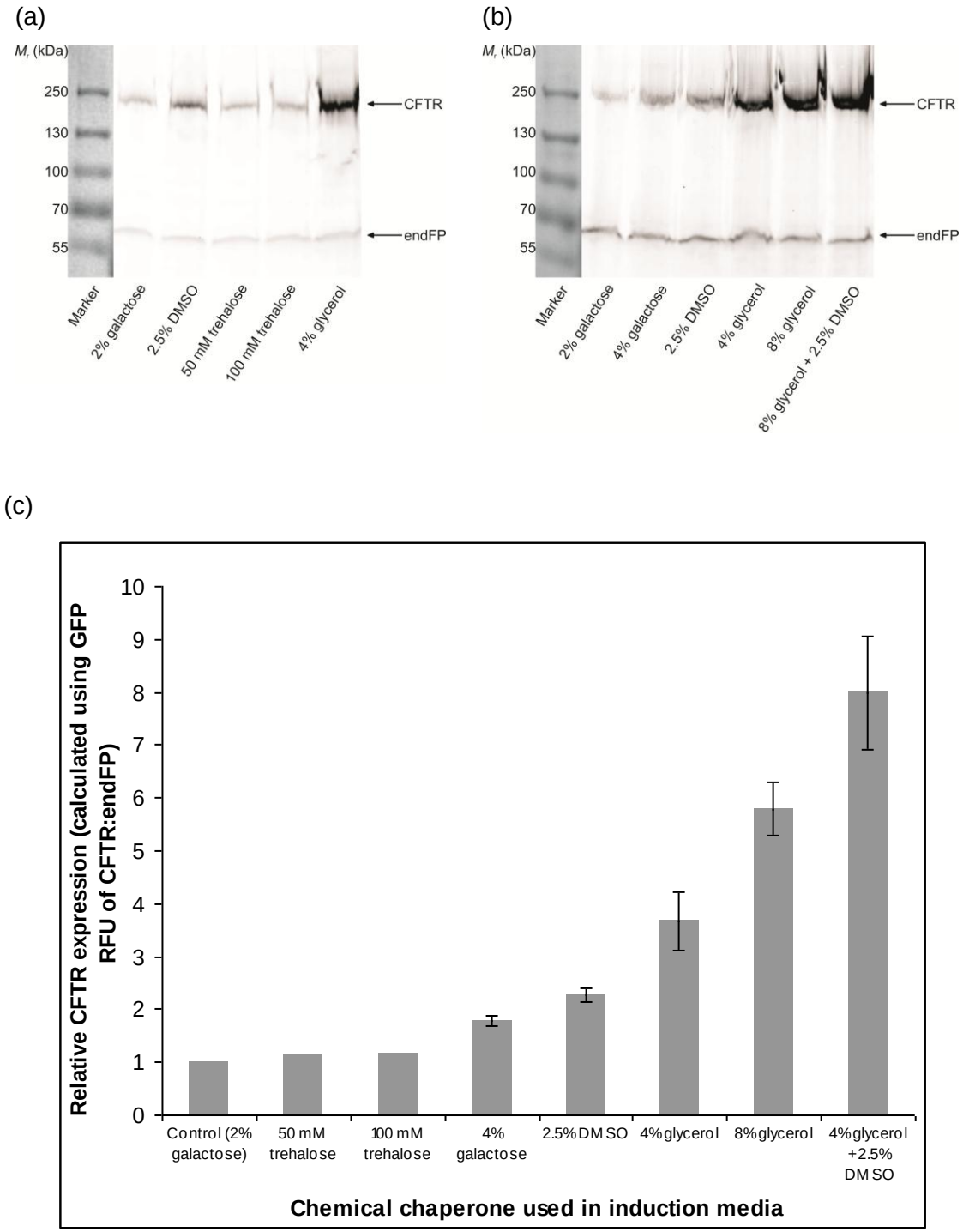


Figure 2.1. Effect of trehalose, additional galactose, DMSO and glycerol on CFTR expression. (a&b) representative SDS-PAGE gels viewed under fluorescence (ex. 488 nm, em. 530 nm). (c) CFTR:endFP ratio relative to control culture (2% galactose) was calculated by measuring GFP fluorescence (RFU) of CFTR and endogenous FP full-length CFTR in each sample and applying densitometry analysis using ImageJ. Error bars (where present) represent the standard deviation from a minimum of three independent experimental repeats.

2.4.2 CFTR:end FP peaked at ~14 hours post-induction

To evaluate the effect of harvesting cells at various post-induction timepoints, mCFTR expressing *S. cerevisiae* cells were induced with 2% galactose and 8% glycerol and samples were taken at various timepoints from 8-26 hours. Expression of CFTR and endFP were assessed using in-gel fluorescence. CFTR:endFP was calculated and the peak ratio was observed at ~14 hours with peak CFTR expression at ~15 hours (Fig. 2.2). After this timepoint CFTR:endFP decreased and very little CFTR or endFP remained after 20 hours (Fig. 2.2).

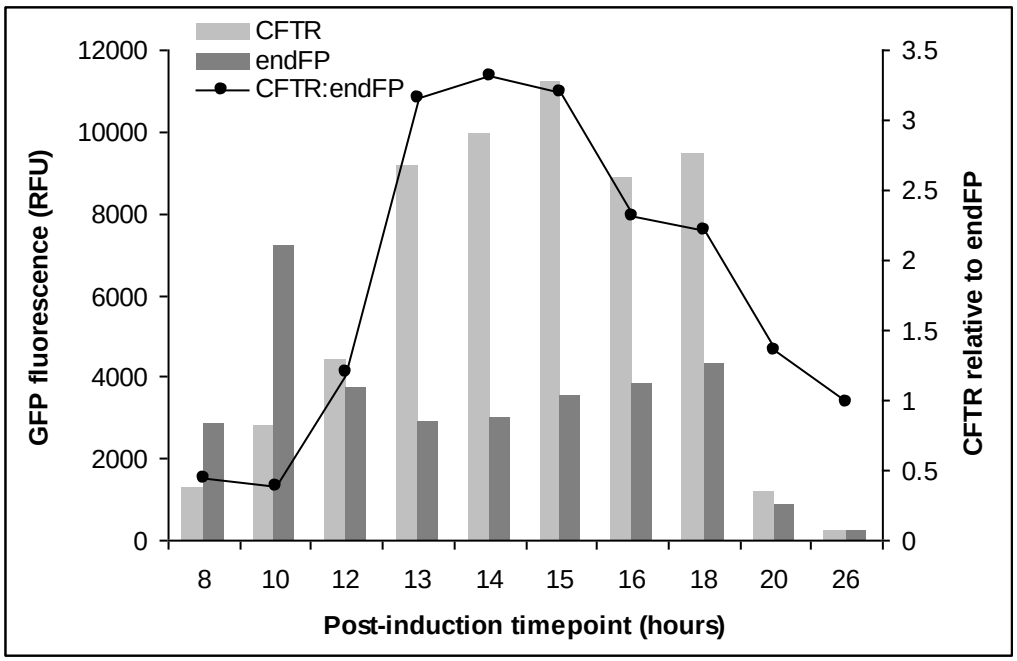
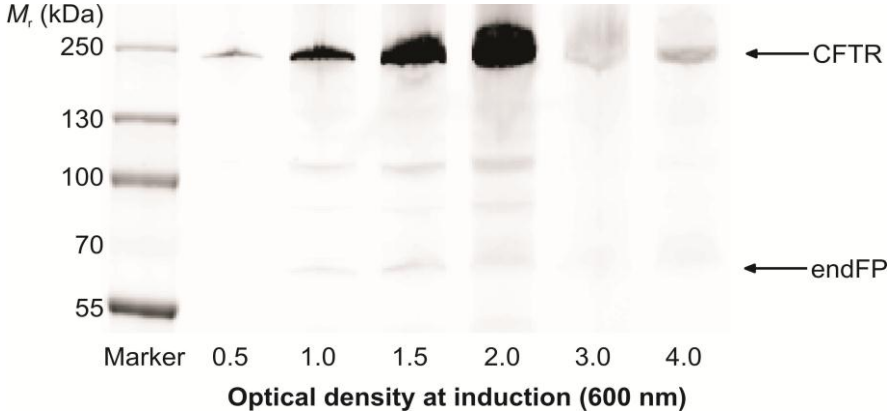


Figure 2.2. Effect of time of induction on CFTR expression. (a) SDS-PAGE gel viewed under fluorescence (ex. 488 nm, em. 530 nm). (b) CFTR:endFP ratio was calculated by measuring GFP fluorescence (RFU) of CFTR and endFP in each sample and applying densitometry analysis using ImageJ.

2.4.3 *Optimal CFTR expression is observed at an induction OD_{600} of 2.0*

To ascertain the optimal cell density at which CFTR expression is induced in relation to recovery of the full-length protein, cultures were induced at OD_{600} values ranging from 0.5 to 4.0 and harvested at the same time point post-induction (14 hours). Peak CFTR expression was seen in cultures induced at an OD_{600} of 2.0 (Fig. 2.3a). After induction at an OD_{600} of 3.0, less than 25% of maximum CFTR was observed (Fig. 2.3a). CFTR:endFP was highest in cultures induced at an OD_{600} of 0.5 and exhibited a negative correlation to induction cell density with only ~35% of CFTR:endFP at the highest cell density of 4.0 (Fig. 2.3b). Cultures induced at an OD_{600} of 2.0 showed CFTR:endFP at ~89% of maxiumum (Fig. 2.3b).

(a)



(b)

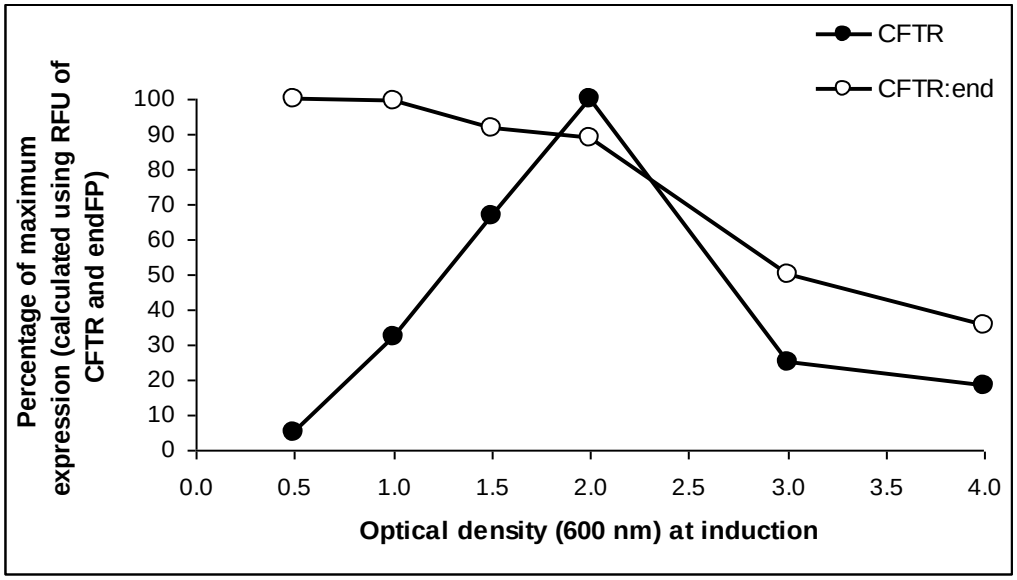


Figure 2.3. Effect of cell density (detected by OD600) at induction on CFTR expression. (a) Proteins were resolved by SDS-PAGE and view under fluorescence (ex. 488 nm, em. 530 nm). (b) Solubilisation (%) was calculated by measuring GFP fluorescence (RFU) of full-length CFTR and endFP in each sample and applying densitometry analysis using ImageJ.

2.4.4 NaCl improves solubility of CFTR in the mild detergent, DDM.

Detergent solubilisation efficiency of cCFTR, mCFTR and plCFTR crude membranes was analysed using DDM, LPG and NaPFO in Tris and phosphate buffers. LPG solubilisation efficiency was >93% in all cases (Fig. 2.4). DDM solubility varied from ~27% for cCFTR up to ~50% for mCFTR in MRB (Tris buffer). The efficiency of DDM solubilisation was increased to >80% in PBS for mCFTR and plCFTR (Fig. 2.4) (solubilisation of cCFTR was not performed with DDM in PBS).

Interestingly NaPFO solubilised <20% in all cases and efficiency was <5% for cCFTR (Fig. 2.4). It has since been shown that lithium PFO (LiPFO) is significantly more efficient (Urbatsch, I, Texas Tech University, USA, personal communication) than NaPFO, probably because of the better solubility of the Li salt form of the detergent at 4 ^{o}C.

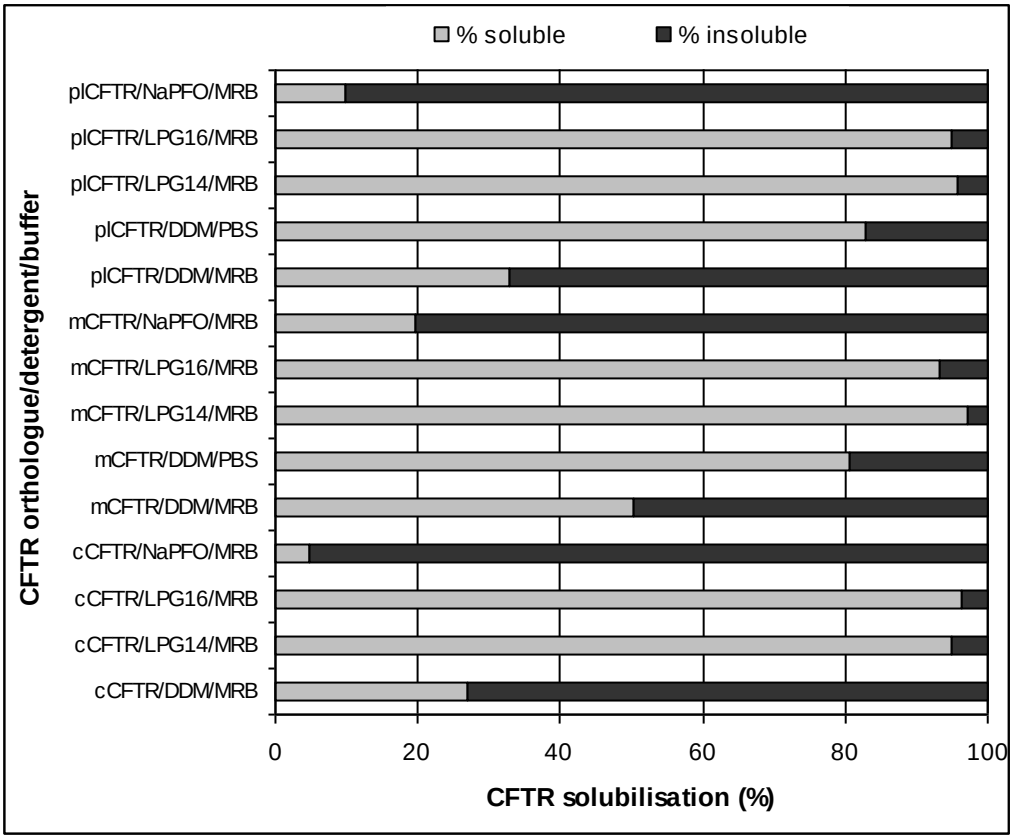


Figure 2.4. Detergent solubilisation of cCFTR, mCFTR and plCFTR in MRB (pH7.5) or PBS (pH 7.0) solubilised in DDM, LPG14, LPG16 or NaPFO. Soluble and insoluble proteins were resolved by SDS-PAGE and view under fluorescence (ex. 488 nm, em. 530 nm). Solubilisation (%) was calculated by measuring GFP fluorescence (RFU) of full-length CFTR in each fraction and applying densitometry analysis using ImageJ.

The effect of pH on DDM solubilisation efficiency of mCFTR was investigated using PBS at pH 7.0, 7.8, 8.5 and 9.0. The highest efficiency was observed at pH 9.0 with ~83% of CFTR in the soluble fraction (Fig. 2.5). Solubilisation in the other samples varied very little from ~74% to ~76% efficiency (Fig. 2.5).

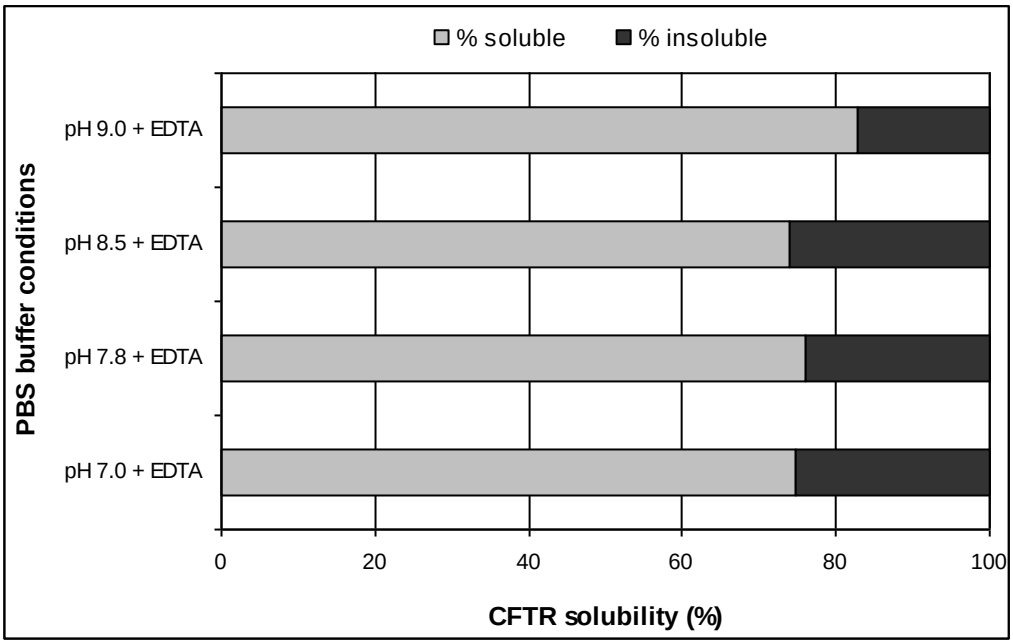


Figure 2.5. Effect of pH on CFTR solubilisation efficiency. Soluble and insoluble proteins were resolved by SDS-PAGE and view under fluorescence (ex. 488 nm, em. 530 nm). Solubilisation (%) was calculated by measuring GFP fluorescence (RFU) of full-length CFTR in each fraction and applying densitometry analysis using ImageJ.

To further explore the DDM solubilisation of CFTR we tested a range of NaCl concentrations in PBS and also 1 M NaCl in Tris-buffer using plCFTR crude membranes. Increasing solubilisation efficiency correlated with an increase in NaCl concentration in PBS increasing solubility from ~25% at 150 mM NaCl to ~61% at 1 M NaCl (Fig. 2.6). With the addition of 1 M NaCl to the Tris-buffer, solubilisation efficiency was increased to ~87% (Fig. 2.6).

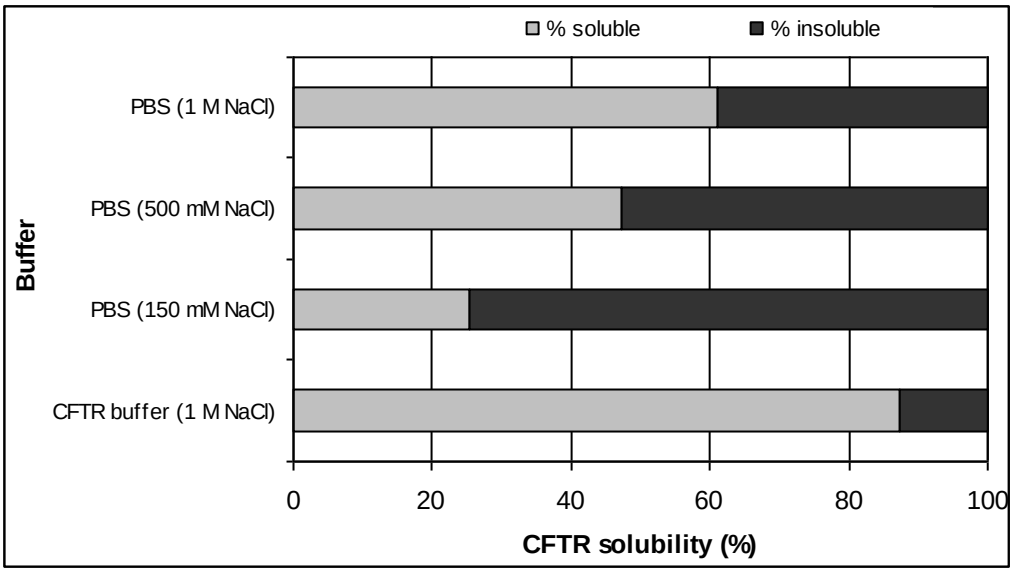


Figure 2.6. Effect of NaCl on CFTR solubilisation efficiency. Soluble and insoluble proteins were resolved by SDS-PAGE and view under fluorescence (ex. 488 nm, em. 530 nm). Solubilisation (%) was calculated by measuring GFP fluorescence (RFU) of full-length CFTR in each fraction and applying densitometry analysis using ImageJ.

2.5 Discussion

Expression of CFTR in heterologous expression systems is necessary in order to obtain sufficient quantities for structural and functional studies. Attempts to express CFTR in yeast systems have previously proven problematic with low levels of expression and degradation of the protein (Huang *et al.*, 1996, Kiser *et al.*, 2001, Zhang *et al.*, 2002b, Ketchum *et al.*, 2004). In this study we have demonstrated the importance of optimising several factors affecting recombinant protein expression in *S. cerevisiae* and their effect on production of full-length CFTR. The importance of using chemical chaperones, in particular glycerol, was the first step towards maximising CFTR expression and, owing to the improvement in detectable protein, this enabled further optimisation of the system to be investigated. Despite the findings of (Newstead et al. 2007) who found the addition of 10% glycerol actually had a negative effect on expression of some membrane proteins, we observed a considerable increase in expression of the full-length CFTR protein with the addition of up to 8% glycerol. This was somewhat expected and supports previous findings that glycerol rescues folding of the F508del mutant of CFTR (Brown *et al.*, 1996, Sato *et al.*, 1996, Papp and Csermely, 2006). We also showed that DMSO and trehalose improved CFTR expression although to a lesser extent than glycerol. This was again consistent with previous data (Kandror *et al.*, 2004, Römisch, 2004). Alongside the more well studied chemical chaperones, we also studied the effect of using additional galactose when inducing CFTR expression. Interestingly, 4% galactose increased expression by 1.9-fold which was surprising as the 2% galactose used in the standard induction media should be in sufficient excess to provide an ample carbon source for the yeast cultures over the induction period. This suggests that galactose may also exhibit chaperone properties in addition to its role as a substrate for metabolism in yeast.

In yeast, CFTR expression (relative to an endogenous fluorescent marker) showed peak expression at ~14 hours. Following this time, expression levels began to decrease with very little detectable full-length CFTR present after 20 hours. Levels of endFP were also very low after 20 hours probably due to overall cell death or stasis in the stationary phase. Similar results were also apparent when exploring the effects of cell density at induction where expression of CFTR (after 14 hours post induction) increased up to an initial induction OD_{600} of 2.0 but with a remarkable decrease above this cell density. An increase in degradation is also observed with induction at higher cell densities and at an OD_{600} of 3.0 and above CFTR appears to be almost completely degraded. To obtain optimal levels of CFTR whilst limiting degradation, an OD_{600} of 1.5-2.0 at induction is most favourable as beyond this point it appears that the cell

stress response has detrimental effects on expression of CFTR and endFP and it is likely that at this high cell density cell death will occur.

Detergents which had previously been used to solubilise CFTR were used (Ramjeesingh *et al.*, 1997, Huang *et al.*, 1998, Ketchum *et al.*, 2004) in addition to the mild, non-denaturing detergent, DDM which has not been previously reported as an efficient solubilising detergent for yeast-expressed CFTR. In tune with this, solubilisation in DDM was initially low compared to LPG, but we were able to improve solubilisation considerably with the addition of 1 M NaCl to solubilisation buffers. This high level of salt may be tolerated by CFTR, indeed crystal structures for isolated CFTR domains (NBD2 PDB: 3GD7; unpublished, (Schmidt *et al.*, 2011)) were obtained in high salt buffers. Solubilisation was relatively constant with pH range from 7.0 to 8.5 but there was a slight increase from ~75% at pH 7.0 to ~83% at pH 9.0. As the majority of downstream chromatography protocols require a pH of 8.0 or lower, these results are encouraging.

This study has shown the importance of optimising expression of recombinant membrane proteins in *S. cerevisiae* and that expression of CFTR can be improved substantially by performing these experiments. Although these methods and conditions could be applied to other membrane proteins to optimise expression in yeast systems, comparison with the findings of Drew *et al.* (Newstead *et al.*, 2007a, Drew *et al.*, 2008) implies that tailoring of conditions for each new protein may be needed.

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Chapter 3 - Expression and Purification of the Cystic Fibrosis Conductance Regulator Protein in *Saccharomyces cerevisiae*

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Protein in *Saccharomyces cerevisiae*. *J. Vis. Exp.* (61), e3860, doi:10.3791/3860 (2012).

3.1 Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride ion channel, that when mutated, can give rise to cystic fibrosis in humans. There is therefore considerable interest in this protein, but efforts to study its structure and activity have been hampered by the difficulty of expressing and purifying sufficient amounts of the protein[1-3]. Like many 'difficult' eukaryotic membrane proteins, expression in a fast growing organism is desirable, but challenging, and in the yeast *S. cerevisiae*, so far low amounts were obtained and rapid degradation of the recombinant protein was observed[4-9]. Proteins involved in the processing of recombinant CFTR in yeast have been described[6-9]. In this report we describe a methodology for expression of CFTR in yeast and its purification in significant amounts. The protocol describes how the earlier proteolysis problems can be overcome and how expression levels of CFTR can be greatly improved by modifying the cell growth conditions and by controlling the induction conditions, in particular the time period prior to cell harvesting. The reagents associated with this protocol (murine CFTR-expressing yeast cells or yeast plasmids) will be distributed via the US Cystic Fibrosis Foundation, which has sponsored the research. An article describing the design and synthesis of the CFTR construct employed in this report will be published separately (Urbatsch, I.; Thibodeau, P. *et al.*, unpublished). In this article we will explain our method beginning with the transformation of the yeast cells with the CFTR construct - containing yeast plasmid (Fig. 3.1). The construct has a green fluorescent protein (GFP) sequence fused to CFTR at its C-terminus and follows the system developed by Drew *et al.* (2008)[10]. The GFP allows the expression and purification of CFTR to be followed relatively easily. The JoVE visualized protocol finishes after the preparation of microsomes from the yeast cells, although we include some suggestions for purification of the protein from the microsomes. Readers may wish to add their own modifications to the microsome purification procedure, dependent on the final experiments to be carried out with the protein and the local equipment available to them. The yeast-expressed CFTR protein can be partially purified using metal ion affinity chromatography, using an intrinsic polyhistidine purification tag. Subsequent size-exclusion chromatography yields a protein that appears to be >90% pure, as judged by SDS-PAGE and Coomassie-staining of the gel.

The video component of this article can be found at http://www.jove.com/video/3860/

3.2 Protocol

3.2.1 Preparation of Media and Buffers

1. YNB: For one litre, suspend 6.9 g yeast nitrogen base without amino acids and 0.77 g complete supplement mixture without uracil in 1 l water. Autoclave. Store at 4 °C.
2. YNBA: For 400 ml, suspend 2.76 g yeast nitrogen base without amino acids, 0.38 g complete supplement mixture without uracil and 8g bacteriological agar in 350 ml water. Autoclave. Mix 8 g glucose with 50 ml water and heat gently until dissolved. Sterilise through a 0.2 µm filter and add to the YNBA whilst the agar is molten. Store at room temperature.
3. 20% glucose medium: For 500 ml, mix 100 g glucose with 500 ml YNB and heat gently until dissolved. Pass through a 0.2 µM filter into a sterile Duran bottle. Store at room temperature.
4. 20% galactose medium: For 2 litres, mix 400 g galactose with 2l YNB and heat gently until dissolved. Pass through a 0.2 µm filter into a sterile Duran bottle. Store at room temperature.
5. Protease inhibitor stocks: CFTR is highly susceptible to proteolysis[4]. The authors found the following inhibitors to be effective in limiting proteolysis, though readers may wish to tailor this list to meet their own requirements. Store in 100 µl aliquots at -20 °C to reduce freeze-thaw problems. All inhibitors should be diluted from the stock solutions to the working concentration as below:

Inhibitor	Stock Concentration	Stock Preparation	Working Concentration
AEBSF	200 mM	Dissolve 48mg in 1ml distilled water	0.2 mM
Benzamidine	300 mM	Dissolve 36mg in 1ml distilled water	0.3 mM
Chymostatin	4 mM	Dissolve 2.5mg in 1ml dry DMSO	4 µM
E-64	7 mM	Dissolve 2.5mg in 1ml distilled water	7 µM
Leupeptin	20 mM	Dissolve 10mg in 1ml distilled water	20 µM

Pepstatin A	15 mM	Dissolve 10mg in 1ml dry DMSO	15 µM
PMSF	1 M	Dissolve 174mg in 1ml dry DMSO	1 mM

6. DTT (1 M): Dissolve 154 mg dithiothreitol in 1ml distilled water. Store at -20 °C. Use at 1:1000 dilution in buffers indicated.
7. EDTA (0.5 M): Mix 29.22 g ethylenediaminetetraacetic acid with 100 ml water. Add 10 N NaOH dropwise until all of the EDTA has dissolved and the pH reaches 8. Make up to 200 ml with water and pass through a 0.2 µm filter into a sterile Duran bottle. Store at room temperature.
8. CRB (300 mM Tris-HCl, pH 7.4, 0.56 M sorbitol, 1 mM EDTA): Dissolve 18.17 g Tris-base in 350 ml water and adjust pH to 7.4 by addition of HCl. Add 51.35 g sorbitol and make volume up to 500 ml with water. Autoclave. Add 1 ml EDTA stock solution and store at 4 °C.
9. CFTR buffer (50 mM Tris, pH 8.0, 10% v/v glycerol): Dissolve 6.06 g Tris-base in 500 ml water. Adjust pH to 8 with HCl, add 100 ml glycerol and make up to 1 L with water. Autoclave and store at 4 °C.
10. 2x load dye: 50 mM Tris-HCl (pH 7.6), 5% glycerol, 5 mM EDTA (pH 8.0), 0.02% bromophenol blue, 4% SDS, 0.05 M DTT. Make 10 ml, aliquot and store at -20 °C.

3.2.2 Screening Transformants

This protocol assumes that the CFTR-GFP-8His fusion gene has been inserted into a yeast plasmid downstream to a *GAL1* galactose promoter (Fig. 3.1) and that the plasmid has been transformed into FGY217 cells, a Pep4 deletion mutant of *S.cerevisiae*[10]. Cells can be grown on YNBA plates and stored for several weeks at 4 °C. For longer term storage, glycerol stocks should be made and stored at -80 °C. Methods for cloning and transformation are described in detail by Drew *et al.* (2008)[10].

1. Pick 5-10 well-separated colonies from a transformation plate. Transfer each colony to a separate sterile 50 ml Falcon tube containing 9 ml YNB and 1ml of 20% glucose medium. For this step, it is important to have a final concentration of 2% glucose (w/v) in the culture to maintain cell growth. Grow overnight for 16 hours at 250 rpm, 30 °C in an orbital shaking incubator.

2. Make glycerol stocks for each of the screened colonies. Aseptically add 0.8 ml of the overnight cultures to 0.2 ml sterile glycerol in labelled screw-top vials, vortex briefly and store at -80 °C.
3. Dilute the remaining overnight cultures to a final volume of 50 ml in YNB, including 250 µl of 20% glucose medium. For this step, it is important to dilute the glucose concentration to approximately 0.1% (w/w) in the culture because high glucose can repress the *GAL1* promoter[10]. Grow cultures in labelled 250 ml Erlenmeyer baffled flasks to an OD_{600} of 0.7-0.8 at 250 rpm, 30 °C in an orbital shaking incubator.
4. Induce the cultures by addition of 5 ml 20% galactose medium to each flask and grow on for 16 hours.
5. Confirm the expression of CFTR using fluorescence microscopy. Take 100 µl of culture and add 100 µl glycerol to limit cell mobility in solution. Analyse cells on a Delta Vision RT restoration microscope (or similar), using a blue laser under a FITC filter (excitation wavelength of 490 nm and emission wavelength of 528-538 nm). Positive expression of the CFTR-GFP fusion protein should be visible as a ring of fluorescence at the plasma membrane of the yeast cells. Untransformed yeast cells may be used as a control.
6. Transfer the cultures into 50ml Falcon tubes. Harvest the cells by centrifugation at 3500 x g, 4 °C for 10 minutes in a bench top centrifuge. Whilst the centrifuge is running, prepare 2 ml microfuge tubes with screw tops containing approximately 500 µl acid-washed glass beads and place on ice. Discard the supernatants and resuspend each pellet in 500-800 µl ice-cold CRB with protease inhibitors. Transfer the suspensions to the microfuge tubes containing the beads and keep on ice.
7. Lyse the cells by vigorously shaking/vortexing each microfuge tube for 10 periods of 30 seconds, resting on ice in between periods. A beadbeater can be employed as an alternative, e.g. a BioSpec mini beadbeater operated for 3 min.
8. Place the tubes into a benchtop microfuge and centrifuge at 3,500 x g, 4 °C for 5 minutes. Transfer the supernatants containing the crude membrane population to clean microfuge tubes and place on ice. Add 500 µl fresh ice-cold CRB with protease inhibitors to each tube and repeat the process to accumulate the membranes.
9. Collect the crude membranes by spinning at maximum speed, 4 °C in a benchtop microfuge for 2 hours. Discard the supernatant and resuspend each pellet in 50 µl ice cold CFTR buffer.
10. In clean microfuge tubes, mix 15 µl of each suspension with 15 µl 2x load dye by pipetting up and down. Incubate at room temperature for 10 minutes. Do not boil the samples, as this will cause CFTR and other membrane proteins to form SDS-insoluble aggregates and also denature the GFP tag.

11. Load the samples along with PageRuler Plus prestained protein standards (Fermentas) onto a 4-20% Tris/glycine gradient gel (NuSep) and run at 150 V for 40 minutes or until the dye-front is at the bottom of the gel.
12. Identify the highest expressing cells by in-gel fluorescence. Place into a fluorescence imaging system such as a Typhoon scanner. Scan the gel using the blue laser at an excitation wavelength of 488 nm and an emission wavelength of 526 nm. The CFTR-GFP fusion should be visible at approximately 220 kDa. There will also be a weak fluorescent band visible at about 70 kDa which is probably an intrinsic yeast FAD containing membrane protein (such as succinate dehydrogenase subunit A)[11,12].
13. Stain the gel with Coomassie, destain and scan the gel for comparison to the fluorescence scan using a convenient image viewing software package. The Coomassie-stained gel allows a relative assessment of CFTR-GFP expression levels in different clonal lines after normalization for the amount of total protein loaded onto each track of the gel.
14. Streak out the highest expressing cell line from its glycerol stock onto a fresh YNBA plate and incubate at 30 °C for 2-3 days. This plate may then be stored for up to 2 weeks at 4 °C.

3.2.3 Large-scale Fermenter Culture

1. Prepare pre-cultures for the fermenter. Scrape a 1 cm^2 area of cells from the YNBA plate using a sterile loop and add to 45 ml YNB and 5 ml 20% glucose medium, such that the OD_{600} is approximately 0.1. Grow in 250 ml Erlenmeyer baffled flasks at 250 rpm, 30 °C in an orbital shaking incubator until the OD_{600} reaches 1.
2. Split the culture between two 2 l Erlenmeyer baffled flasks each containing 450 ml YNB and 25 ml 20% glucose medium. Grow these on at 250 rpm, 30 °C in an orbital shaking incubator until the OD_{600} reaches 1.2.
3. Whilst these pre-cultures are growing, set up the fermenter. Make 11.2l of YNB as described, but dissolve an additional 8.28 g YNB and 0.95 g drop out supplement to compensate for the addition of glycerol at induction. Aseptically add the 11.2 l YNB and 75 ml 20% glucose medium to a sterile 20 l fermenter vessel and adjust the running temperature to 30 °C.
4. Aseptically add the precultures to the fermenter and set the stirring speed to approximately 800 rpm and maintain the temperature at 30 °C. Compressed air should flow at approximately 15 dm^3min^{-1}. Once the fermenter culture reaches an

OD_{600} of 1.2, induce by aseptically adding 1.5 l YNB 20% galactose solution and 1.2 l glycerol. Reduce the temperature to 25 °C and grow the culture for 16 hours.

5. Transfer the fermenter contents into chilled 1 l centrifuge pots on ice using a peristaltic pump. Harvest the cells by centrifugation at 3,500 x g, 4 °C for 30 minutes in a large capacity rotor (e.g. 6 litre). Resuspend the cells in 150 ml ice cold CRB with protease inhibitors. From here on, all work should be carried out at 4 °C.
6. Lyse the cells by passing through a Constant Systems cell disrupter in 4 passes at 25, 30, 32 and 35 kPa, collecting the lysate on ice in each case. Alternatively, use a bead beater (Biospec) with an equal volume of acid-washed 0.5 mm glass beads and agitate for 3 minutes on full power. Transfer the lysate to 50ml Falcon tubes and pellet the cell debris by centrifugation at 3,500 x g, 4 °C for 15 minutes in a benchtop centrifuge.
7. Transfer the supernatant to chilled centrifuge tubes. Centrifuge at 14,000 x g, 4 °C for 30 minutes in a centrifuge to remove mitochondria.
8. Transfer supernatant to chilled ultracentrifuge tubes. Centrifuge at 200,000 x g, 4 °C for 90 minutes in an ultracentrifuge to collect microsomes.
9. Carefully decant and discard the supernatant and add 2 ml ice cold CFTR buffer with protease inhibitors and 1 mM DTT to each tube. Gently resuspend the pellets using a paintbrush, top up each tube with CFTR buffer and mix using a vortex mixer.
10. Centrifuge the suspension at 200,000 x g, 4 °C for 60 minutes in an ultracentrifuge, discard the supernatant and resuspend pellets in 2 ml ice cold CFTR buffer with protease inhibitors (no DTT) using a paintbrush.
11. Pool the resuspended microsomes, adjust the final volume to 50 ml with CFTR buffer and mix well. Reserve a 1 ml aliquot for SDS-PAGE gel analysis, as described. The microsomes can now be flash frozen in liquid nitrogen and then stored at -80 °C until needed.
12. CFTR can be extracted from microsomes using one of the following detergents: lithium perfluorooctonoate acid (LiPFO), tetradecanoly-lysophosphatidylglycerol (LPG14), n-dodecyl-β-D- maltoside (DDM). Mix the microsomes with CFTR buffer, protease inhibitors and 5% detergent (w/w). If DDM is used, also add 300 mM NaCl to the buffer. Agitate at 4 °C for 15 minutes on a tube rotator.
13. Centrifuge the samples at 100,000 x g, 4 °C for 1 hour in an ultracentrifuge. Retain the supernatant and take a small aliquot for SDS-PAGE gel analysis. CFTR may now be purified from the solubilised material by immobilised metal affinity chromatography followed by size exclusion chromatography.

3.3 Representative Results

Transformation of yeast with the CFTR-containing plasmid is not 100% efficient. A representative small-scale screen of CFTR expression in selected colonies from a transformation experiment will yield about 1 in 4 colonies expressing the protein. A typical result from a screen of 5 colonies picked from a plate is shown in panel A of Fig. 3.3. One of the colonies shows a strong level of expression of the CFTR-GFP fusion protein which typically runs between the 250 kDa and 130 kDa markers, as shown. The CFTR-GFP fluorescence levels will vary considerably between experiments, with colony 4 in Fig. 3.3 showing at least 10x greater fluorescence than the intrinsic fluorescent band at about 70 kDa. If expression levels of CFTR-GFP appear to give less fluorescence than the 70 kDa band, then it is probably worth re-transforming and choosing a colony with higher levels of CFTR-GFP expression. As shown in Fig. 3.3A, it is unlikely, even with a high expression level of CFTR-GFP, that the CFTR-GFP band will be discernable in the cell extract by Coomassie staining. Once selected colonies have been grown in larger scale experiments, and microsomes isolated, the presence of CFTR-GFP within the microsomes will need to be assessed, as shown in Fig. 3.3B. The results of this experiment are important, not only to assess the efficiency of the induction of expression, but also to check that the microsomes have been prepared carefully and that proteolysis has been minimized. The results shown in Fig. 3.3B imply that in this experiment the CFTR-GFP expression is somewhat lower (as judged relative to the intrinsic 70 kDa band) than in the small-scale experiment shown in panel A. However this impression is biased by the overexposure of the fluorescence detector in this measurement. This was because the experimenter was checking for the presence of proteolytic fragments of the CFTR construct. There is some evidence in this experiment for some fluorescent proteolytic fragments between the 130 kDa and 100 kDa markers, but these are very weak compared to the full-length CFTR-GFP band. With the protease inhibitors described here, we find little evidence for proteolytic degradation of CFTR after cell breakage. If significant proteolysis is observed, we recommend making fresh protease inhibitor stock solutions. We have also found that commercial protease inhibitor cocktail tablets are not as effective for this system. Growth of cells beyond 16 hr (post-induction) will give rise to decreased CFTR expression as shown in Figure 3.4. This is probably due to turnover of the protein, perhaps due to upregulation of the yeast protein quality control machinery[6-9,13]. It is therefore advisable to monitor CFTR expression levels after induction with galactose, if possible, as the optimal time to harvest the cells may vary from one laboratory to another.

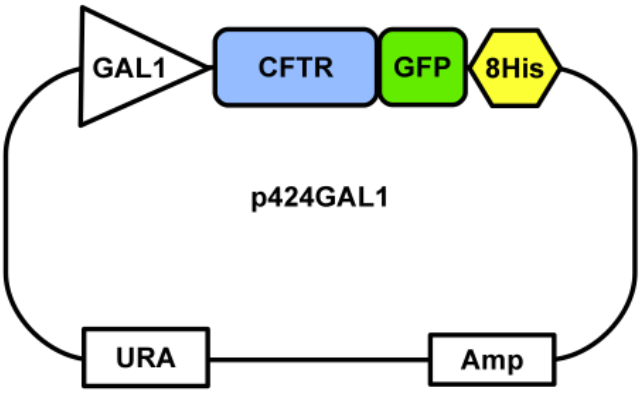

Figure 3.1. The CFTR construct-containing yeast plasmid. The CFTR-GFP-8His fusion is inserted into the 2μ p424GAL1 expression vector, under the control of a galactose (*GAL1*) promoter. The vector also contains a uracil selection marker (URA) and an ampicillin resistance gene (Amp).

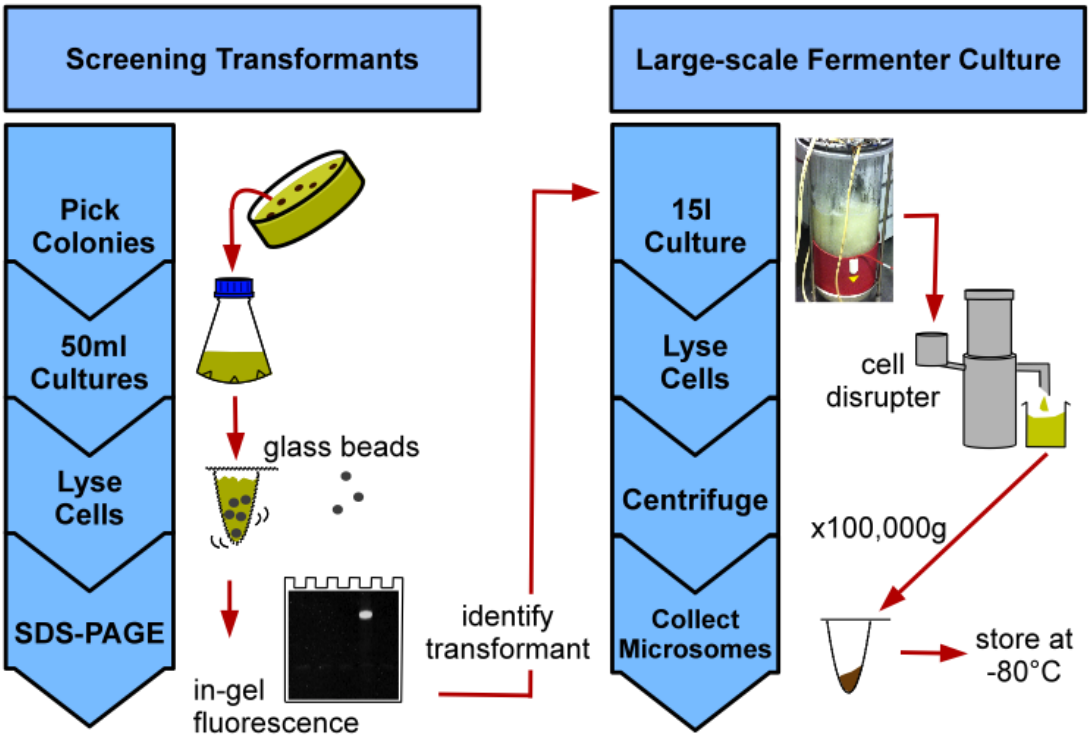

Figure 3.2. A flowchart summarising the visualised protocol.

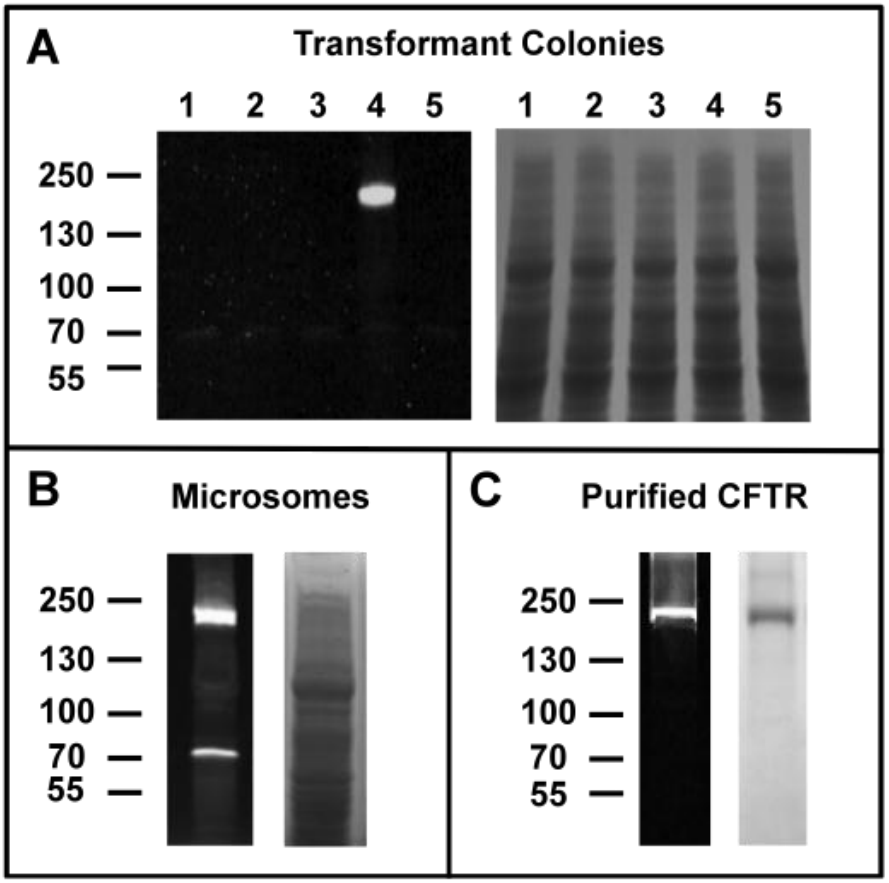

Figure 3.3. Representative SDS-PAGE gels of CFTR expression and purification. Panel A shows five randomly picked transformant colonies (lanes 1-5) that were screened for CFTR expression. **Panel B** shows microsomes that were isolated from a 15 l fermenter culture. **Panel C** shows purified murine CFTR obtained after two-stage purification using affinity chromatography followed by size exclusion chromatography. All gels are shown under illumination conditions exciting fluorescence from the GFP domain (left) and after Coomassie stain (right). The relative locations of molecular weight standards are listed on the left (kDa).

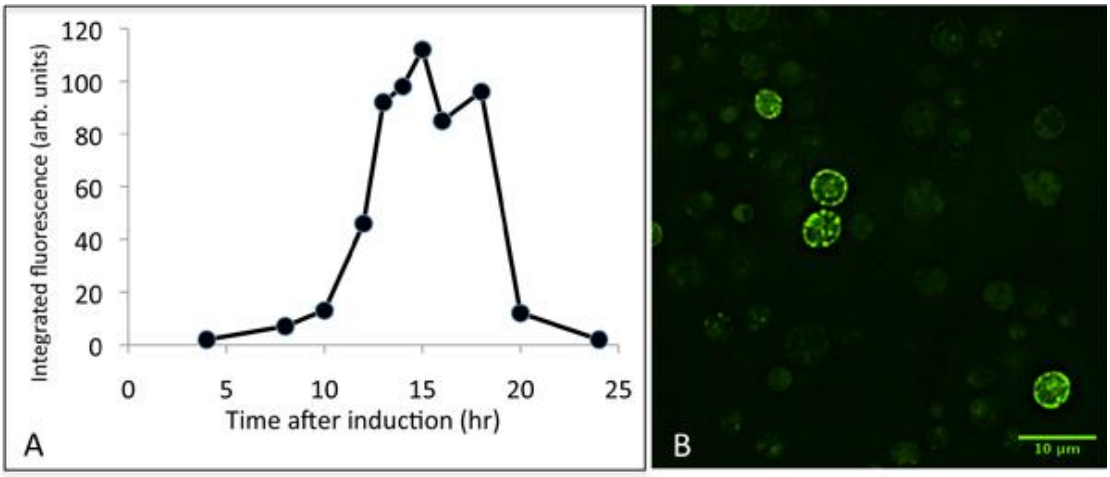

Figure 3.4. Representative data for the expression of CFTR in yeast. Panel A shows a timecourse of CFTR expression after induction with galactose. Cell extracts were analysed by SDS-PAGE, and the GFP fluorescence for the CFTR-GFP protein band was integrated. **Panel B** shows typical results for fluorescence microscopy of GFP-expressing cells 16 hr post induction. Typically, only a fraction of the cells express CFTR at high levels.

3.4 Discussion

This paper provides a method for the expression of murine CFTR protein in yeast cells, which should facilitate research on cystic fibrosis. The aim is to link this paper with the release of the murine CFTR DNA construct, which will be available through the Cystic Fibrosis Foundation (http://www.cff.org/research/CFFT/). Other orthologs should become available later. Transformation of the yeast cells with the CFTR-containing vector is straightforward, but it is important to screen for colonies expressing high levels of CFTR. Variable expression levels may arise from several factors, but the number of copies of the plasmid per cell probably accounts for a significant degree of variation. Critical steps described here should allow production of CFTR-expressing yeast cells and CFTR-containing microsomal membranes. Once the transformation, growth, harvesting and lysis of yeast cells have been mastered, purification of the protein should be possible, and in Figure 3.3 we have given an example of the purity that should be achievable in this case as a useful benchmark. It is not our intention in this manuscript to provide detailed methodology for purification of the protein. However, there are some critical downstream purification steps that are specific to the *S.cerevisae* expression system, such as cell lysis and microsome purification, and these have been included in detail in this manuscript. It should be mentioned, however, that apart from the two methods we have used, alternative yeast cell disruption methods can be employed, such as the use of a French pressure cell. The recombinant protein has a TEV-cleavable C-terminal GFP domain that allows the protein to be tracked after induction (Fig. 3.4). Yeast have an intrinsic 70 kDa protein (probably succinate dehydrogenase[12]) that fluoresces under the same conditions[11], and this can provide a useful internal calibration standard for the relative expression levels of CFTR in whole cell extracts or microsomes (Fig. 3.3). It is clear from the data shown in Figure 3.4 that the timing of cell harvesting after induction with galactose is crucial. Yields of CFTR drop precipitously after about 16 hr of induction, so that there is barely any detectable CFTR in yeast cells after 24 hr of induction.

The yield of purified protein is about 1-2 mg CFTR protein per 15 litre fermenter culture. Recovery can be estimated as about 70% of the total CFTR-GFP protein up to the microsome stage, and about 25% recovery of purified protein. Characterisation of the *S. cerevisiae*-expressed CFTR is ongoing. As seen in Fig. 3.4, the protein's location in the cell can be monitored by fluorescence microscopy. Although much of the fluorescence is found around the periphery of the cell as expected[10], some of the protein displays a punctate localization, either in, or just inside the plasma membrane which could be due to CFTR recycling through a late Golgi/endosomal pathway[14] or

perhaps a compartment downstream of the budding of transport vesicles from the ER[4]. Treatment with PNGaseF, an enzyme that deglycosylates proteins, showed minimal change in the migration of the CFTR protein band on SDS-PAGE, implying that it is unglycosylated, or has minimal glycosylation[15]. Experiments on the phosphorylation state of the protein are underway. In some of the detergents tested so far, the purified protein displays ATPase activity (that is inhibited by a CFTR-specific inhibitor[16]) at rates that are similar to those previously published[2,15]. Measurement of CFTR channel activity will require reconstitution of the purified protein, which would imply a final purification step in a detergent that has a relatively high critical micelle concentration (cmc)[17]. Yeast microsomes containing CFTR can be solublised with several commonly employed detergents[18], including detergents such as dodecyl maltoside[2], which are generally considered to be 'mild'. However most high cmc detergents have proven to be inefficient for solubilsation, so far, suggesting that exchange into these detergents should be considered at a late stage in any purification scheme.

Disclosures

No conflicts of interest declared.

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Chapter 4 - Purification of the Cystic Fibrosis Transmembrane Conductance Regulator Protein Expressed in *Saccharomyces cerevisiae*

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Expressed in *Saccharomyces cerevisiae*. *J. Vis. Exp.* (), e51447, doi:10.3791/51447 (2014).

4.1 Abstract

Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein cause cystic fibrosis (CF), an autosomal recessive disease that currently limits the average life expectancy of sufferers to <40 years of age. The development of novel drug molecules to restore the activity of CFTR is an important goal in the treatment CF, and the isolation of functionally active CFTR is a useful step towards achieving this goal.

We describe two methods for the purification of CFTR from a eukaryotic heterologous expression system, *S. cerevisiae*. Like prokaryotic systems, *S. cerevisiae* can be rapidly grown in the lab at low cost, but can also traffic and post-translationally modify large membrane proteins. The selection of detergents for solubilisation and purification is a critical step in the purification of any membrane protein. Having screened for the solubility of CFTR in several detergents, we have chosen two contrasting detergents for use in the purification that allow the final CFTR preparation to be tailored to the subsequently planned experiments.

In this method, we provide comparison of the purification of CFTR in dodecyl-β-D-maltoside (DDM) and 1-tetradecanoyl-*sn*-glycero-3-phospho- (1'-*rac*-glycerol) (LPG-14). Protein purified in DDM by this method shows ATPase activity in functional assays. Protein purified in LPG-14 shows high purity and yield, can be employed to study post-translational modifications, and can be used for structural methods such as small-angle Xray scattering and electron microscopy. However it displays significantly lower ATPase activity.

The video component of this article can be found at http://www.jove.com/video/51447/

4.2 Introduction

Cystic fibrosis (CF) is the most common genetic disorder in Europe and North America with an incidence of about 1 in 2,500 live births. CF occurs when mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein cause loss of its function at the plasma membrane of epithelial cells[1]. The most serious consequence of this defect is irreversible lung damage, which shortens the life expectancy of sufferers to <40 years of age[2,3].

CFTR is an ATP-binding cassette (ABC) transporter that has evolved to become an ion channel[1,4]. Despite its quite altered function in the plasma membrane of cells, it still retains significant sequence homology with other ABC transporters. Intriguingly, the specialized parts of CFTR (*i.e.* its regulatory region and its N- and C-termini) share no significant sequence similarity with other metazoan ABC transporters, hence there are no clues as to the origins of these sequences in CFTR. On the basis of its primary structure, CFTR is classified as a C-family member of the ABC transporter family, but there is no strong evidence for a residual functional linkage to this sub-family. There have been some reports of glutathione transport activity for CFTR[5-7], which would be consistent with the roles of other C-family members[8,9], although other reports suggest that reduced glutathione may inhibit the CFTR ATPase activity, rather than showing the substrate-induced stimulation that characterize the ABC transporters[10]. Measurement of ion conductance is sufficiently sensitive to allow the channel activity of single CFTR molecules to be studied[1] and CFTR channel properties have been monitored as a function of time, temperature, ATP concentration, membrane potential and phosphorylation state, as well as in the presence of a host of small molecule inhibitors, potentiators and modifiers. These studies have also added significantly to our knowledge of how ABC transporters function. Nevertheless, expression of CFTR in significant amounts and its subsequent purification has proven to be particularly challenging and success has been limited to a few laboratories[10-13].

The need to develop more effective drugs is pressing, yet this process has been hindered by the lack of purified CFTR for screening small molecules. Solving the CFTR expression and purification problem would enable high-throughput drug screening aimed at correcting the primary defect in CF and would also open up a route for high-resolution structural studies to inform rational drug design. Moreover, even relatively basic biochemical characteristics of the protein, such as its functional oligomeric state, interacting proteins and ATPase activity remain poorly characterized. We have previously reported a protocol for the large-scale expression of GFP- and His-tagged

murine CFTR in *S. cerevisiae*[14] and now further describe protocols for the purification of CFTR. We have used these methods to purify five orthologues of CFTR, and present data for the purification of chicken CFTR as an example. The selection of detergents for solubilization and purification is a critical step in the purification of any membrane protein. Having screened for the solubility of CFTR in several detergents, we have chosen two contrasting detergents for use in the purification. Dodecyl-β-D-maltoside (DDM) is a non-ionic detergent that has been extensively used for both structural and functional studies of membrane proteins[15-21]. The ionic detergent 1-tetradecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (LPG-14) is highly efficient in the solubilization of CFTR and has previously been used in the purification of functional membrane proteins[10,22,23], including purification of CFTR from *S. cerevisiae*[24].

4.3 Protocol

4.3.1 Preparation of Buffers

1. To make the 100x stock of protease inhibitor (PI) cocktail dissolve 96 mg AEBSF, 3.5 mg chymostatin, 10 mg E64, 16.5 mg leupeptin, 16.5 mg pepstatin, 348 mg PMSF and 4 mg bestatin in 20 ml DMSO. Make 1 ml aliquots and store at -20 °C. To make a 100x stock of benzamidine, dissolve 720 mg in 20 ml ultra pure water (ddH_2O) and store in 1 ml aliquots at -20 °C. This quantity is sufficient for one purification. In all buffers, PI and benzamidine stocks are used at a 1 in 100 dilution.
2. Prepare 'mPIB' (0.3 M Tris pH 8, 0.3 M sucrose, 2 mM DTT) and 'CFTR' (50 mM Tris pH 8, 20 % (v/v) glycerol, 1 mM DTT) buffers and chill to 4 °C. Before use, add 1:100 of the protease inhibitor cocktail and 1:100 benzamidine according to the volume of mPIB used to resuspend the cell pellet (*e.g.* use 3.5 ml PI and 3.5 ml benzamidine in a total volume of 350 ml mPIB).
3. Prepare solubilization buffers. Lyso-phosphatidyl glycerol-14 (LPG) solubilization buffer (50 mM Tris pH 8, 10 % (v/v) glycerol, 50 mM NaCl, 1 mM DTT, protease inhibitors (PIs) and 4% (w/v) LPG) and dodecyl maltoside (DDM) solubilization buffer (50 mM Tris pH 8, 20 % (v/v) glycerol, 1 M NaCl, 1 mM DTT, protease inhibitors, 4% (w/v) DDM). Buffer can be sonicated in a sonicator bath (35 W, 40 kHz) to assist with dispersal of the detergent, but avoid vortexing the mixture, as this creates bubbles. Chill to 4 °C before use.
4. CFTR purification buffer for the LPG purification is 50 mM Tris, 10% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.1% (w/v) LPG-14 and protease inhibitors. Prepare 350 ml of this buffer, and 150 ml of the same buffer plus 1 M imidazole. Adjust pH of both buffers to 8.0.
5. The buffer for purification in DDM consists of 50 mM Tris pH 8.0, 20% (v/v) glycerol, 1 M NaCl, 1 mM DTT, 0.1% (w/v) DDM. Prepare 350 ml of this buffer, and 150 ml of the same buffer plus 1 M imidazole. Adjust pH of both buffers to 8.0.
6. For gel permeation chromatography (GPC) buffer containing LPG, prepare 50 mM Tris pH 8.0, 10% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.05% (w/v) LPG-14. For GPC using DDM prepare a buffer of 50 mM Tris pH 8.0, 20% (v/v) glycerol, 1 M NaCl, 1 mM DTT, 0.1% (w/v) DDM. All buffers and ddH_2O used on the GPC column should be filtered (0.2 µm filter) and degassed before use.
7. SDS-PAGE sample buffer (2x the working concentration): 50 mM Tris-HCl pH 7.6, 5% (v/v) glycerol, 5 mM EDTA, 0.02% (w/v) bromophenol blue. Make 700 µl

aliquots and store at -20 °C. Before use, add 200 µl of 20% (w/v) sodium dodecyl sulfate (SDS) and 100 µl of fresh 0.5 M DTT. Incubate for at least 10 min with sample at room temperature before loading on gel. Do not heat; this will denature the GFP and may cause CFTR to aggregate.

8. To make lipid stocks for reconstitution, dissolve a 4:1 (w/w) mixture of *E. coli* lipids and cholesterol in chloroform and methanol (2:1 v/v), and dry in a glass vial under N_2 gas for 2 hr to form a lipid film. Add GPC buffer (with no NaCl) to a lipid concentration of 40 mg/ml and use repeated vortexing and sonication (35 W, 40 kHz) to clarify the solution.
9. For the ATPase assay, prepare 100x stocks of ATPase inhibitors by dissolving sch28080 to 1 mM in DMSO, NaSCN to 1 M in ddH_2O and oligomycin to 2.5 mM in 100% (v/v) ethanol. Store in aliquots at -20 °C. Make 100 ml of ATPase buffer with 50 mM Tris pH 7.4, 150 mM NH_4Cl, 5 mM $MgSO_4$ and 0.02% (w/v) NaN_3. This can be stored at room temperature and used for several assays. Prepare a 5 mM ATP stock immediately prior to use and keep on ice. (N.B. Use Na_2ATP to prevent excessive background signal from phosphate in the assay). Prepare the SDS stop solution (12% (w/v) SDS in ddH_2O).
10. For the Chifflet detection prepare buffer A (3% (w/v) ascorbate, 0.5% (w/v) ammonium molybdate, 0.5 M HCl) immediately before use and buffer B (2% (w/v) sodium citrate, 2% (w/v) sodium meta-arsenite, 2% (v/v) acetic acid).

4.3.2 Isolation of Yeast Microsomes

1. *S.cerevisiae* expressing chicken CFTR are grown as described in O'Ryan *et al.* (2012)[14]. Store the material from a 20 L fermentation in two aliquots at -80 °C for up to 6 months.
2. Defrost one aliquot of cells rapidly and resuspend in 3 ml chilled mPIB per gram of cells.
3. Disrupt cells in a bead mill using glass beads of 425-600 µm diameter. Use 5 x 1 min periods of cell disruption separated by 1 min rest periods. (The rest periods are essential to ensure that the cells are not heated during disruption.)
4. Monitor cell disruption by centrifugation of a 1 ml sample of the cell lysate from the bead mill. Centrifuge (12,000 x g, 4 °C, 5 min) in a bench top centrifuge. Dilute the supernatant to 1:50 with mPIB in a cuvette and measure the A380. If A380 > 0.1, or has stopped increasing despite several repeated bead-beating cycles, proceed to the following step.

5. Centrifuge the total cell lysate (12,000 x g, 4 °C, 20 min). Retain the supernatant. Discard the pellet (containing unbroken cells and mitochondria), but if there is any doubt about the efficiency of cell breakage, then retain the pellet also.
6. Centrifuge the supernatant from the previous step (200,000 x g, 4 °C, 1.5 hr). Discard the supernatant and resuspend the pelleted microsomal membranes in CFTR buffer. **If the microsomes are intended for purification using DDM, supplement the CFTR buffer with 1 M NaCl**.
7. Repeat the centrifugation of the resuspended membrane fraction (100,000 x g, 4 °C, 1 hr) and discard the supernatant.
8. Resuspend the pelleted microsomes in a minimum volume of CFTR buffer (final volume 5-15 ml, total microsomal protein 70-200 mg). A Bradford assay may be used to determine the total concentration of microsomal proteins[25]. In addition the fluorescence emission spectrum of the membranes should be measured (excitation = 485 nm, emission = 500-600 nm) and should have a distinct GFP fluorescence peak (maximum at 512 nm). CFTR can be specifically detected on an SDS-PAGE gel, scanned under GFP fluorescence conditions (**Figure 4.1**).
9. Flash-freeze the resuspended microsomes by plunging into liquid nitrogen and store at -80 °C, or continue to the next step.

4.3.3 Solubilisation of Microsomes

1. If frozen, defrost microsomes immediately before use in a water bath set to 10 °C.
2. For the solubilisation of membranes, dilute the microsomes with an equal volume of the relevant solubilisation buffer to give a final detergent concentration of 2% (w/v) and a microsomal protein concentration 5 mg/ml. Incubate this mixture for 1 hr at 4 °C with agitation (tube rotator). Retain 200 µl for analysis.
3. Centrifuge the mixture (100,000 x g, 4 °C, 45 min). Remove the supernatant containing the solubilised membrane proteins, pass it through a 0.45 µm syringe filter and store on ice. Measure the fluorescence of the supernatant.
4. Resuspend the insoluble fraction in 1% (w/v) SDS solution to a volume equal to the soluble fraction. Measure the fluorescence in this fraction and retain an aliquot of 50 µl for SDS-PAGE analysis.

4.3.4 Nickel-affinity Purification of CFTR

1. Link two 5 ml nickel sepharose columns in series. Wash with 2 column volumes (CV) 20% (v/v) ethanol, followed by 2 CV ddH_2O, then wash the column with 2 CV of solubilization buffer, containing 1 M imidazole. Repeat with 2 CV of solubilization buffer lacking imidazole.
2. Add imidazole to a final concentration of 5 mM to the solubilized material and manually load the material onto the column or into a sample loop if using an automated liquid chromatography device.
3. Load the solubilized material onto the column at a flow rate of 0.5 ml/min, and wash with 2 CV of imidazole-lacking buffer at the same flow rate to remove unbound material. Collect fractions in 50 ml Falcon tubes.
4. For the first wash, use 3 CV of purification buffer with 40 mM imidazole at a flow rate of 1 ml/min. Collect 2 ml fractions.
5. For the second wash, use 3 CV of purification buffer with 100 mM imidazole. Collect 2 ml fractions.
6. Elute CFTR from the HisTrap column with 3 CV of purification buffer with 400 mM imidazole. Collect 2 ml fractions.
7. Monitor fluorescence in eluted fractions.
8. Retain aliquots of peak fractions for SDS-PAGE analysis. Flash freeze remaining peak fraction samples and store at -80 °C, or continue to the next purification step.

4.3.5 Gel Permeation Chromatography (GPC) Purification of CFTR

1. Equilibrate the column (Superose 6 10/300 GL) with 1.2 CV ddH_2O followed by 1.2 CV GPC buffer.
2. During step 1, concentrate the Ni-affinity purified fractions with the highest GFP fluorescence using a 100,000 MWCO centrifugal filter at 4 °C. If purifying in DDM, avoid concentrating the sample above a protein concentration of 0.3 mg/ml protein as this will cause significant sample loss. Remove the retentate from the concentrator and centrifuge at 100,000 x g for 30 min at 4 °C to pellet large particles.
3. Inject this sample onto the column and elute with an isocratic gradient of 1.2 CV GPC buffer. Collect 0.5 ml fractions.
4. Measure GFP fluorescence to identify those fractions containing CFTR. Retain a small volume (*e.g.* 50 µl) of each for analysis by SDS-PAGE.

5. Freeze fractions in liquid nitrogen and store at -80 °C.

4.3.6 Reconstitution of CFTR

1. Add lipids to the purified CFTR at lipid-to-protein ratio 100:1 (w/w) and incubate at 4 °C for 1 hr. Similarly set up a lipid-only control, substituting the purified protein with the same volume of GPC buffer.
2. Remove detergent from the protein/lipid mixture using hydrophobic adsorbent beads. Wash adsorbent beads in 5 CV ddH_2O, 5 CV 70% (v/v) ethanol, 5 CV ddH_2O and 5 CV GPC buffer lacking the detergent. Add 200 mg of washed adsorbent beads per ml of purified protein and incubate at 4 °C overnight with gentle agitation.
3. Collect the reconstitution sample from the adsorbent beads into a fresh tube using a thin-ended pipette tip.

4.3.7 Measurement of ATPase Activity

1. Determine the rate of CFTR-specific ATPase activity using a modified Chifflet assay[26,27] in a 96-well plate format. With sodium phosphate stock solution (0.65 mM) prepare 0-20 nmol phosphate in a final volume of 50 µl as standards. Use a 1:1 mixture of CFTR buffer and ATPase buffer to dilute the phosphate stock.
2. Incubate both reconstituted CFTR and blank liposomes with 1:100 (v/v) ATPase inhibitors on ice for 10 min. Use at least 5 µg of reconstituted CFTR.
3. Add ATP to a final concentration of 2 mM and incubate at 25 °C for 1 hr. Stop the reaction by adding 40 µl of 10% (w/v) SDS to each well (including the standards).
4. Add 100 µl of buffer A and incubate for 10 min. Add 100 µl buffer B to each well and measure the absorbance at a wavelength of 800 nm in a 96-well plate-compatible UV/Vis spectrophotometer.
5. Convert absorbance at 800 nm into an amount of liberated phosphate using the phosphate standards. Calculate the rate of ATP hydrolysis after subtracting background signal (liposome-only wells).
6. For non-reconstituted CFTR follow the same protocol using CFTR buffer for the background readings.

4.4 Representative Results

The protocol described above is an efficient means to isolate CFTR-enriched microsomes, with almost complete recovery of CFTR during the cell breakage and preparation of the crude microsomes (**Figure 4.1**). Other cell breakage methods may also be employed effectively. We have utilized a French pressure cell, and other high-pressure/cavitation devices (also in combination with impacting against a ruby target) with equal efficiency. For convenience and low initial cost of the equipment, we find the bead-beating method the best.

Using LPG to solubilize and purify CFTR yielded 80 µg protein/L culture at >90% purity (**Figure 4.2**). The high yield was due to efficient solubilisation of CFTR by LPG (compare **Figure 4.2b**, lanes 2 and 4). In addition, efficient and tight binding to the column resulted in minimal loss of CFTR in the unbound fraction and the absence of CFTR in the wash fractions (**Figure 4.2**, lanes 3, 5 and 6). The eluted protein had a purity of >90%, estimated by Coomassie-stained SDS-PAGE gels and using densitometry of the CFTR and contaminant bands. Gel permeation chromatography (GPC) separated LPG-purified CFTR from low-molecular weight contaminants (**Figure 4.4**, lower panel).

The protocol for CFTR purification using DDM gives purity of about 60% and yield of roughly 50 µg/L (**Figure 4.3**). Electron microscopy (EM) of negatively stained fractions from the GPC eluting at about 10 ml (**Figure 4.4**) showed that DDM-purified CFTR contains aggregates of 20-30 nm diameter as well as smaller particles of 10 nm diameter (data not shown). It is possible that the small aggregates can reversibly associate and dissociate as ultra filtration with a 1 MDa cut-off filter failed to remove the EM-detectable aggregates. LPG-purified material did not adsorb to a glow-discharged grid, hence was studied by cryo-EM of unstained fractions. This showed a very homogeneous particle population of a relatively small size (6-8 nm diameter, data not shown).

Finally, the ATPase activity of the purified proteins was measured (**Figure 4.5**). As a member of the ABC protein family, CFTR has two nucleotide binding domains (NBDs) capable of binding and/or hydrolyzing ATP. The data indicate that the purified protein was not able to hydrolyze ATP in the LPG-solubilized state and showed weak ATPase activity in the presence of DDM (**Figure 4.5**, unfilled bars). After the addition of lipids, and detergent removal, ATPase activity was 4-fold higher for samples that had been purified in DDM (13 nmol ATP/min/mg protein). The addition of lipids and removal of LPG similarly restored activity to CFTR that had been isolated using LPG, but with a

final lower rate (1.5 nmol ATP/min/mg protein) than the DDM-purified and reconstituted material.

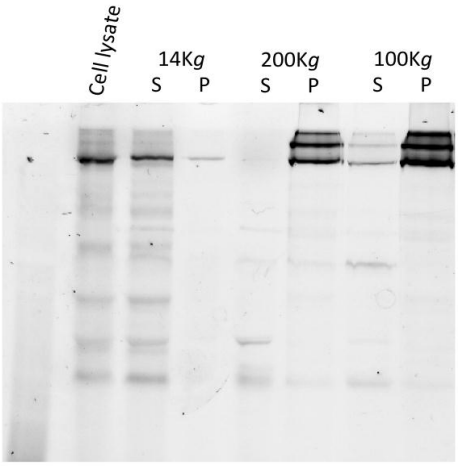

Figure 4.1. Monitoring levels of chicken CFTR in cell lysate (CL), supernatants (S) and pellets (P) during various centrifugation steps used for microsome isolation and washing. SDS-PAGE gels were visualized using the in-gel fluorescence of the GFP tag. The supernatant after cell breakage and centrifugation at 14,000 x g contains virtually all the CFTR (including degradation products). Ultracentrifugation at 200,000 x g sediments all the full-length CFTR leaving some fragments in the supernatant. Ultracentrifugation at 100,000 x g of salt-washed microsomes pellets nearly all the CFTR with the removal of some further fragments.

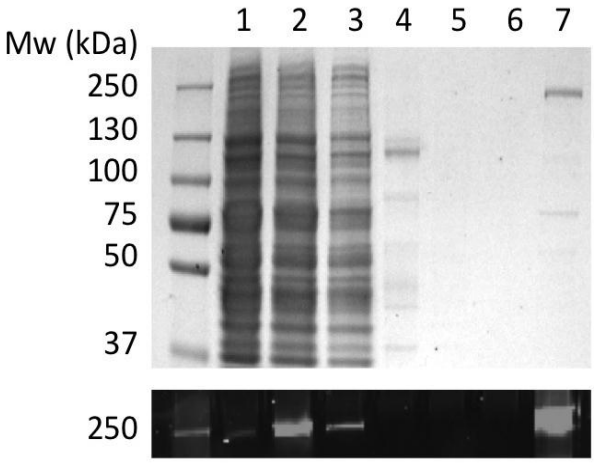

Figure 4.2. Purification of chicken CFTR in LPG by immobilized metal ion affinity chromatography. Fractions were analyzed by SDS-PAGE followed by Coomassie staining (upper panel) and fluorescence detection of the GFP tag (lower panel). *Tracks:* (1) Microsomes. (2) LPG solubilised microsomes. (3) Unbound material. (4) Insoluble material. (5) & (6) 40 and 100 mM imidazole washes. (7) Material eluted with 400 mM imidazole.

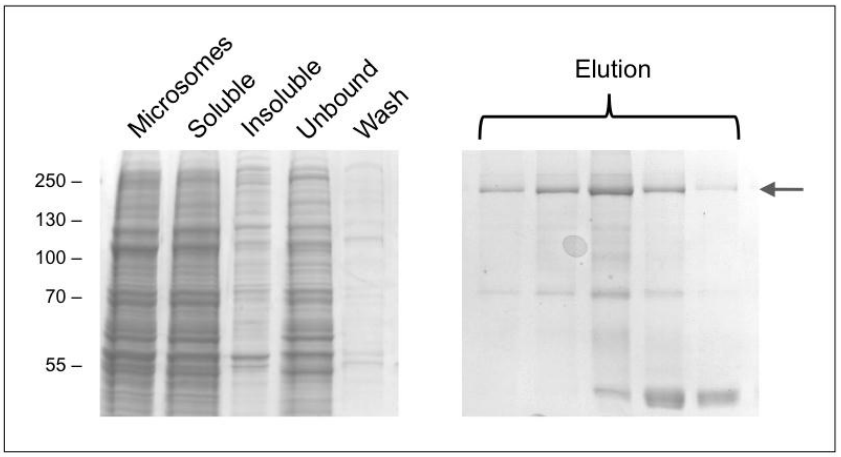

Figure 4.3. Purification of chicken CFTR in DDM by immobilized metal ion affinity chromatography. Fractions were analyzed by SDS-PAGE followed by Coomassie staining. The left hand panel shows fractions prior to elution. Several consecutive elution fractions are shown in the right hand panel with CFTR indicated by the arrow. Later fractions are enriched in a 40 kDa contaminant, which has been identified by mass spectrometry as ribosomal protein L3.

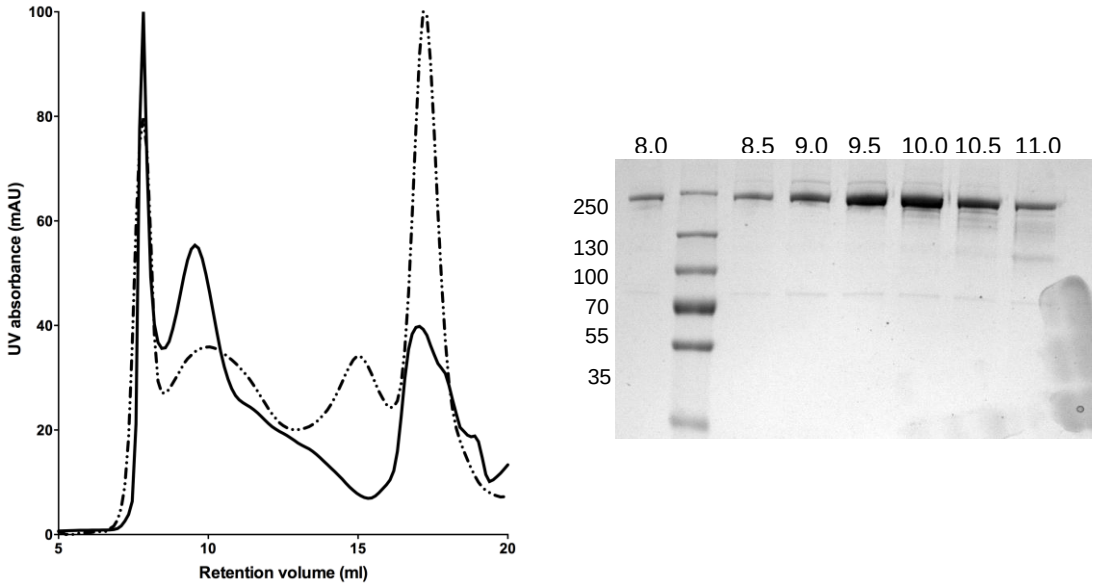

Figure 4.4. Purification of chicken CFTR by gel permeation chromatography. CFTR purified by Ni-affinity chromatography was concentrated and applied to a GPC column. The elution profile for CFTR (left panel) purified in buffer containing LPG-14 (solid line) or DDM (dashed line) are overlaid. SDS-PAGE (right panel) revealed that CFTR eluted between 8 and 11 ml.

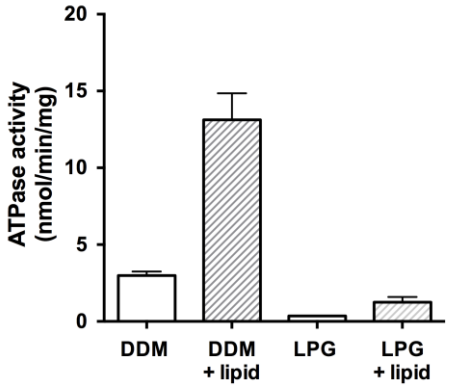

Figure 4.5. ATPase activity of purified chicken CFTR fractions. Protein purified in DDM or LPG was assayed using a modified Chifflet assay26 in the presence of a cocktail of ATPase inhibitors to eliminate any background ATPase activity from F-, P- and V-type ATPases (unfilled bars). The rate of ATP hydrolysis was also measured after detergent removal and lipid addition (filled bars). The plot shows the mean and standard deviation (*n*=*3*). Differences between mean values for ATPase activity in presence and absence of lipid, and difference between activity in DDM and LPG are significant to $p<0.05$.

4.5 Discussion

We have previously described a method for the overexpression of murine CFTR[14]. Since the publication of that protocol, we have expressed and purified several different orthologs of CFTR using the same system. All orthologs tested so far purified well in the LPG detergent, whilst the DDM purification showed more variation across different orthologs (data not shown). This flexibility illustrates the strength of the yeast approach: it is possible to screen many constructs with relative rapidity in order to select one for a particular purpose.

Washing the yeast microsomes with buffer containing 1 M NaCl prior to solubilisation with DDM results in a cleaner microsome preparation and reduces contaminants at later stages. This step is unnecessary in the LPG protocol as the final CFTR sample is >90% pure without the microsome wash. Furthermore, purification in DDM requires several alterations to the buffers for solubilisation and purification, namely the addition of extra glycerol and salt. Together, these additions considerably increased the binding of the DDM-solubilised protein to the column.

The DDM purification methodology has scope for improvement, in particular the removal of a 40 kDa major contaminant that, judged by mass spectrometry, is due to the yeast ribosomal subunit L3, which appears to have an inherent affinity for the nickel resin. There is no obvious polyHis sequence in the L3 protein, but examination of its 3D structure when bound to the ribosome (PDB = 1FFK) shows that the folded L3 subunit has a potential polyHis cluster. That this band is less problematic in LPG-purified material may be due to the harsher LPG detergent.

Though the purification in DDM appears to be poorer than that in LPG, milder detergents such as DDM may be more compatible with functional and structural analyses and have already been used in several X-ray crystallographic studies of membrane proteins[15-21]. Furthermore, our results indicated that the use of LPG leads to loss of ATPase function in CFTR relative to purification in DDM. Hence we would recommend the LPG-based purification protocol for the generation of CFTR where the purity is crucial, for example in applications such as the characterization of post-translational modifications, or in the generation of antibodies, the LPG-based protocol would be chosen. On the other hand in applications where the activity and fully native state of the protein is essential, we would propose the DDM-based protocol as a better option.

To conclude, this protocol describes a reproducible method for the isolation of CFTR in the zwitterionic detergent LPG-14 or the non-ionic detergent DDM. As such it indicates a greater range of purification conditions for CFTR than have previously been reported[10-13]. In addition milligram quantities of purified CFTR can be obtained using these procedures when combined with a high volume yeast growth system such as a 20 L fermenter and a high capacity cell harvesting system such as a 6 L low speed centrifuge rotor. The CFTR obtained has a cleavable GFP tag which allows easy monitoring of the protein in various biochemical and biophysical assays.

The reagent described in this manuscript (chicken CFTR–containing plasmid or frozen yeast cells) can be obtained through the Cystic Fibrosis Foundation (USA).

Disclosures

The authors have no competing financial interests nor other conflicting interests with respect to this work.

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Chapter 5 - A gene optimisation approach to enhance expression of human CFTR in *Saccharomyces cerevisiae*

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5.1 Abstract

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein is of great medical interest as mutations in the *CFTR* gene are the cause of the disease cystic fibrosis. Functional and structural analysis of the CFTR protein have been somewhat hampered by the difficulties presented when expressing membrane proteins. In this study we investigate the use of gene optimised recombinant human CFTR in the *Saccharomyces cerevisiae* expression system. Using a codon optimised CFTR gene, we were able to increase expression ~4-fold in comparison to wild-type CFTR. The addition of a yeast Kozak-like sequence around the ATG start codon of CFTR increased expression ~28-fold compared to the gene lacking the sequence. Timecourse expression analysis of GFP tagged protein revealed peak CFTR, relative to an endogenous fluorescent protein, at ~18 hours following induction of protein expression. Cells analysed by fluorescence microscopy exhibited an increase in CFTR at the periphery of cells up to ~20 hours with internalisation after this timepoint. Optimal DDM solubilisation efficiency was seen in the 20 hour samples. The use of a gene optimisation approach enabled relatively high levels of expression of human CFTR in the yeast system. Not only were expression levels improved but the protein appeared to be correctly processed by the yeast machinery and trafficked towards the plasma membrane of cells and we were able to efficiently extract CFTR from the membrane using the mild detergent, DDM.

5.2 Introduction

With over 70,000 patients worldwide, cystic fibrosis (CF) is one of the most prevalent life-threatening genetic diseases in the modern world. Research into CF has lead to a greater understanding of the disease and recent advances in medical treatments have increased life expectancy of sufferers. Over half of CF patients today are expected to live into their 40's. Despite this progress, there is still a lack of information pertaining to the precise structural and functional mechanisms underlying the disease. In order to study the protein responsible for CF, CFTR, it is first necessary to express and purify the protein. We have previously published a method for expression of milligram quantities of murine CFTR (O'Ryan *et al.*, 2012). This method demonstrates that it is possible to express relatively high quantities of CFTR in a yeast expression system and, using a similar approach, our aim was to express and purify the more medically significant human CFTR (hCFTR) protein to similar levels for further study.

CFTR is an integral membrane protein which is susceptible to degradation and has a propensity to form aggregates (Ward *et al.*, 1995, Zhang *et al.*, 2002b). Previous attempts to express hCFTR have been successful primarily in mammalian or insect cells (Bear *et al.*, 1992, Ramjeesingh *et al.*, 1997, Zhang *et al.*, 2009) and expression of the wild-type (WT) protein has proven difficult in yeast systems (Huang *et al.*, 1996, Kiser *et al.*, 2001, Zhang *et al.*, 2002b, Sun *et al.*, 2006, Fu and Sztul, 2009). This is probably due, at least in part, to the tight regulation of protein synthesis and ER associated degradation machinery in yeast (Zhang *et al.*, 2002b). In mammalian cells, correctly folded, core-glycosylated, immature CFTR is released from the ER via coat protein complex II (COPII) coated vesicles and translocated to the Golgi complex where it undergoes further glycosylation (Riordan, 1999, Bannykh *et al.*, 2000, Amaral, 2005). Mature, fully glycosylated CFTR is packaged into vesicles and trafficked to the plasma membrane (Bannykh *et al.*, 2000). Despite interactions with several chaperones in the ER (e.g. Hsp70, Hsp90 and calnexin), a large proportion of WT-CFTR is incorrectly folded and is processed by the ER quality control system and undergoes ubiquitination prior to being retrotranslocated to the cytosol for proteasome degradation (Riordan, 1999, Ahner *et al.*, 2007, Rosser *et al.*, 2008, Grove *et al.*, 2009, Rosser *et al.*, 2009). Yeast exhibit protein processing functions similar to those characteristic of eukaryotes, including post-translational modifications (PTM's) (Porro *et al.*, 2005). Under the right conditions, correctly folded, glycosylated CFTR should traffic to the plasma membrane of *S. cerevisiae* cells.

A major obstacle of heterologous overexpression of recombinant proteins is that codon bias of mammalian genes often differs to that of the host system (Yadava and Ockenhouse, 2003, Angov *et al.*, 2011). The availability of tRNAs is essential for translation of immature CFTR and codon bias plays a major role in the efficiency of protein synthesis (Ikemura, 1982, Sharp *et al.*, 1988, Hani and Feldmann, 1998). Rare codons found in mammalian genes, such as hCFTR, have the potential to stall polypeptide formation in yeast and can result in truncated protein, amino acid substitutions or frameshifts (Burgess-Brown *et al.*, 2008). One approach to overcome this is to codon-optimise genes by replacing codons which are present at low frequency in the heterologous host genome with more favourable codons present at a greater frequency (Gustafsson *et al.*, 2004, Burgess-Brown *et al.*, 2008).

Previous codon optimisation strategies have referred to the Kazusa database of codon usage (http://www.kazusa.or.jp/codon/) (Zhang *et al.*, 2002a, Amoah *et al.*, 2007) to identify rare codons. Whilst this database contains information on codon usage of genes in *S. cerevisiae* it does not consider the relative abundance of codons based on whether genes are poorly or highly expressed which would impact on the availability of tRNAs for translation. A more accurate analysis of codon usage was performed by (Bai *et al.*, 2011) to codon optimise the mouse P-glycoprotein (Pgp or *mdr3*) gene for expression in *Pichia pastoris* (*P. pastoris*). Analysis of genes which are highly expressed in *P. pastoris* and *S. cerevisiae* highlighted several low frequency (<10%) codons in the host genomes which were present in the WT-Pgp gene. Where possible, these rare codons were substituted in the codon optimised Pgp gene (Opti-Pgp). Expression of Opti-Pgp was increased substantially compared to that of the WT protein and when purified, retained its folding state and ATPase activity. This protocol could be used to improve expression of other membrane proteins, such as CFTR (Bai *et al.*, 2011).

The consensus sequence flanking the ATG start of higher eukaryotic genes was first identified as GCC(A/G)CCATG in 1986 (Kozak, 1986) and has since been used to improve recombinant protein expression in a variety of systems (Sano *et al.*, 2002, Zhang *et al.*, 2006, Du *et al.*, 2008). The sequence is recognised by the ribosome in the host cell and translation of the nascent polypeptide chain is initiated (Kozak, 1982). The strength of the Kozak sequence, along with other initiation factors, influence expression levels of recombinant proteins in eukaryotic systems (Kozak, 1986). The Kozak consensus sequence in *S. cerevisiae* differs significantly from that found in mammalian genes. The consensus sequence derived from highly expressed genes in yeast is rich in A-residues, which are particularly prevalent at the -1 and -3 positions,

and a G-residue at the +4 position (Hamilton *et al.*, 1987). The use of yeast Kozak-like sequences has proven useful to express mammalian genes in these systems (Zhang *et al.*, 2006).

Using the optimised *S. cerevisiae* culture conditions described previously (Rimington *et al.*, 2014a) we transformed the codon optimised human CFTR gene (Opti-h*cftr*) into yeast with and without a Kozak sequence and compared it to the wild-type gene with Kozak (WT-h*cftr*) in the same system. Expression levels of Opti-hCFTR (+Kozak) protein was increased versus Opti-hCFTR (-Kozak) and versus WT-hCFTR determined by measuring GFP fluorescence of recombinant CFTR relative to an endogenous yeast fluorescent protein (endFP). Previous work to ascertain the most favourable culture conditions for expression of CFTR orthologues in the *S. cerevisiae* strain, FGY217, identified the optimal induction time for CFTR expression at ~14-15 hours (O'Ryan *et al.*, 2012, Rimington *et al.*, 2014a). This data was obtained from cells expressing mouse and chicken CFTR. Due to possible differences in protein structure and stability between CFTR orthologues (Price *et al.*, 1996, Ostedgaard *et al.*, 2007, Stahl *et al.*, 2012) we considered it necessary to revisit the CFTR timecourse analysis specifically for Opti-hCFTR. A timecourse experiment was performed as previously described (Rimington *et al.*, 2014a) extending sampling time to 36 hours. Whole-cell fluorescence microscopy was also utilised to monitor expression and localisation of GFP-tagged Opti-hCFTR. We also evaluated the detergent solubilisation efficiency for Opti-hCFTR at each timepoint with the mild, non-ionic detergent, dodecyl-β-D-maltopyranoside (DDM).

5.3 Materials and methods

Following the Pgp optimisation protocol, a codon optimised hCFTR gene (Opti-hCFTR) was designed for expression in *P. pastoris* and *S. cerevisiae*. The Opti-hCFTR construct was further enhanced by the addition of protein tags and other construct features. A SUMOstar (SUMO*) tag was added to the N-terminus of CFTR. The SUMO (Small Ubiquitin-like Modifier) family of proteins covalently attach to other proteins post-translationally and have been shown to aid in folding, stability and cellular trafficking (Geiss-Friedlander and Melchior, 2007). The SUMO* fusion tag is a modified version of the original SUMO tag used for recombinant protein overexpression in prokaryotic cells. SUMO* has been engineered to resist proteolytic cleavage by endogenous SUMO proteases found in yeast. SUMO* fusions exhibit improved expression, folding and solubility in eukaryotic expression systems (Liu *et al.*, 2008, Rozen-Gagnon *et al.*, 2012). Purification tags were added at both the N and C terminal ends for downstream protein purification. A TEV-cleavable eGFP* tag was included at the C-terminus to enable monitoring of expression of WT-hCFTR and Opti-hCFTR and cellular localisation of Opti-hCFTR.

As previously stated, the most important positions in the yeast consensus sequence, to augment initiation of translation, are the A-residues at position -3 and -1 and a G-residue at +4 (Hamilton *et al.*, 1987) and these were incorporated into the Kozak-like sequence cloned into the Opti-hCFTR construct. The WT-hCFTR gene was also cloned and incorporated the Kozak-like sequence and eGFP* tag but excluded the RGS-10His and Sumo* tags. To investigate the effect of the Kozak sequence on Opti-hCFTR expression, an additional Opti-hCFTR construct, lacking the Kozak-like sequence was also cloned. The constructs were cloned into the *S. cerevisiae* strain, FGY217 (provided by Dr. David Drew, Imperial College of London) in which expression of CFTR was under control of the galactose inducible promoter, GAL1, which is heavily repressed in the presence of glucose (Johnston *et al.*, 1994, Drew *et al.*, 2008). This enables tight regulation of recombinant protein induction time.

5.3.1 Cell strains and vectors

The bacterial and yeast strains, along with the vectors used during this study are summarised in (Table 5.1).

Table 5.1. Summary of bacterial strains, yeast strains and cloning vectors used throughout study.

Name/designation	Genotype/features	Supplier/origin
Bacterial strains		
One Shot® Top10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16*	*Invitrogen*
XL10-Gold Ultracompetent Cells	*endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^R F'[proAB $lacI^q$ZΔM15 Tn10(Tet^R Amy*	*Stratagene*
Yeast strains		
FGY217	*MATa, ura3-52, lys2∆201, pep4∆*	*David Drew*
Vectors		
pDDGFP-2	*GAL1 promoter, URA3 gene, TEV cleavable C-terminal 8His-eGFP, ampicillin resistant*	*David Drew*
pTR	*GAL1 promoter, URA3 gene, TEV cleavable C-terminal 8His-A206KeGFP, ampicillin resistant*	*In-house*

5.3.2 E. coli culture media and agar

Lysogeny (Luria) broth (LB) - (*10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl in sterile dH_2O)* was used for all standard liquid *E. coli* cultures.

LB agar (LBA) - (LB, 20 g/L bacteriological agar, 100 µg/mL ampicillin) was used to prepare plates for growth of *E. coli* colonies.

Super optimal broth with catabolite repression (SOC) medium - *(20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM glucose)* was used for transformations using One Shot® Top10 cells.

NZY+ broth - *(10 g/L NZ amine (casein hydrolysate), 5 g/L yeast extract, 5 g/L NaCl, 1.25 mM $MgCl_2$, 1.25 mM $MgSO_4$, 2 mM glucose. Adjusted to pH 7.5 using NaOH) was used for tranformations using XL10-Gold Ultracompetent Cells.*

5.3.3 Yeast culture media and agar

CFTR agar -uracil (-URA agar) - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L glucose,* 20 g/L bacteriological agar*)*

CFTR culture media (0.15% or 2% glucose) - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 0.75 g/L D-glucose (for 0.1%) or 20 g/L glucose (for 2%))*

CFTR induction media - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L D-galactose, 8% glycerol (v/v))*

6X CFTR induction media - *(41.4 g/L yeast nitrogen base (YNB), 4.62 g/L complete supplement mixture (CSM) without uracil, 120 g/L D-galactose, 48% glycerol (v/v) in sterile dH_2O)*

5.3.4 Buffers and stock solutions

CFTR buffer (CB) - *(50 mM Tris-HCl pH 8.0, 1 M NaCl, 20% glycerol, 1 mM dithiothreitol (DTT))*

100X Protease inhibitor cocktail (PIC) - *(20 mM AEBSF, 600 µM bestatin, 400 mM chymostatin, 700 µM E-64, 2 mM leupeptin, 1.5 mM pepstatin A, 100 mM PMSF in dry DMSO plus 300 mM benzamidine in sterile dH_2O)*

2X SDS solubilisation buffer (SB) - *(50 mM Tris-HCl pH 7.6, 5% glycerol (v/v), 5 mM EDTA, 0.02% bromophenol blue in 700 µl aliquots. 200 µl of 20% (w/v) sodium dodecyl sulphate (SDS) and 100 µl of 0.5 M DTT added just prior to use)*

10X SDS running buffer - *(30 g/L Tris-HCl, 144 g/L glycine and 10 g/L SDS in sterile dH_2O)*

Detergent solutions - *(4% (w/v) DDM in CB)*

5.3.5 Codon optimisation

Codon optimisation of the hCFTR gene (GenBank accession number, M28668.1) was performed by Dr. Ina L Urbatsch using the method previously published for *Pgp (*Bai *et al.,* 2011*)*. The Opti-hCFTR gene was synthesised by GeneArt (Regensburg, Germany) with codon usage adjusted to the codon bias of *P. pastoris* and *S.*

cerevisiae. Several codons were identified in the WT-hCFTR nucleotide sequence which are present at low frequency in highly expressed genes in *P. pastoris* and *S. cerevisiae* (unpublished data). These codons were substituted for those present at higher frequencies in the two yeast strains.

5.3.6 Plasmid construction

A modified construct of the p424GAL1 2µ yeast expression vector, pDDGFP-2 (table 5.1), containing the URA3 gene, an octa-His tag and an enhanced GFP (eGFP) tag (obtained from David Drew) was further adapted to replace the octa-His tag with a StrepII purification tag. Site directed mutagenesis was performed following standard protocols or, where necessary, single primer reactions as described in (Edelheit *et al.*, 2009) for the tag replacement. To prevent GFP dimerisation, an A206K mutation was introduced into the eGFP gene (Shaner *et al.*, 2005, Shaner *et al.*, 2007) using site-directed mutagenesis. The newly modified vector was designated pTR (table 5.1). Presence of the StrepII tag and A206K mutation were confirmed by DNA sequencing of pTR.

5.3.7 Molecular cloning

Cloning of the Opti-hCFTR, Opti-hCFTR (-Kozak) and WT-hCFTR genes was facilitated by restriction cloning or homologous recombination as previously described (Drew *et al.*, 2008, O'Ryan *et al.*, 2012). In this study, the Kozak-like sequence AAAAGAATGG was incorporated into the Opti-hCFTR and WT-hCFTR constructs. Using restriction site cloning, Opti-hCFTR with a FLAG tag at residue 901 and C-terminal eGFP*-StrepII tags (modified and provided by Dr. John Kappes) was cloned using *Bam*HI and *Xho*I restriction sites, removing the inbuilt eGFP*-StrepII tags from pTR in the process. Ligations were performed overnight at 16 °C using a vector:insert ratio of 1:3. Following incubation, 5 µl of the ligation product was transformed into One Shot® TOP10 Chemically Competent *E. coli* cells or XL10-Gold Ultracompetent Cells (Table 5.1) using the manufacturers standard protocols. Plasmid DNA was extracted from single colonies from the transformation plates using a QIAprep Spin Miniprep Kit (*Qiagen*). Opti-hCFTR (-Kozak) and WT-hCFTR were amplified by PCR with the addition of 35 bp overhangs to facilitate homologous recombination as described (Gietz and Woods, 2006, Gietz and Schiestl, 2007, Drew *et al.*, 2008). PCR products were

purified using a QIAquick PCR Purification kit (*Qiagen*). The presence of the Kozak-like sequence was confirmed by DNA sequencing of WT-hCFTR and Opti-hCFTR. A summary of the final constructs is provided in (Table 5.2).

Table 5.2: Summary of constructs used for cloning of CFTR genes.

Construct name	N-term	Gene	C-term
Opti-hCFTR	Kozak-RGS-10His-Sumo*-	Opti-hCFTR-	eGFP*-StrepII
WT-hCFTR	Kozak-	WT-hCFTR	eGFP*-8His
Opti-hCFTR (-Kozak)	RGS-10His-Sumo*-	Opti-hCFTR-	eGFP*-8His

5.3.8 Transformation of CFTR genes into S. cerevisiae

Opti-hCFTR was transformed into competent FGY217 *S. cerevisiae* cells, which are auxotrophic for uracil, by incubating 1 µg of plasmid DNA with 50 µg carrier DNA (sheared salmon sperm DNA) and 50 µl of competent FGY217 cells (prepared as described in (Drew *et al.*, 2008)) for 30 minutes at 30 °C. Following the addition of 50% PEG 3350 to a final concentration of 33% (w/v), transformation mixtures were incubated for a further 30 minutes at 30 °C. Cells were heat shocked for 15 minutes at 42 °C before being pelleted and resuspended in 400 µl of sterile dH_2O. WT-hCFTR and Opti-hCFTR (-Kozak) were transformed using the method described in (Drew *et al.*, 2008). Transformed cells were spread on -URA agar plates using 100 µl and 300 µl of cell suspension. Plates were incubated for 3 days at 30 °C.

5.3.9 hCFTR expression

For small-scale expression analysis, 5 ml of CFTR culture medium (2% glucose) was inoculated with ~50 colonies from fresh transformation plates to create a mass population culture (MPC). 500 µl of the MPC's were inoculated into 10 ml of CFTR culture medium in 50 ml sterile Falcon tubes. The cultures were grown for ~8 hours at 225 rpm at 30 °C. The cultures were diluted to an optical density at 600 nm (OD_{600}) of 1.5 in 10 ml and were then centrifuged at 4,500 x *g* for 5 minutes at 4 °C. The supernatants were discarded and cells were washed with 25 ml of sterile dH_2O. The cell pellets were resuspended in 10 ml of CFTR induction media and were incubated for 15 hours at 225 rpm at 30 °C.

5.3.10 Harvesting and cell lysis

All steps were carried out at 4 °C or on ice. Cells were harvested 15 hours post induction by transferring cultures to 50 ml Falcon tubes and centrifuging at 4,500 x *g* for 5 minutes at 4 °C. The supernatants were discarded and cells were resuspended in 500 µl of CB + PIC. The cell suspensions were transferred to 1.5 ml screw-top tubes containing ~400 µl of acid washed glass beads (425-600 µm diameter). Cells were lysed by 4 x 1 minute at 3450 oscillations/min in a BioSpec Mini-Beadbeater-16 (Bartlesville, OK, USA) with 1 minute on ice between each round of homogenisation. Following lysis, tubes were centrifuged at 4,500 x *g* for 5 minutes at 4 °C to pellet glass beads and unbroken cells and large cell debris. 450 µl of the supernatants were transferred to sterile 1.5 ml microfuge tubes. Crude membranes were pelleted by ultracentrifugation at 100,000 x *g* for 1 hour at 4 °C. The supernatants were discarded and crude membrane pellets were resuspended in 50 µl of CB + PIC. CFTR expression was analysed by SDS-PAGE.

5.3.11 SDS-PAGE analysis

Unless otherwise stated, 25 µl of each sample was added to an equal volume of 2X SB. Samples were loaded onto NuSep nUView 10% Tris-glycine gels and electrophoresed at 150 V for ~45 minutes in 1X SDS running buffer.

5.3.12 In-gel fluorescence of CFTR

GFP-tagged CFTR was analysed following SDS-PAGE using a ChemiDoc™ MP Imaging System (*Bio-Rad*) fitted with a Blue LED Module Kit with an excitation wavelength of 488 nm and an emission wavelength of 520 nm with images captured using a Supercooled CCD camera and saved for further analysis using Image Lab™ Software (*Bio-Rad*) and ImageJ.

5.3.13 Timecourse of Opti-hCFTR expression

An overnight culture was prepared of Opti-hCFTR by inoculating 50 ml of CFTR culture medium (2% glucose) with ~5 colonies from a fresh streak plate obtained from a high-expressing single colony glycerol stock as described (O'Ryan *et al.*, 2012). The culture was grown overnight at 225 rpm at 30 °C. The culture was diluted to an OD_{600} of 0.1 in 500 ml of CFTR culture medium (0.15% glucose) in a 2 L-baffled Erlenmeyer flask. At an OD_{600} of 1.5, glucose concentration was tested using Medi-Test Glucose Test Strips (*BHR Diagnostics*) to ensure the concentration was <0.005%. CFTR expression was induced by the addition of 100 ml of 6X CFTR induction medium. The culture was incubated at 225 rpm at 25 °C. 25 ml sample aliquots were taken at 0, 12, 14, 16, 18, 20, 24, and 36 hour post induction timepoints. Cells were harvested by centrifugation for 5 min at 4,500 x *g* at 4 °C. The supernatants were discarded and cell pellets were resuspended in 1 ml of CB + PIC and stored at -80 °C until required for analysis. Cells were lysed as previously described for small-scale cultures using ~400 µl of acid washed glass beads and transferring 900 µl of the supernatant prior to pelleting crude membranes. Opti-hCFTR expression was analysed by SDS-PAGE (as previously described) and fluorescence microscopy.

5.3.14 Fluorescence microscopy

Cells were harvested from 500 µl of each of the 25 ml timecourse samples by centrifugation for 5 min at 4,500 x *g* at 4 °C. The supernatants were discarded and cell pellets were resuspended in 50 µl of 50% glycerol (v/v) in sterile dH_2O. Microscope slides were prepared by transferring 5 µl of resuspended cells onto a 76 mm x 26 mm x 1.0-1.2 mm microscope slide (*Thermo Scientific*) and carefully placing a cover slip (thickness 1.5) over the sample. Images were collected on an Olympus BX51 upright microscope using a 60x/ 1.40 U Plan Apo objective and captured using a Coolsnap ES camera (*Photometrics*) through MetaVue Software (*Molecular Devices*). Cells were exposed for 200 ms using a FITC filter. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

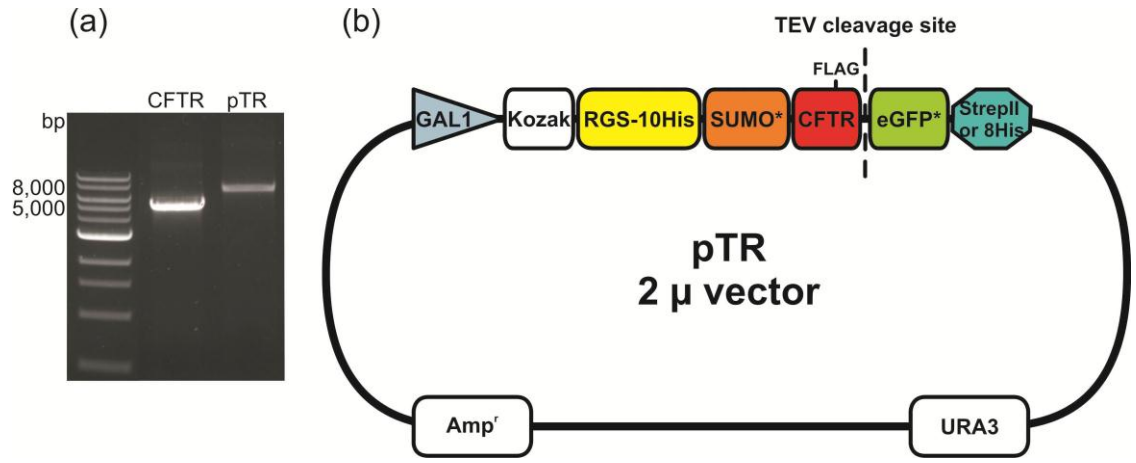
5.3.15 DDM solubility of Opti-hCFTR timepoints

100 µl of Opti-hCFTR crude membranes (with total protein concentrations of ~1.5-2.5 mg/ml) from each timepoint was added to 100 µl of 4% DDM in CB + PIC. Samples were incubated with end-over-end rotation for 1 hour at 4 °C. Insoluble material was pelleted by ultracentrifugation at 100,000 x *g* for 1 hour, soluble material was transferred to fresh tubes and insoluble pellets were resuspended in equivalent volumes of CB + PIC. The soluble and insoluble fractions were analysed by SDS-PAGE (as previously described).

5.4 Results

5.4.1 Plasmid construction, molecular cloning and transformation of CFTR genes

Following cloning of Opti-hCFTR by restriction site cloning (Fig. 5.1a) the construct (Fig. 5.1b) was transformed into FGY217. WT-hCFTR and Opti-hCFTR (-Kozak) were cloned by homologous recombination. DNA sequencing confirmed the presence of the A206K mutation and strepII tag in the new vector and also the Kozak-like sequence in the WT-hCFTR and Opti-hCFTR constructs (Fig. 5.1c).



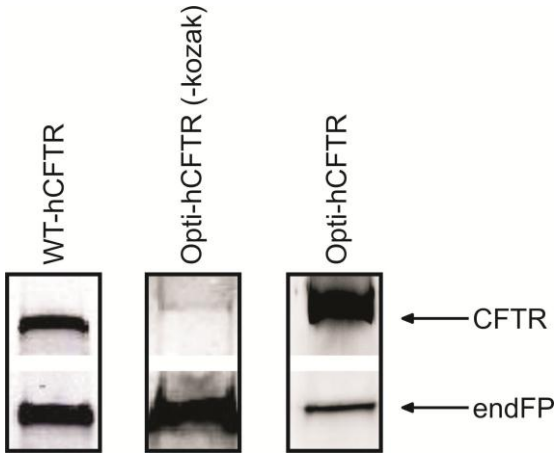
(c)

Figure 5.1. Restriction site cloning of Opti-hCFTR into pTR. (a) PCR amplified Opti-hCFTR and linearised pTR 1% agarose gel exposed by UV light. (b) Ligated vector and insert generate the Opti-hCFTR expression vector to be transformed into FGY217. (c) DNA sequencing confirmed the presence of the A206K mutation, Kozak-like sequence and the StrepII tag.

5.4.2 hCFTR expression

To examine the effect of codon optimisation and the introduction of the yeast Kozak-like sequence to the Opti-hCFTR construct, expression of CFTR was analysed relative to an endogenous FAD-containing fluorescent protein (endFP), likely to be the yeast succinate dehydrogenase flavoprotein subunit SDH1 (Fig. 5.2a). Expression of Opti-hCFTR was increased ~4-fold compared to that of WT-hCFTR (Fig. 5.2b). Insertion of the Kozak-like sequence increased Opti-hCFTR expression ~28-fold (Fig. 5.2b).

(a)



(b)

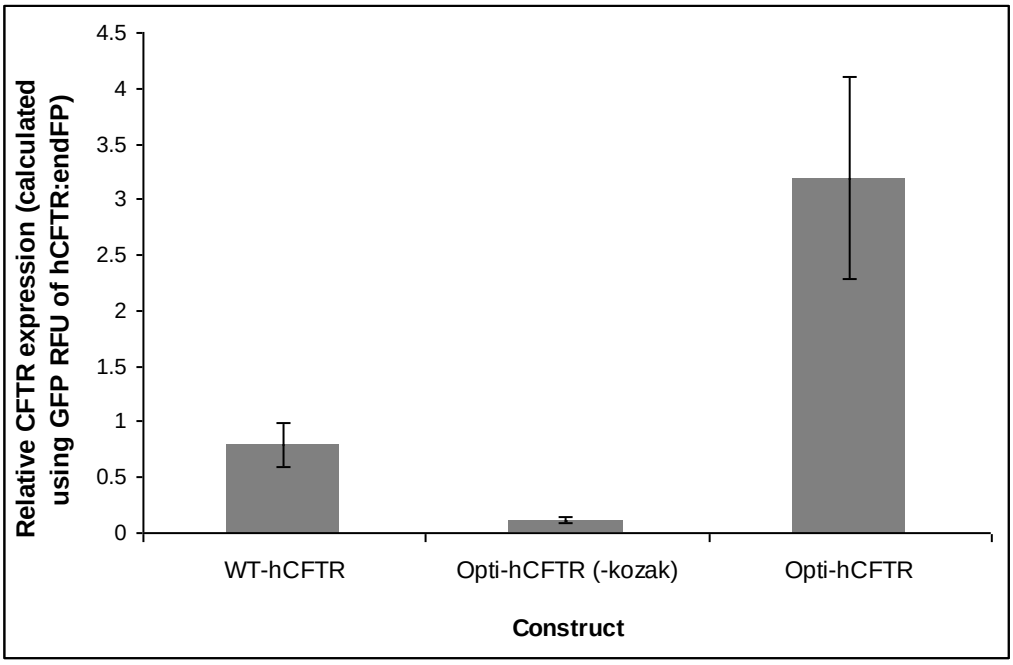
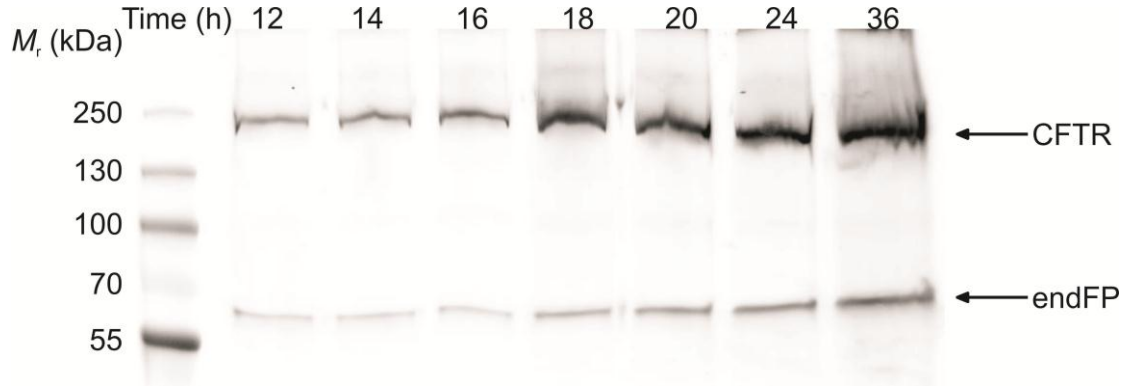


Figure 5.2. Comparison of expression of WT-hCFTR, Opti-hCFTR (-Kozak) and Opti-hCFTR. (a) Representative SDS-PAGE gels viewed under fluorescence of varying exposures to enable visualisation of bands (ex. 488 nm, em. 530 nm). Framed sections denote separate gels. (b) hCFTR:endFP ratio was calculated by measuring GFP fluorescence (RFU) of hCFTR and endFP from separate gels and applying densitometry analysis using ImageJ.

5.4.3 *Optimal Opti-hCFTR expression was observed at ~18 hours*

S. cerevisiae cells expressing Opti-hCFTR were induced with 2% galactose and 8% glycerol and samples were taken at various timepoints from 0-36 hours. Expression of CFTR and endFP were assessed using in-gel fluorescence (Fig. 5.3a). CFTR:endFP was calculated and peak CFTR expression was observed at ~18 hours (Fig. 5.3b). Full-length CFTR was still present in the 36 hour timepoint samples (Fig. 5.3a&b).

(a)



(b)

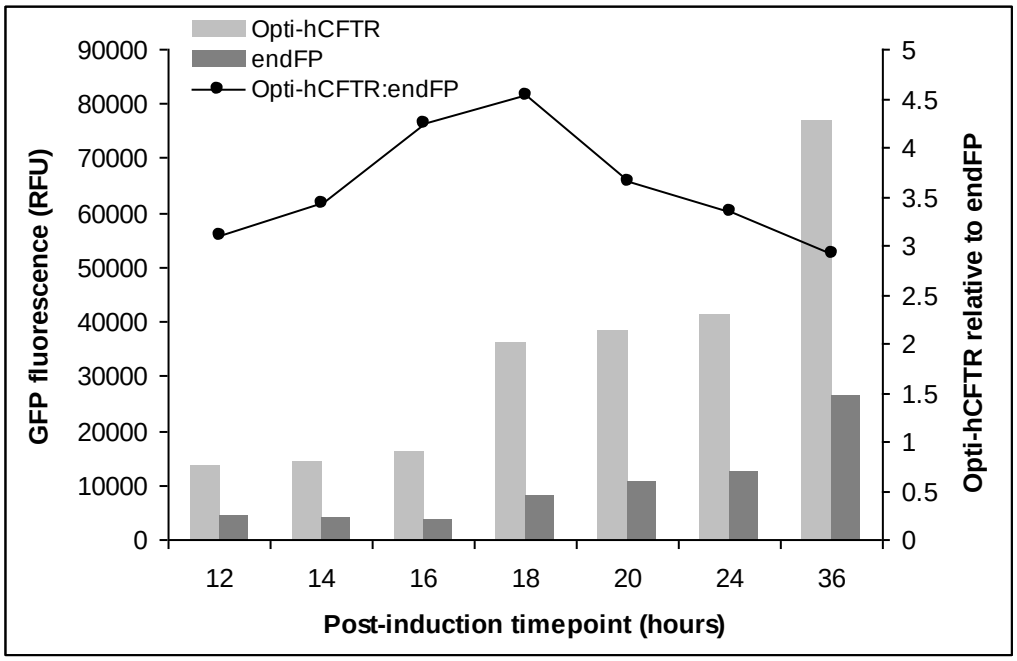


Figure 5.3. Timecourse analysis of Opti-hCFTR expression. (a) SDS-PAGE gel viewed under fluorescence (ex. 488 nm, em. 530 nm). (b) GFP fluorescence (RFU) of Opti-hCFTR and endFP was calculated by applying densitometry analysis to band intensities from SDS-PAGE gel using ImageJ. Opti-hCFTR:endFP ratio was calculated for each sample.

5.4.4 Opti-hCFTR localisation using fluorescence microscopy

Opti-hCFTR expression levels and cellular localisation were analysed using fluorescence microscopy. GFP-tagged Opti-hCFTR was observed with punctate localisation at or near the plasma membrane. There was an increase in this pattern of fluorescence up to the 20 hour timepoint, after which, fluorescence became more internalised within the cells (Fig. 5.4).

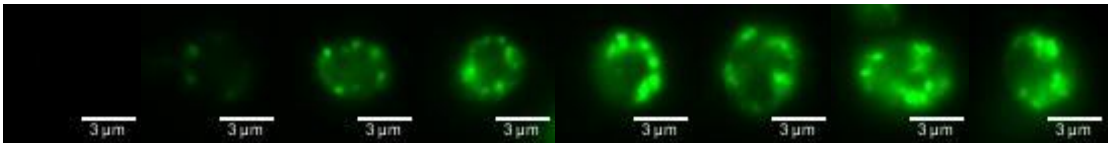


Figure 5.4. Fluorescence microscopy of Opti-hCFTR expressing S. cerevisiae cells harvested during timecourse experiment. Cells were resuspended in 50% glycerol (v/v) and viewed with a 60x objective under a FITC filter following 200 ms exposure. Images were captured with a Coolsnap ES camera through MetaVue and analysed using ImageJ.

5.4.5 DDM solubility of timecourse samples

Detergent solubilisation efficiency of CFTR present in samples from each timepoint in the analysis was assessed. Solubilisation efficiency was calculated by measuring in-gel fluorescence of soluble and insoluble fractions. Peak solubility (~41%) was observed in the 20 hour timepoint sample. The 24 and 36 hour timepoints exhibited ~30% solubilisation efficiency (Fig. 5.5).

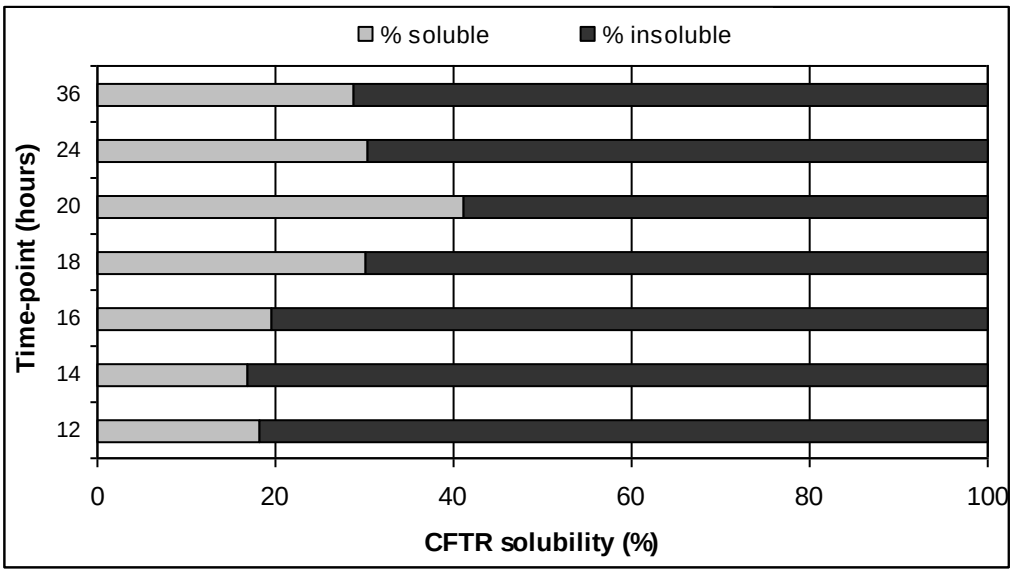


Figure 5.5. Detergent solubilisation of Opti-hCFTR solubilised in DDM. Soluble and insoluble proteins were resolved by SDS-PAGE and view under fluorescence (ex. 488 nm, em. 530 nm). Solubilisation (%) was calculated by measuring GFP fluorescence (RFU) of full-length CFTR in each fraction and applying densitometry analysis using ImageJ.

5.5 Discussion

Heterologous overexpression of membrane proteins is notoriously difficult and expression of hCFTR in yeast has previously proven particularly challenging (Huang *et al.*, 1996, Kiser *et al.*, 2001, Zhang *et al.*, 2002b, Sun *et al.*, 2006, Fu and Sztul, 2009). However, with careful optimisation of constructs and culture conditions specific for the yeast strain used, high levels of full-length protein expression can be achieved (Rimington *et al.*, 2014a).

We have previously published a method for overexpression of the murine CFTR protein in *S. cerevisiae* (O'Ryan *et al.*, 2012). Our aim in this study was to express the human CFTR protein in sufficient quantities for biophysical and functional analysis. To achieve this, we employed a gene optimisation strategy using genes which were codon optimised to the codon bias of *S. cerevisiae* and *P. pastoris* and which also encompass several features to enhance protein expression and downstream purification. Our previous work identified the need for optimisation of yeast cultures to attain good levels of CFTR expression (Rimington *et al.*, 2014a). Using a similar approach, we assessed expression, cellular localisation and detergent solubility of Opti-hCFTR by timecourse analysis.

Gene optimisation has proven a useful tool for expression of membrane proteins and has been shown to substantially improve expression (Bai *et al.*, 2011). The WT-hCFTR nucleotide sequence was codon optimised by comparing codon bias in highly expressed genes in *S. cerevisiae* and *P. pastoris* and adjusting codon usage accordingly. The increase in expression we observed in Opti-hCFTR of ~4-fold compared to WT-hCFTR is comparable to results obtained by the Urbatsch group (Bai *et al.*, 2011) in which expression of codon optimised Pgp in *P. pastoris* was increased 2- to 3-fold compared to the WT protein.

A further feature we chose to investigate during this study was the effect of a yeast Kozak-like sequence around the ATG start codon of CFTR genes. Kozak-like sequences have been shown to improve expression of recombinant proteins in heterologous expression systems (Alonso *et al.*, 2002, Jäger *et al.*, 2013). The addition of the yeast Kozak-like sequence had a remarkable effect and expression of Opti-hCFTR was ~28-fold higher with the sequence present than in the construct lacking the Kozak-like sequence. This data suggests that a yeast Kozak-like sequence is of high importance for heterologous expression of CFTR and can potentially be employed to tune the expression levels of a target protein.

Previous data identified optimal culture conditions for CFTR expressing FGY217 *S. cerevisiae* cells for the murine, chicken and platypus orthologues (Rimington *et al.*, 2014a) and established that peak CFTR expression, relative to endFP, was ~14-15 hours post-induction for Opti-mCFTR (O'Ryan *et al.*, 2012, Rimington *et al.*, 2014a). Interestingly, Opti-hCFTR appeared to be more stable and full-length protein was observed up to 36 hours after induction whereas Opti-mCFTR was almost completely degraded by the cells at this time point. Maximum Opti-hCFTR expression relative to endFP was at ~18 hours and remained fairly high throughout the timecourse.

Fluorescence microscopy of GFP-tagged Opti-hCFTR expressing *S. cerevisiae* cells showed that they exhibited increasing fluorescence in the vicinity of the plasma membrane up to ~18 hours indicative that the protein had been correctly folded and trafficked from the Golgi. After ~20 hours, Opti-hCFTR became more internalised within the cell and solubilisation efficiency of Opti-hCFTR also began to decline after this point, possibly due to aggregation of the protein within cytoplasmic inclusion bodies. It is also possible that Opti-hCFTR transits more slowly from the ER and Golgi compartments as the yeast cells enter early stationary phase, hence there is an accumulation of protein in these internal compartments. This would also explain the lower solubilisation efficiency as a large proportion of the protein would not have undergone complete processing within the ER or Golgi resulting in greater quantities in the insoluble fractions.

This research demonstrates the usefulness of yeast as an expression system for hCFTR. Amongst the many advantages of yeast as a heterologous expression system for recombinant hCFTR is the relative ease with which they can be genetically manipulated to exploit their key features resulting in the availability of numerous strains and mutants to optimise this system. Yeast cultures are relatively fast and cost effective to grow in the lab and can be grown to very high cell densities producing high yields of protein in comparison to other systems. CFTR has a fast turnover rate within mammalian cells (Prince *et al.*, 1994) however the tight regulation of expression with the use of inducible promoters such as GAL1 in yeast allows for controlled protein induction and relatively higher yields (Johnston *et al.*, 1994, Drew *et al.*, 2006, Drew *et al.*, 2008). As homeostatic regulation of inorganic ions is an essential function of the plasma membrane of yeast cells (Jennings and Cui, 2008), increased transport of chloride ions by active CFTR channels would likely be toxic to cells. However, the ability of yeast to express high levels of recombinant protein at lower temperatures results in reduced channel activity of the protein preventing this toxicity. This lower

temperature also allows protein folding to occur at a steadier rate helping to alleviate stress on the host machinery (Griffith *et al.*, 2003).

The results presented in this paper demonstrate that *S. cerevisiae* can be used as an efficient system to express recombinant full-length Opti-hCFTR which appears to be trafficked towards the periphery of cells indicating that the protein is correctly processed by the ER and Golgi machinery. Protein obtained from this system can be efficiently extracted from crude membranes using the mild detergent DDM. The relatively high yields of Opti-hCFTR generated by this system will enable further biochemical and biophysical analysis of the protein which could help shed light on the underlying mechanism that cause CF.

5.6 References

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Chapter 6 - Comparison of CFTR purification methods and thermal stability analysis of purified CFTR

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6.1 Abstract

Expression and purification of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein from heterologous expression systems has proven problematic. CFTR is susceptible to degradation and is prone to aggregation when overexpressed in these systems and obtaining highly purified functional protein can be difficult. The aim of this study was to compare the effectiveness of four affinity tags used for purification of human CFTR expressed in *Saccharomyces cerevisiae*. We compared the use of nickel, FLAG, StrepII and GFP affinity chromatography methods either as one- or two-step purifications and assessed CFTR yields following binding and elution. The purity of CFTR in the elutions was also calculated. Protein from the two methods which produced the highest purity of CFTR, a two-step nickel-FLAG (~80% purity) and a single-step GFP purification (~90% purity) was analysed using a thermal stability CPM binding assay and the denaturation midpoint (T_m) for the two methods was compared. The T_m for nickel-FLAG purified CFTR was ~40.7 °C whilst for GFP purified CFTR it was ~43 °C indicating greater stability of CFTR purified by the GFP method. Correct folding of CFTR was confirmed by transmission electron microscopy and single particles of ~10 nm were visible. The CPM binding assay could prove useful in a high-throughput platform for screening of drugs or small molecules to identify treatments for highly unstable forms of CFTR, such as the F508del mutation.

6.2 Introduction

Membrane proteins are notoriously difficult to express and purify from heterologous expression systems (Huang *et al.*, 1998, Kiser *et al.*, 2001, Zhang *et al.*, 2002b). However, extraction from the lipid membrane and subsequent purification is usually necessary for structural and functional analysis. CFTR is a eukaryotic membrane protein in which defects cause the disease Cystic Fibrosis (CF). Despite the identification of the CFTR gene in 1989 (Kerem *et al.*, 1989, Riordan *et al.*, 1989, Rommens *et al.*, 1989) structural and functional data for CFTR is still limited and so too are treatments for CF. Obtaining high yields of purified CFTR has proven problematic owing to its propensity to form aggregates and its susceptibility to degradation once extracted from the plasma membrane (Ward *et al.*, 1995, Zhang *et al.*, 2002b). To help overcome these difficulties, CFTR must be solubilised in detergents which emulate the lipid membrane environment and stabilise the protein by providing protection for its hydrophobic regions (Garavito and Ferguson-Miller, 2001, Seddon *et al.*, 2004). The preferred detergent for downstream analysis is one which maintains correct folding and structure of CFTR so it retains function and activity (Lin and Guidotti, 2009). We have previously reported a method for purification of the Opti-chicken CFTR (cCFTR) orthologue expressed in *S. cerevisiae* cells using n-dodecyl-β-D-maltopyranoside (DDM) and lysophosphatidylglycerol (LPG) (Pollock *et al.*). cCFTR yields up to 50 µg/L cell culture in DDM and 80 µg/L in LPG can be produced following this method, however the purity of cCFTR is only ~60% for the DDM purification. This can be increased to ~90% in LPG but ATPase activity of cCFTR is severely diminished (<1 nmol/min/mg) in LPG. DDM solubilised cCFTR exhibited a low, but measurable rate of ATP hydrolysis (~3 nmol/min/mg) which was increased ~4-fold (to ~13 nmol/min/mg) upon detergent removal and reconstitution of cCFTR into proteoliposomes (Pollock *et al.,* Cant, 2013). The fact that cCFTR exhibited higher ATPase activity indicates that the protein retains its correct conformational state and its activity as an ion channel when solubilised in DDM as opposed to LPG.

Although the use of DDM as a detergent for CFTR solubilisation has previously proven difficult (Ramjeesingh *et al.*, 1997), the Riordan group have employed its use quite successfully to purify CFTR solubilised in 1% DDM from baby hamster kidney (BHK) cells using nickel affinity chromatography (Rosenberg *et al.*, 2004). Whilst this method required an initial alkaline pH treatment to remove peripheral membrane proteins, eluted CFTR was reasonably pure and exhibited ATP hydrolysis of ~60 nmol/mg/min which is comparable to data previously published on CFTR purified from insect cells (Li *et al.*, 1996).

A limitation of the DDM solubilisation and subsequent purification described in our previous method was the relatively low purity of CFTR at ~60% with a major contaminating yeast protein present at ~44 kDa, identified by mass spectrometry analysis as Ribosomal Protein L3 (RPL3). Improving the DDM purification protocol and increasing the purity of CFTR is necessary to enable functional and structural studies of the protein. Whilst obtaining highly purified CFTR is a priority, the quality of protein obtained must also be considered. Monitoring protein unfolding can provide information on the conformation state of purified CFTR and by calculating the denaturation midpoint (T_m), and therefore the thermal stability, a range of variables such as buffer conditions, pH, detergents, and purification methods could be screened with relative ease (Alexandrov *et al.*, 2008, Kean *et al.*, 2008). The T_m has previously been used to assess the effect of the disease causing mutation, F508del, on the isolated nucleotide binding domain 1 (NBD1) of CFTR. Using differential scanning calorimetry and circular dichroism, a decrease in T_m of 6-7 °C was observed for the (F508del)NBD1 indicating a decrease in its thermal stability compared to wild type NBD1 (Protasevich *et al.*, 2010). Changes in T_m could be used to determine the stability of CFTR obtained from different purification methods.

One approach to monitoring thermal stability of protein has been the use of fluorescent dyes to label regions of proteins exposed during heating at a constant temperature and this method has been successfully employed to screen conditions for purification of predominantly soluble proteins (Ericsson *et al.*, 2006, Mezzasalma *et al.*, 2007). However, similar approaches have been used for developing thermal stability assays for membrane proteins (Yeh *et al.*, 2006, Kean *et al.*, 2008). The Thermofluor assay uses a fluorescent dye which becomes non-fluorescent upon binding to the hydrophobic regions of membrane proteins as they unfold and conditions which stabilise, or destabilise, the protein of interest are detected as shifts in the T_m calculated by changes in fluorescence. A disadvantage of this method is that the presence of some detergents can interfere with the assay as the dye can bind to free detergent micelles and obscure binding to a protein hydrophobic regions (Kean *et al.*, 2008). An alternative dye, which is non-fluorescent until it reacts with thiols (cysteine residues in most proteins), is 7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin (CPM) which binds to cysteine residues as they are exposed as a result of thermally induced protein unfolding. The CPM dye has previously been used to screen buffer conditions for stability profiling of membrane proteins (Alexandrov *et al.*, 2008) and has the potential to be used in a similar manner to assess the stability of purified CFTR.

We have previously described methods for expressing and solubilising codon-optimised (Opti-) murine CFTR (mCFTR) and human CFTR (hCFTR) using DDM (O'Ryan *et al.*, 2012, Rimington *et al.*, 2014a, Rimington *et al.*, 2014b). In this study, we investigate the efficiency of subsequent purification of Opti-hCFTR, referred to as CFTR from this point on, using four affinity tags and their associated chromatography matrix. The small peptide tags used were His, a deca-histidine tag in this case (10His), FLAG (DYKDDDDK) and StrepII (NWSHPQFEK) which were incorporated into the CFTR constructs as previously described (Rimington *et al.*, 2014b). Immunoprecipitation facilitated by the GFP tag present at the C-terminus of the CFTR constructs was also performed.

Following assessment of CFTR yield and purity, the thermal stability of protein from the two most promising methods, GFP and nickel-FLAG purifications, was analysed to draw comparisons of the two methods. Further characterisation of protein obtained from the GFP purification methods was further characterised using transmission electron microscopy to ascertain the aggregation state of the CFTR protein. Mass spectrometry analysis was also performed on GFP purified CFTR to confirm the presence of CFTR and identify contaminating yeast proteins.

6.3 Methods

6.3.1 Yeast culture media and agar

CFTR agar -uracil (-URA agar) - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L glucose,* 20 g/L bacteriological agar*)*

CFTR culture media (0.15% or 2% glucose) - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 0.75 g/L D-glucose (for 0.1%) or 20 g/L glucose (for 2%))*

CFTR induction media - *(6.9 g/L yeast nitrogen base (YNB), 770 mg/L complete supplement mixture (CSM) without uracil, 20 g/L D-galactose, 8% glycerol (v/v))*

6X CFTR induction media - *(41.4 g/L yeast nitrogen base (YNB), 4.62 g/L complete supplement mixture (CSM) without uracil, 120 g/L D-galactose, 48% glycerol (v/v) in sterile dH_2O)*

6.3.2 Buffers and stock solutions

CFTR buffer (CB) - *(50 mM Tris-HCl pH 8.0, 1 M NaCl, 20% glycerol, 1 mM dithiothreitol (DTT))*

500 mM NaCl CFTR buffer (medium-salt CB) - *(50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20% glycerol, 1 mM DTT)*

100X Protease inhibitor cocktail (PIC) - *(20 mM AEBSF, 600 µM bestatin, 400 mM chymostatin, 700 µM E-64, 2 mM leupeptin, 1.5 mM pepstatin A, 100 mM PMSF in dry DMSO plus 300 mM benzamidine in sterile dH_2O)*

2X SDS solubilisation buffer (SB) - *(50 mM Tris-HCl pH 7.6, 5% glycerol (v/v), 5 mM EDTA, 0.02% bromophenol blue in 700 µl aliquots. 200 µl of 20% (w/v) sodium dodecyl sulphate (SDS) and 100 µl of 0.5 M DTT added just prior to use)*

10X SDS running buffer - *(30 g/L Tris-HCl, 144 g/L glycine and 10 g/L SDS in sterile dH_2O)*

6.3.3 *CFTR solubilisation and purification*

Microsomes were prepared from either baffled flask or fermenter cultures as previously described (O'Ryan *et al.*, 2012, Rimington *et al.*, 2014a, Rimington *et al.*, 2014b). Equal volumes of microsomes (5 mg/mL total protein concentration) and 4 % (w/v) DDM in CB for nickel, FLAG and StrepII purifications or medium-salt CB for GFP immunoprecipitations were incubated end-over-end at 4 °C on a laboratory tube rotator for 90 minutes. The solubilisation mixture was centrifuged at 100,000 x *g* for 1 hour to pellet insoluble material.

6.3.4 *Measuring GFP fluorescence of CFTR*

GFP fluorescence was monitored in solution using a Cary Eclipse fluorescence spectrophotometer (Varian) with an excitation wavelength of 485 nm and an emission spectra of 500-600 nm and slit widths of 5 nm.

6.3.5 *On-column TEV cleavage of CFTR*

CFTR was eluted from GFP resin with ~2 units TEV-Express protease (Expedeon) or Pro-TEV Plus (Promega) per µg of CFTR (estimated by GFP fluorescence) in 0.5 or 1 mL CB + PIC by incubating with end-over-end rotation on a laboratory tube rotator overnight at 4 °C. CFTR was eluted in a single fraction and resin was washed to ensure all CFTR was collected. TEV-Express was removed from samples using GST SpinTrap columns (GE Healthcare Life Sciences) following the manufacturers instructions.

6.3.6 *SDS-PAGE analysis*

Unless otherwise stated, 25 µl of each sample was added to an equal volume of 2X SB. Samples were loaded onto NuSep nUView 10% Tris-glycine gels and electrophoresed at 150 V for ~45 minutes in 1X SDS running buffer. Gels were analysed under fluorescence prior to coomassie staining with InstantBlue stain

(Expedeon) or silver staining with Silver Stain Plus (Bio-Rad) following the manufacturer's instructions.

6.3.7 In-gel fluorescence of CFTR

GFP-tagged CFTR was analysed following SDS-PAGE using a ChemiDoc™ MP Imaging System (Bio-Rad) fitted with a Blue LED Module Kit with an excitation wavelength of 488 nm and an emission wavelength of 520 nm with images captured using a Supercooled CCD camera and saved for further analysis using Image Lab™ Software (Bio-Rad) and ImageJ.

6.3.8 Ni Sepharose purification

Solubilised microsomes were added to pre-equilibrated Ni Sepharose High Performance resin (GE Healthcare) using ~10 µl resin per mL of microsomes. The binding mixture was supplemented with 1 mM PMSF and 50 mM imidazole and samples were batched mixed with end-over-end rotation for 90 minutes at 4 °C. The binding mixture was transferred to a 1 ml FPLC column (*Generon*) and washed with 10 column volumes (CV's) of 100 mM imidazole in CB with a flow rate of ~1 mL/min. CFTR was eluted with 400 mM imidazole in CB in 1 mL fractions. For samples undergoing subsequent FLAG purification, DTT was omitted from the elution buffer. GFP fluorescence in the elution fractions was monitored and fractions were collected until GFP was no longer detected. Samples from each stage of the purification and peak fractions were analysed by SDS-PAGE for relative yield and purity.

6.3.9 FLAG purification

For one-step FLAG purification, DTT was removed from solubilised microsomes using a centrifugal concentrator with a 100 kDa molecular weight cut off (MWCO) filter by washing with 10 CV's of CB (-DTT). DTT-free microsomes or Ni-purified material was added to pre-equilibrated ANTI-FLAG M2 affinity gel (Sigma-Aldrich) using ~50 µl resin per mL of material. The binding mixture was supplemented with 1 mM PMSF and samples were batched mixed with end-over-end rotation for 90 minutes at 4 °C. The

binding mixture was transferred to an FPLC column (*Generon*) or a gravity flow column (*BioRad*) and washed with 10 CV's of CB (-DTT) with a flow rate of ~0.5-1 mL/min. In some cases on-column TEV cleavage was performed to remove the GFP tag (as described above). CFTR was eluted by competitive binding with 100 µg/mL FLAG peptide in CB (-DTT) with or without in 0.5 mL fractions following a 30 minute incubation period. GFP fluorescence in the elution fractions was monitored and fractions were collected until GFP was no longer detected. For preparation in which GFP removal was performed, CFTR was eluted in four 0.5 mL fractions per mL of starting material. Samples from each stage of the purification and peak fractions were analysed by SDS-PAGE for relative yield and purity.

6.3.10 StrepII purification

Ni-purified material was added to pre-equilibrated Strep-Tactin Superflow Plus resin (Qiagen) using ~25 µl resin per mL of material. The binding mixture was supplemented with 1 mM PMSF and samples were batched mixed with end-over-end rotation overnight at 4 °C. The binding mixture was washed with 10 CV's of CB. CFTR was eluted by competitive binding with 2.5 mM desthiobiotin in CB in 0.5 mL fractions. GFP fluorescence in the elution fractions was monitored and fractions were collected until GFP was no longer detected. Samples from each stage of the purification and peak fractions were analysed by SDS-PAGE for relative yield and purity.

6.3.11 GFP purification

Solubilised microsomes were added to pre-equilibrated GFP-Trap_A resin (ChromoTek) using ~10 µl resin slurry per mL of material. The binding mixture was supplemented with 1 mM PMSF and samples were batched mixed with end-over-end rotation overnight at 4 °C. The binding mixture was transferred to a 1 ml FPLC column (*Generon*) and washed with 10 CV's of medium-salt CB with a flow rate of ~1 mL/min. CFTR was eluted by on-column TEV cleavage (as described above) in 0.5 mL fractions. Samples from each stage of the purification and peak fractions were analysed by SDS-PAGE for relative yield and purity.

6.3.12 *Thermal stability of GFP-Trap_A purifed hCFTR*

The thermal stability of protein obtained from the GFP and FLAG purifications was compared by measuring unfolding of CFTR and subsequent binding of CPM to cysteine residues. CPM fluorescence was monitored using either a StepOnePlus Real Time PCR system (*Life Technologies*) using the SYBR Green reagent settings and a temperature increment of 0.5 °C in a 25 µl reaction in sealed thin-walled white PCR tubes or using a Cary Eclipse Fluorescence Spectrophotometer (*Varian*) with an excitation wavelength of 387 nm and an emission wavelength of 463 nm with a heating rate of ~2.5 °C/min controlled by an external water bath (*Grant Instruments*) in a 50 µl reaction mix in a 1 cm quartz cuvette (*Hellma Analytics)*. Reaction mixes were incubated on ice for 5 minutes using 200 ng of CPM dye and 5-10 µg/mL protein in either 25 µl or 50 µl reaction mixes. The samples were heated and CPM fluorescence measurements were recorded from 20 to 70 °C. As the heating rate was not constant using the Varian system with external water bath heating, the actual temperature in the cuvette was monitored throughout the experiment and temperature adjustments were applied to the data. The unfolding transition temperature, or denaturation midpoint (T_m) was calculated from data normalised from first derivative plots ($\Delta F/\Delta T$ plotted against temperature) following subtraction of buffer only controls. The peak, and consequently the T_m was calculated using nonlinear regression analysis using GraphPad Prism 6.02 (*GraphPad Software Inc.*).

6.3.13 *Negative staining and analysis of GFP purified CFTR by TEM*

To generate a hydrophilic surface and increase adhesion of protein, 400-mesh copper grids with a continuous carbon film (*Electron Microscopy Sciences*) were glow discharged for 30 seconds. Grids were placed onto a 5 µl droplet of GFP purified CFTR at ~5 µg/mL for 1 minute. Excess liquid was removed by wicking with filter paper (Whatman No. 1) followed by washing with sterile dH_20 (2 x 5 µl droplets). Grids were negatively stained by placing on a 5 µl droplet of 4% (w/v) uranyl acetate for 30 seconds. Excess stain was removed by wicking with filter paper (Whatman No. 1). Grids were analysed with a FEI Tecnai Biotwin Transmission Electron Microscope operating at 100 kV. Micrographs were collected digitally using a Gatan Orius SC1000 11 Megapixel CCD camera and analysed using ImageJ (*http://rsb.info.nih.gov/ij*). Particles were selected using a box size of 18 x 18 nm.

6.3.14 Analysis of GFP purified CFTR by mass spectrometry

To confirm the presence of CFTR and to identify contaminants in the extracted and purified samples, mass spectrometry was performed. DDM solubilised, GFP purified CFTR (~100 ng protein) was subjected to SDS-PAGE with loading samples only allowed to migrate ~0.5 cm into the gel. The gel was coomassie stained with InstantBlue stain (*Expedeon*) to enable excision of the total protein band. Biological Mass spectrometry analysis was performed by the Protein Identification Service in the Faculty of Life Sciences at the University of Manchester. Peptide fragments were compared to the Human UniProt (version 2013-05) database and the Yeast (ORFs) (version 2010-01-06) database. Protein identifications were been made using Mascot (version 2.2.06; *Matrix Science*).

6.4 Results

6.4.1 Comparison of purification methods for Opti-hCFTR

Following purification of CFTR by single-step nickel, FLAG or GFP affinity chromatography, or two-step nickel-FLAG, or nickel-StrepII affinity chromatography, the yield of full-length CFTR after binding and after elution was calculated relative to the starting material (Fig. 6.1). The highest levels of binding were observed for DDM-solubilised microsomes bound to nickel sepharose resin at ~59% and the FLAG resin at ~58%. The GFP-Trap_A resin bound ~31% of CFTR. Following elution, CFTR present from the single-step nickel, FLAG and GFP was ~20%, ~30% and ~8.3% respectively. Binding of Ni-purified protein to the FLAG resin was very low, at ~7% and binding to the Strep-Tactin resin was ~33%. As these two methods were the second step of two-step purifications, the final yields were reduced to ~1.3% and ~0.7% respectively based on the yield obtained from the first step nickel purification.

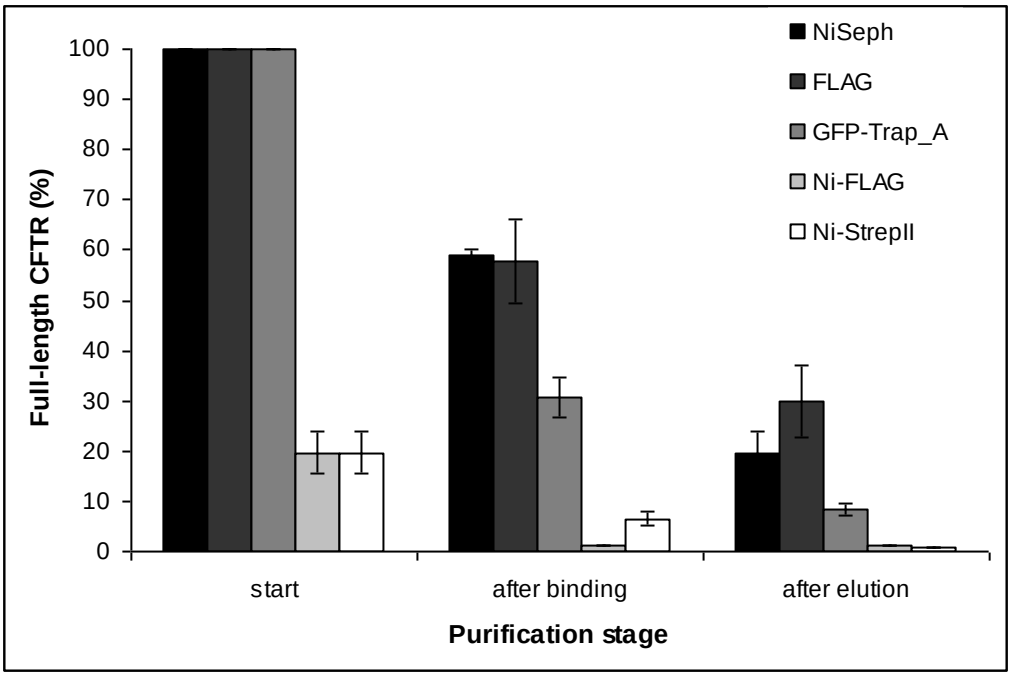
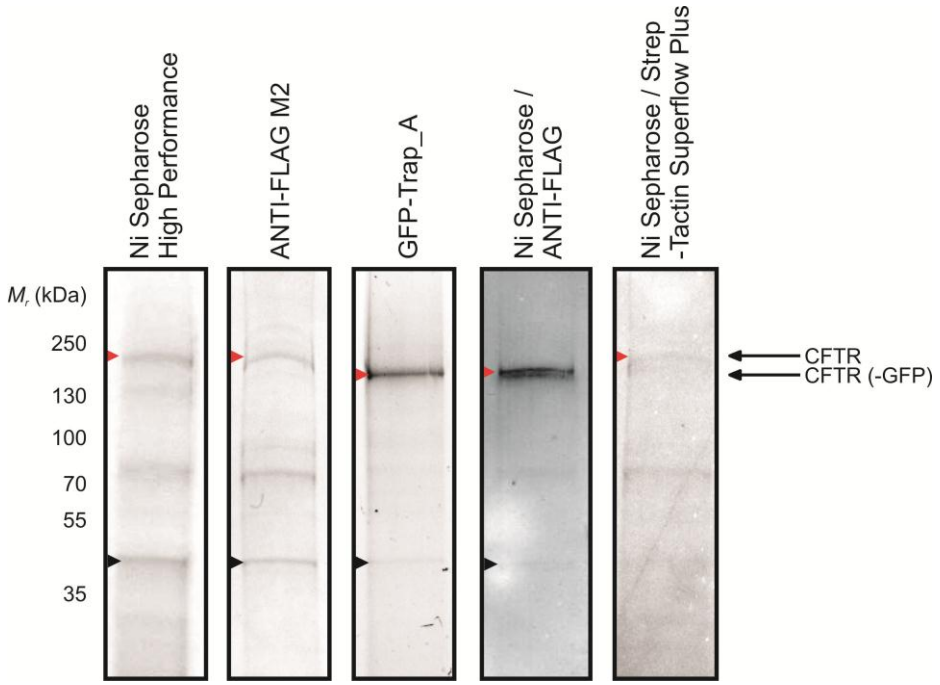


Figure 6.1. Purification comparison of DDM-solubilised Opti-hCFTR. Purification was performed using nickel, FLAG, StrepII or GFP affinity chromatography. Concentration of full-length CFTR was calculated following SDS-PAGE analysis under fluorescence for GFP-tagged protein, or by estimation from coomassie or silver-stained gels. Full-length CFTR was calculated relative to the protein present at the start of each purification. Error bars (where present) represent the standard deviation from a minimum of three independent experimental repeats.

To assess the purity of CFTR (Fig. 6.2a, red arrows) eluted from each purification method, densitometry using ImageJ was performed following SDS-PAGE (Fig. 6.2a) to resolve the proteins in the peak elution fractions. The purity of CFTR in the single-step purifications (nickel, FLAG and GFP affinity chromatography) was ~34%, ~29% and ~91% respectively. The two-step purifications (nickel followed by either FLAG or Strep affinity chromatography) resulted in ~80% and 22% purity respectively (Fig. 6.2b). A common contaminating protein is seen at ~44 kDa (Fig. 6.2a, black arrows) in the nickel, GFP and nickel-StrepII purifications which is absent from the FLAG and nickel-FLAG purifications. Despite on-column TEV cleavage to remove the GFP tag from the nickel-FLAG protein, some GFP-tagged protein remained as the cleavage was not 100% efficient.

(a)



(b)

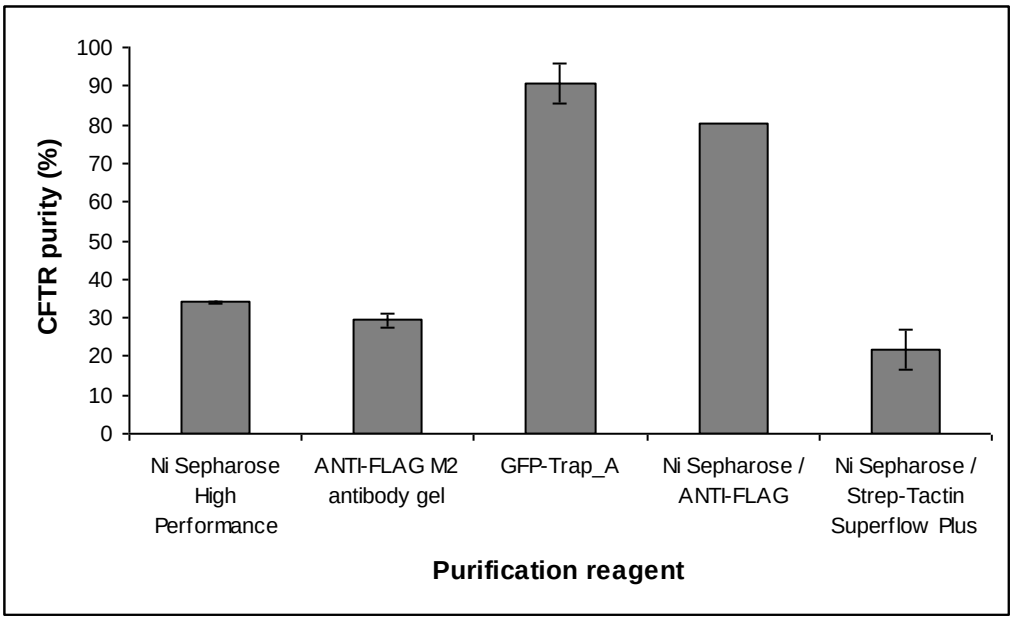
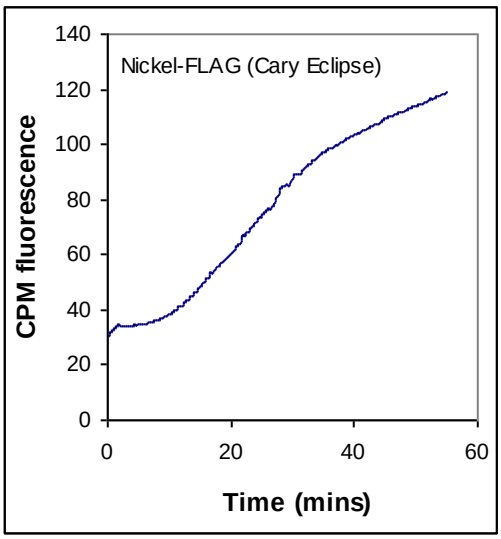


Figure 6.2. Comparison of purity of Opti-hCFTR purified by nickel, FLAG, nickel-StrepII, nickel-FLAG or GFP purification method. (a) Representative SDS-PAGE gel lanes from each purification method showing CFTR (red arrows), major contaminants and degradation products imaged following coomassie staining for nickel, FLAG, GFP and nickel-StrepII and silver staining for nickel-FLAG purifications. The RPL3 contaminant is indicated by the black arrows in the nickel, FLAG, GFP and nickel-FLAG purifications. (b) Purity of full-length CFTR was calculated by applying densitometry analysis using ImageJ. Error bars (where present) represent the standard deviation from a minimum of three independent experimental repeats.

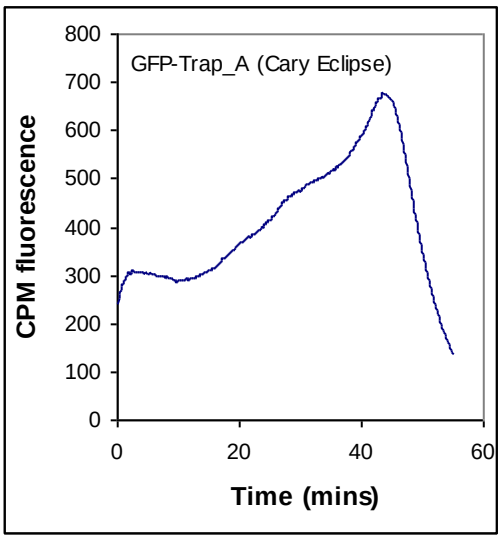
GFP purified CFTR has a higher T_m than nickel-FLAG purified CFTR

To assess and compare the thermal stability of CFTR purified using either one-step GFP or two step nickel-FLAG purification, protein unfolding was monitored using a CPM binding assay in either a StepOnePlus PCR System or a Cary Eclipse Fluorescence Spectrophotometer. The Cary Eclipse raw data plot of the CPM assay using nickel-FLAG purified CFTR exhibits increasing fluorescence over time, and therefore increasing temperature (Fig. 6.3a) whilst there is a decrease in CPM fluorescence in the latter stages of the assay using GFP purified CFTR (Fig. 6.3b). The data obtained from the StepOnePlus PCR machine using GFP purified CFTR exhibited a similar decrease in CPM fluorescence (Fig. 6.3c) and following subtraction of buffer only controls (Fig. 6.3d) this decrease remained apparent using the normalised data (Fig. 6.3e). The increase in CPM fluorescence above ~63 °C for the nickel-FLAG purified trace is likely due to denaturation of GFP and subsequent labelling of its natively buried cysteine residues (PDB: 1GFL). The T_m of GFP purified CFTR was calculated from the first derivative plot of fluorescence change ($\Delta F/\Delta T$) against temperature (T) as 43.0 °C (± 0.2 °C). The T_m of nickel-FLAG purified CFTR was 40.7 °C (Fig. 6.3f).

(a)



(b)



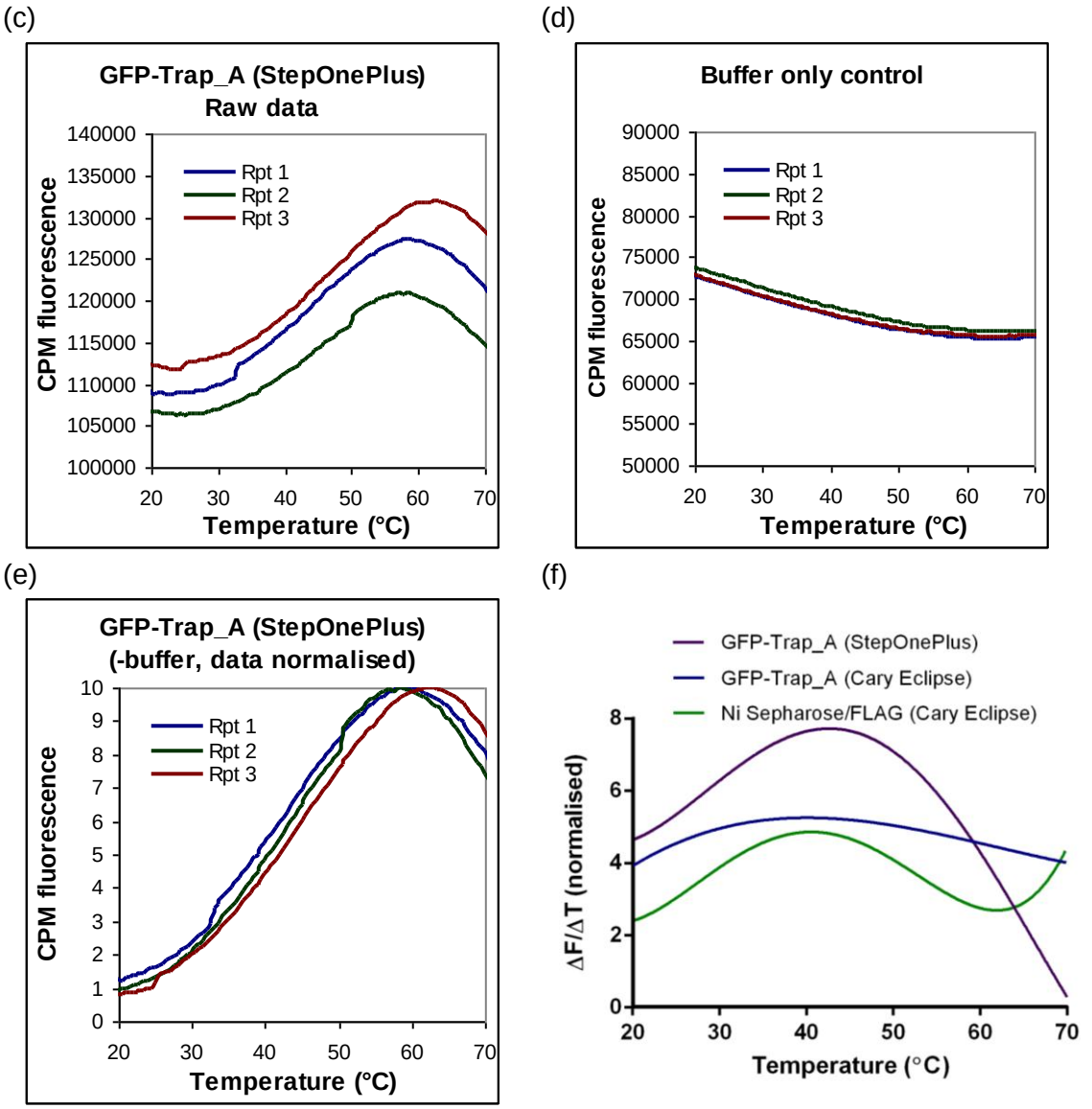


Figure 6.3. Thermal stability of DDM solubilised Opti-hCFTR purified by either one-step GFP or two-step nickel-FLAG purifications. Following the addition of 200 ng of CPM dye protein unfolding was monitored by measuring CPM fluorescence from 20 to 70 °C resulting from binding to exposed cysteine residues as the protein unfolded. Examples of raw data traces in a Cary Eclipse Fluorescence Spectrophotometer were obtained for (a) nickel-FLAG purified CFTR and (b) GFP-Trap_A purified CFTR. Raw data is also shown for a StepOnePlus PCR system for (c) GFP-Trap_A purified CFTR. (d) Measurements were also recorded in the StepOnePlus PCR system for buffer only controls (e) which were subtracted from the readings for CFTR and the data normalised. The first derivative plot of fluorescence change ($\Delta F/\Delta T$ versus T) was calculated from the normalised data and, following non-linear regression analysis trendlines were assigned using GraphPad Prism. The T_m was calculated as 42.8 °C for GFP purified CFTR (n = *3*) analysed using a StepOnePlus PCR System and 43.2 °C using a Cary Eclipse Fluorescence Spectrophotometer. The estimated T_m for nickel-FLAG purified CFTR was 40.7 °C.

6.4.2 Analysis of GFP purified CFTR using TEM

To examine the dispersity and aggregation state of DDM solubilised, GFP purified CFTR, transmission electron microscopy was performed. CFTR existed as a mixture of single particles with a diameter of ~10 nm and larger aggregates of ~20-30 nm (Fig. 6.4).

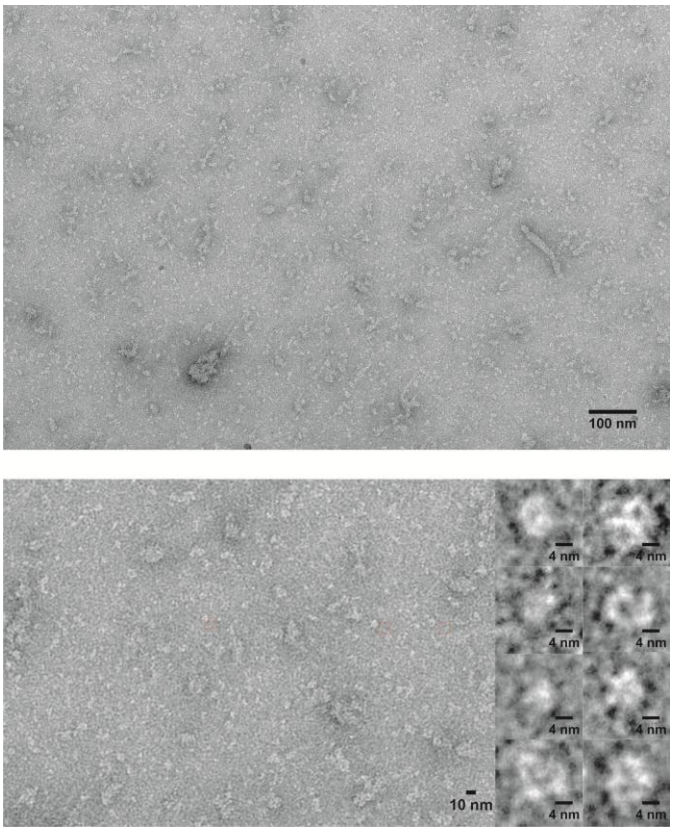


Figure 6.4. Transmission electron microscopy analysis of negatively stained GFP purified CFTR. Single particles of ~10 nm and aggregates of ~20-30 nm exist in a polydispersed mixture.

6.4.3 *Analysis of GFP-Trap_A purifed hCFTR by mass spectrometry*

The presence of human CFTR was confirmed following analysis by mass spectrometry. Several contaminating yeast proteins were identified, including the protein component of the large (60S) ribosomal subunit, RPL3. The protein contaminants with the most identified peptides are listed in (Table 6.1).

Table 6.1. Top 14 protein contaminants with the most identified peptides and their corresponding molecular weight identified by mass spectrometry analysis of DDM solubilised, GFP purified CFTR compared to the Human UniProt (version 2013-05) database and the Yeast (ORFs) (version 2010-01-06) database.

Identified protein	Accession number	M_r
CFTR - Cystic fibrosis transmembrane conductance regulator	CFTR_HUMAN	168 kDa
SSA1 - member of heat shock protein 70 (HSP70) family	YAL005C	70 kDa
ACC1 - Acetyl-CoA carboxylase	YNR016C	250 kDa
PDR12 - ATP-binding cassette (ABC) transporter	YPL058C	171 kDa
PMA1 - H+-ATPase	YGL008C	100 kDa
IST2 - Cortical ER protein	YBR086C	106 kDa
SSB2 - ATPase, member of the HSP70 family	YNL209W	67 kDa
FKS1 - Catalytic subunit of 1,3-beta-D-glucan synthase	YLR342W	215 kDa
ATP1 - Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	YBL099W	59 kDa
RPL3 - Protein component of the large (60S) ribosomal subunit	YOR063W	44 kDa
SSA3 - ATPase, member of the HSP70 family	YBL075C	71 kDa

6.5 Discussion

Obtaining sufficient quantities of CFTR for functional and structural analysis has been a limitation for the field of CF research. Highly purified CFTR has previously been obtained using LPG combined with diheptanoylphosphatidylcholine (DHPC) to solubilise CFTR expressed in insect cells (Ketchum *et al.*, 2004). Our previous published methods have described the purification of murine and chicken CFTR following detergent solubilisation in both DDM and LPG yielding purities of up to ~60% and ~90% respectively (Pollock *et al.,* O'Ryan *et al.*, 2012). As DDM solubilised CFTR exhibited ATPase activity and appeared to be correctly folded we investigate CFTR purification using this milder, non-denaturing detergent. Our aim was to improve the purity of DDM solubilised CFTR. We investigated CFTR yields and purity following affinity chromatography using nickel, FLAG, StrepII and GFP in either one- or two-step purifications.

By monitoring CFTR yields after binding and elution for each purification method we found the highest binding efficiency was observed in the Ni Sepharose and ANTI-FLAG M2 preparations and, following elution, the highest recovery of CFTR was from the FLAG purification. The efficiency of binding to the GFP-Trap_A resin was low in comparison at just over half of that of the Ni and FLAG purifications. Recovery of CFTR from the GFP purification was lower than with either the Ni or FLAG methods but was greater than for either of the two step purification methods with <1% of CFTR recovered from the two-step nickel-StrepII purification which was considerably lower than the FLAG, GFP and nickel-FLAG methods.

As highly pure protein is required for many downstream functional and structural studies, is was crucial to assess the purity of CFTR from each purification method. The purity of the protein recovered from the one-step GFP purification was >90%, and was >95% in some cases. This level of purity was only previously achieved from this expression system using LPG solubilised CFTR (Pollock *et al.*). One advantage of this purification method is the cleavage of the GFP tag during elution which was a requirement for this study as GFP fluorescence would generate high background noise in the CPM thermal stability assay. Should the GFP tag be required, the protein could be eluted from the GFP-Trap_A resin by competitive binding with pure GFP although this would cause excess GFP to be present in the elution fractions which could interfere with downstream analysis and quantification using GFP fluorescence. The purity of CFTR from the nickel and FLAG preparations was much lower than for the GFP purified material. This was improved to ~80% using a two-step nickel-FLAG

purification, but the final yield was <1.5% of total DDM solubilised CFTR at the start of the purification. The yield and purity of CFTR recovered from the StrepII purification was very low suggesting that this method may not be suitable for purification of CFTR although it may be possible to optimise the protocol to improve the results.

We have previously established that that the addition of up to 1 M NaCl substantially increases the efficiency of CFTR extracted from microsomes during DDM solubilisation (Rimington *et al.*, 2014a, Rimington *et al.*, 2014b). However, the maximum recommended NaCl concentration for the GFP purification is 500 mM which resulted in a slight reduction of solubilisation efficiency. As the purity of CFTR is >90% using this method, this compromise of lower solubilisation efficiency is acceptable and the use of a lower concentration of NaCl may actually prove beneficial resulting in milder buffer conditions throughout the purification and indeed, 500 mM NaCl and DDM solubilisation has previously been used to extract active CFTR from BHK cells (Rosenberg *et al.*, 2004). Based on the results, purification using GFP immunoprecipitation appears to be the most favourable method for obtaining highly pure CFTR. Whilst this method does not yield the highest amount of protein, the purity obtained from this one-step method means that no further purification steps are required.

To characterise GFP purified CFTR, the protein was analysed for stability, folding and dispersity. Some comparisons were also made to CFTR purified by the nickel-FLAG method which yielded protein which was ~80% pure. This method has also been previously used to purify CFTR expressed in BHK cells (personal communication, Dr Ellen Hilderbrandt, Texas Tech University, (Cant, 2013)).

Thermal stability of CFTR recovered from the two highest purity preparations was compared by monitoring unfolding of CFTR and subsequent binding of CPM to cysteine residues exposed during the unfolding transition. The thermal denaturation profiles obtained indicate an increased stability for CFTR recovered from the GFP purification compared to the two-step nickel-FLAG purified CFTR with respective T_m values of 43 °C (±0.2 °C) and 40.7 °C. The lower T_m for the nickel-FLAG purified protein could be due to the removal of DTT prior to the FLAG purification which may cause destabilisation of the protein as a result of non-native interactions. However, the T_m calculated (40.7 °C) is consistent with previous results for the same construct expressed and nickel-FLAG purified from BHK cells (Cant, 2013). Moreover, DTT was absent from all CPM binding assays as it prevents binding of the CPM dye. Another explanation for the improved stability of the GFP purified CFTR is that there are less

steps involved and highly purified CFTR is isolated from yeast contaminating proteins at an earlier stage. The removal of potential proteases, preventing them from interacting with CFTR, could allow the protein to retain folding and stability therefore increasing the T_m. The increase in CPM fluorescence observed above ~63 °C for the nickel-FLAG purified CFTR is most likely a result of GFP denaturation and is not apparent in the TEV cleaved, GFP-free CFTR obtained from the GFP purification. It should be noted, that due to the inconsistent temperature ramp rate using the Cary Eclipse system, this instrument is not optimal for performing the CPM thermal stability assay as a constant rate of temperature increase is required (Kean *et al.*, 2008). However, in this study, the use of the Cary Eclipse system was necessary for the GFP-tagged nickel-FLAG purified CFTR as the GFP tag generated too much background signal using the StepOnePlus PCR System. As the GFP purified CFTR resulted in a very similar thermal stability profile using both instruments with a variance of only 0.4 °C, the difference observed in T_m for CFTR from the different purification methods is most likely due to changes in protein stability.

TEM of GFP purified CFTR revealed a mixture of single particles with a diameter of ~10 nm and some visible aggregates (~20-30 nm) which may exhibit a reversible association. These results are consistent with data obtained for Opti-chicken CFTR as previously described (Pollock *et al.*) indicating correctly folded protein in the samples.

Analysis of the GFP purified CFTR was analysed by mass spectrometry to confirm the presence of CFTR and to identify contaminating yeast proteins. CFTR purity was calculated as up to 95% following GFP purifications and the presence of the human CFTR protein was confirmed by analysing against the Human UniProt (version 2013-05) database and the Yeast (ORFs) (version 2010-01-06) database. Our previously published purification methods identified a major contaminant at ~44 kDa which was identified as the protein component of the large (60S) ribosomal subunit, RPL3 and resulted in CFTR purity of ~60% using DDM solubilisation (Pollock *et al.,* Cant *et al.*, 2014). Although RPL3 is still present following GFP purification, it is barely detectable by SDS-PAGE analysis and the purity of CFTR was calculated as >90%, a vast improvement on previous methods. We had previously hypothesized that RPL3 may co-purify with CFTR during nickel affinity chromatography due to a potential polyHis cluster present when bound to the ribosome (Pollock *et al.,* Cant *et al.*, 2014). The reduction of RPL3 following the GFP immunoprecipitation and apparent lack of the contaminant following FLAG and nickel-FLAG purification supports this hypothesis. However, another possibility is that RPL3 interacts with other yeast contaminating proteins which are reduced following GFP or FLAG purification. Scale-up of this

method could further improve the purity and yield of CFTR as more efficient, stringent wash steps could be performed using automated systems.

Previous studies in our group have shown that purified, reconstituted chicken CFTR solubilised in the mild, non-denaturing detergent, DDM exhibited higher ATPase activity (~13 nmol/min/mg) than protein solubilised in the harsh ionic detergent, LPG (~1.5 nmol/min/mg) (Pollock *et al.,* Cant, 2013) suggesting that DDM solubilisation preserves CFTR's ability to hydrolyse ATP and the protein is more likely to retain its function as a chloride ion channel compared to CFTR solubilised in LPG. When assayed for ATPase activity, LPG solubilised, nickel purified CFTR exhibited a rate of ATP hydrolysis of ~2.8 nmol/min/mg when reconstituted into proteoliposomes. Provided the rate was increased in a similar manner to the chicken CFTR, DDM purified human protein should exhibit an increased rate of activity. To assess this, it would be necessary to scale up the GFP purification protocol to obtain large enough quantities of CFTR to enable reconstitution and perform ATPase assays.

The one-step GFP purification provides an efficient and rapid method for obtaining highly purified CFTR which appears to be correctly folded and exhibits greater thermal stability compared to nickel-FLAG purified protein. The quantity obtained using the small scale purification is ~40 µg of CFTR per litre of culture. Scale up of this method to a fermenter culture could improve this yield up to ~1-2 mg of highly purified DDM solubilised, GFP purified CFTR for functional and structural studies. Further development of the CPM thermal stability assay could enable its use to screen compounds and/or conditions which stabilise CFTR in its WT and mutant forms and has the potential to be used in high-throughput assays to identify possible treatments for CF.

6.6 References

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Chapter 7 - Final discussion and conclusions

The work presented in this thesis provides insights into the successful use of *S. cerevisiae* for high level expression of recombinant CFTR and its subsequent purification to obtain protein that could be used for functional or structural analysis. The data revisits some earlier issues with our expression system, such as low solubility of CFTR in DDM, the poor expression of human CFTR and also the presence of a major contaminant (RPL3) in DDM purifications. This thesis provides solutions to these problems and explores some preliminary characterisation of purified CFTR.

7.1 Optimisation of the yeast system for CFTR expression

Chapters 2 and 3 identify the importance of optimising culture conditions to obtain relatively high levels of CFTR expression in the yeast system. Expression of full length CFTR had previously proven problematic in *S. cerevisiae* usually resulting in protein degradation and/or aggregation. The data presented in this thesis provides methods to help overcome many of the difficulties faced when expressing recombinant CFTR in yeast and have the potential to be used for other challenging membrane proteins. The use of chemical chaperones, in particular glycerol, during induction had a notable effect on mCFTR expression, with increases of up to ~6-fold, and up to ~7-fold when used in conjunction with DMSO. It is likely that glycerol stabilises CFTR folding intermediates during ER and Golgi processing which are prone to aggregation and degradation (Perlmutter, 2002, Mishra *et al.*, 2007) allowing them to be correctly processed by the ER and Golgi machinery and trafficked towards the plasma membrane of cells (Perlmutter, 2002). Whilst the combined effect of glycerol and DMSO produced the highest levels of expression during these trials, the cost of adding DMSO to cultures would be considerable and the effectiveness of 8% glycerol alone was deemed sufficient and was used for all subsequent cultures.

Establishing the optimal post-induction cell harvest time and the optimal cell density at induction were important parameters to enable consistency between cultures and allow comparisons to be made between different orthologues and CFTR batches. Following these trials, protein expression was induced at an OD_{600} of ~1.5, although the optimum was identified as 2.0, there was a substantial decline in detectable CFTR above this so a lower induction cell density was chosen to help minimise loss of CFTR. Cells from all subsequent cultures were harvested at ~14-16 hours after induction as the highest levels of full-length protein was produced between these timepoints with a substantial decline in detectable CFTR levels after ~18 hours.

The optimal induction conditions identified in chapter 2 enabled expression of reasonably high levels of CFTR which allowed detergent solubilisation trials to be carried out. Solubilisation trials using three CFTR orthologues (cCFTR, mCFTR and plCFTR) highlighted the importance of testing a variety of detergents and buffer conditions to maximise efficiency. The use of up to 1 M NaCl in solubilisation buffers was found to substantially improve CFTR solubilisation from ~27-50% up to ~87% in the mild detergent DDM. This data was very encouraging as, although CFTR was extractable with high efficiency using LPG, this ionic detergent is quite harsh and has been shown to cause unfolding and denaturing of CFTR (Therien and Deber, 2002,

Cant, 2013). Extraction of CFTR from the membrane using DDM is favourable for functional and structural analysis as the protein retains its folded state and channel activity (Lin and Guidotti, 2009) and the data presented in chapter 2 was an important step towards obtaining high enough quantities of functional CFTR for further analysis.

7.2 Scaling up CFTR expression

Chapter 3 uses some of the optimised culture conditions from chapter 2 to describe the experimental procedures that can be employed to express mCFTR following scale-up to a 15 litre fermenter. Expression in a fermenter appeared to be more stable than in small-scale cultures and the optimal cell harvest time was ~15-16 hours following induction. This is likely due to increase aeration generated by the fermenter instrumentation and the greater degree of control the system offers with respect to stirring speed and temperature regulation. This system can generate 1-2 mg of purified mCFTR using LGP solubilised protein and nickel affinity chromatography. At the time of publication of this paper, solubilisation in DDM had proven to be relatively inefficient and the improvements described in chapter 2 were on-going at this stage.

7.3 Purification of functionally active CFTR

Following on from the publication describing the methods for expression of CFTR in *S. cerevisiae*, we published a further paper (chapter 4) which explored purification of cCFTR using LPG and DDM solubilised protein. The DDM solubilisation efficiency was much improved with the use of 1 M NaCl and we able to obtain CFTR with a purity of ~60% which exhibited a rate of ATP hydrolysis of ~13 nmol/min/mg. Elution fractions from these preparations were enriched with a contaminating protein at ~40 kDa which was confirmed by mass spectrometry analysis as RPL3. Removal of RPL3 proved difficult at this stage and it remained in samples following the use of a 100 kDa spin concentrator indicating that there may be an association of RPL3 with CFTR. The presence of the contaminant was a major drawback of the DDM purification up to this point in the project and its removal was a priority in order to obtain highly purified CFTR for further analysis.

7.4 Expression and purification of hCFTR

To truly test the usefulness of the yeast expression system, it was essential to attempt expression of the human CFTR orthologue. Preliminary trials, using the optimal culture conditions established for mCFTR which were also successfully used to express cCFTR and plCFTR, produced very low yields of hCFTR. The construct used for molecular cloning was redesigned as described in chapter 5 and, following the addition of a yeast kozak-like sequence, there was an increase in expression of ~28-fold. A FLAG tag and a StrepII tag were also introduced into the construct to allow investigation of different purification methods to try to improve the purity of DDM solubilised CFTR.

A comparison of the effectiveness of the purification tags was described in chapter 6. The C-terminal GFP tag, although used in the constructs to allow quick and easy visualisation of CFTR, was actually extremely useful for purification of CFTR as it enabled immunoaffinity purification using GFP-Trap_A resin and CFTR purity of up to 95% could be obtained. This was a vast improvement on previous DDM purification methods and this high level of purity had only previously been obtained from this system using LPG solubilised protein. Following purification using this method, there was very little RPL3 remaining in the samples and it would seem that this provided a solution for the removal of this major contaminating protein. Scale-up of the GFP purification method could eliminate RPL3 from preparations completely and further improve the purity of CFTR owing to the more stringent washing steps facilitated by automated HPLC instruments.

7.5 Characterisation of hCFTR

Obtaining sufficient quantities of CFTR enabled some preliminary characterisation of the protein. Chapter 6 investigated the folding state, dispersity and contaminants present in purified CFTR. Data obtained from the CPM binding assay, which monitors protein unfolding by labelling of exposed cysteine residues, suggested that CFTR undergoes an unfolding transition and the T_m for GFP purified CFTR was 43.0 °C (±0.2 °C) which was higher than nickel-FLAG purified protein which had an estimated T_m of 40.7 °C. This difference could be accredited to the fact that there are fewer steps involved in the GFP purification and CFTR is extracted from contaminating proteins and potential proteases at an earlier step. The buffers used in the GFP purification are also less harsh and only contain 0.5 M NaCl, as opposed to the nickel and FLAG buffers which contain 1 M NaCl. In addition, the nickel purification uses imidazole, which is not required for the GFP purification. Essentially, the buffer composition is fairly similar for all stages of the GFP purification, with changes in detergent concentration and the use of TEV protease for elution being the only variances. This could contribute to a more stable protein being obtained however, more repeats would be necessary to confirm this hypothesis. Unfortunately, as TEV cleavage to facilitate removal of the GFP tag from the nickel-FLAG purified protein was not 100% efficient, the GFP remaining in the sample caused too much background in the StepOnePlus PCR system so multiple repeats were not able to be performed in this case.

Analysis of GFP purified CFTR by TEM revealed single particles of ~10 nm and some larger aggregates of ~20-30 nm. The shape and size of the single particles is consistent with previous data presented for DDM purified CFTR following nickel purification (Pollock *et al.*) suggesting the protein is correctly folded supporting the data obtained from thermal stability analysis.

The development of the CPM thermal stability assay using the StepOnePlus PCR system could prove useful for screening of drugs or small molecule interactions with CFTR. In particular, the assay could be used to identify treatments for the F508del mutation, which has been shown to be less stable than WT CFTR (Protasevich *et al.*, 2010, Tosoni *et al.*, 2013, Venerando *et al.*, 2013) and for which currently few effective treatments exist.

7.6 Future directions

The work presented in this thesis describes a novel method for obtaining milligram quantities of CFTR protein using an optimised yeast expression system and purification using immunoprecipitation of GFP-tagged codon optimised CFTR to produce highly purified protein to be used for functional and structural analysis.

The optimisation of *S. cerevisiae* culture conditions described in the earlier chapters of the thesis highlighted the importance of optimising conditions. Another aspect which could be explored is the use of different yeast strains for CFTR expression. It may be possible to further optimise CFTR expression using yeast strains which have reduced, or are even deficient in components of the degradation and/or aggregation pathways.

The comparison of purification methods used provided evidence that CFTR purification can be achieved using a number of methods, to varying extents of efficiency and purity. The tags explored in this thesis represent just a handful of possible affinity tags and there are many more which could be tested to purify the protein. Further improvements to the GFP purification method, including scale-up of the protocol, should lead to greater yields of highly purified CFTR which would allow a wider array of biochemical and biophysical analysis to be performed widening our knowledge further.

The CPM thermal stability assay described in the latter parts of the thesis could form the platform for high-throughout screening assays which could identify correctors for the F508del mutation of CFTR. The advantages of this system are that relatively small quantities of protein are required and many compounds could be screened simultaneously using direct comparisons to controls in multi-well plates. The potential for this assay to identify compounds which have a stabilising effect on the F508del protein could lead to potential treatments for CF.

Chapter 8 - References

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