Development and Characterization of a monoclonal antibody to Calnexin, an ER marker protein

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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ABSTRACT

ΔF508 is the most common disease-causing mutation of the cystic fibrosis (CF) transmembrane-conductance regulator (CFTR) gene. It yields a protein that fails to fold correctly and traffic to the cell surface, yet, under experimental conditions where it can be forced to traffic correctly, it is nearly fully functional. A pharmacological means of effecting correct trafficking of ΔF508 CFTR could cure over 90% of CF patients. An important step in this direction would be to identify the components of the endoplasmic reticulum (ER) that interact with CFTR and can differentiate mutant from wild type proteins, allowing the latter to exit the ER normally, while retaining the former for degradation.

My thesis focuses on the development and characterization of a monoclonal antibody (mAb) designed to make possible the rapid isolation of ER vesicles suitable for the study of CFTR and components that interact with it in its early biogenesis. The mAb that I have developed (1H82) is targeted to the cytoplasmic domain of a type I membrane protein, calnexin, which is specifically localized to the ER. It recognizes both denatured and native calnexin, yields a strong signal in immunoblotting, and has an affinity on the order of 10^8 M^{-1}. Its epitope is localized to a 20 amino acid region about halfway between the transmembrane domain and the C-terminus of calnexin. Since 1H82 is able to bind calnexin in the native environment of ER membrane vesicles it appears to be suitable for its intended purpose as a reagent for future purification of ER vesicles by immunoaffinity isolation.
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1 Introduction

1.1 Hypothesis and specific aims

Approximately 91% of CF patients harbour at least one ΔF508 CFTR allele and about 50% are homozygous for the mutation. ΔF508 causes a defect in intracellular processing, thus preventing the correct localization of the mutant protein. However, any ΔF508 CFTR that has made its way to the plasma membrane is able to function as a plasma membrane Cl⁻ channel. The ultimate goal of my project, therefore, was to elucidate the mislocalization of CFTR by identifying components leading to its retention and degradation.

Certain protein components in the ER retain and degrade the ΔF508 CFTR, thus rendering it incapable of trafficking to the plasma membrane. Chemical cross-linking followed by immunoprecipitation could be performed to identify components that interact closely with CFTR. Components that interact differentially with ΔF508 CFTR relative to the wild type protein would be of particular interest. To identify such components, the ER must be isolated rapidly and the isolated fraction should especially be free of contamination from the plasma membrane. Saucan et al. immunoisolated vesicular carriers operating between the Golgi complex and the basolateral plasmalemma using magnetic beads coated with an antibody against the last 11 amino acids of the poly IgA receptor tail [1]. I would like to implement a similar procedure for the purification of ER. As a first step, it is necessary to develop an antibody against the cytosolic domain of an integral membrane ER resident protein. Calnexin is a unique component of the ER
membrane. Its C-terminus is on the cytosolic side of the membrane, while its N-terminus is on the luminal side. Even though there are anti-calnexin antibodies available, they are either polyclonal or are directed towards the N-terminus [2]. Since our long-term goal would be to characterize protein components that interact closely with CFTR within the ER, large amounts of ER will have to be purified. Using large quantities of the commercial polyclonal antibody would make the experiments very costly. Furthermore, this polyclonal antibody is not affinity purified, and this might yield lower purity ER than is suitable for our goals. Another disadvantage of polyclonal sera is that they vary from one batch to another. A monoclonal anti-calnexin antibody on the other hand would be more stable and available in indefinite supply, and is thus preferred.

In this thesis, I will concentrate on the development and characterization of a monoclonal antibody to calnexin cytoplasmic domain. I hypothesize that the use of this antibody in future work towards isolating and characterizing ER components will hopefully enhance our understanding of the biosynthetic arrest of the ΔF508 CFTR. It is hoped that ways of interfering with the biosynthetic arrest will become evident, and will ultimately allow design of drugs for CF therapy.

1.2 Overview

Cystic fibrosis (CF) is the most common lethal genetic disease affecting 1 in 2500 live births in the Caucasian population [3], and about 5% of Caucasian Americans are carriers of the disease [4]. CF is caused by alterations in a single gene coding for CFTR.
In healthy individuals, hydrolysis of ATP, and phosphorylation by cAMP-dependent protein kinase (PKA) mediates the opening of CFTR, and allows chloride ions to flow through the membrane channel. In CF patients, CFTR channels do not open in response to cAMP elevation. This abnormality in electrolyte transport leads to elevated salt concentration in the sweat, and the measurement of chloride content in perspiration remains the cornerstone of diagnosis [4]. Besides the sweat glands, many other organs are affected by CF. The major cause of mortality in CF patients is respiratory failure due to progressive pulmonary obstruction and chronic bacterial infection of the respiratory tract.

Over 600 mutations in the CF gene have been discovered, and the ΔF508 mutation has the highest frequency of occurrence. ΔF508 causes incorrect folding and ultimately leads to the biosynthetic arrest of the mutant CFTR [5]. Shortly after synthesis, 45 - 80% of the wild-type, and all of the ΔF508 CFTR, is rapidly degraded, while the remaining wild-type CFTR attains a protease-resistant conformation. The conversion of a protease-sensitive form to a more mature, protease-resistant form that is capable of leaving the ER requires ATP [6]. Due to the failure of the ΔF508 CFTR to undergo such a transition, it is trapped in the ER, and is unable to localize to the plasma membrane. However, ΔF508 CFTR is able to function if it can be made to arrive at the plasma membrane [7]. Since defective membrane protein trafficking is the basis of the disease, developing methods to understand this pathway is essential to CF research.

Integral membrane proteins are synthesized by ribosomes bound to the rough ER. Like other integral membrane proteins, membrane targeting and insertion of CFTR
depends on the signal recognition particle (SRP) and SRP receptor [8]. Proper orientation of integral membrane proteins is determined by topogenic sequences. These topogenic sequences include signal sequences, stop-transfer sequences, and internal, uncleaved signal-anchor sequences. Chaperones such as hsc70 and calnexin facilitate the folding of many nascent proteins including CFTR [9, 10]. Only properly folded proteins are transferred from the ER to the Golgi vesicles, and the misfolded polypeptides are degraded in the ER. Experimental evidence suggests that CFTR is a substrate for proteasomal degradation during its maturation within the ER [11].

Protein glycosylation reactions occur in both the ER and the Golgi. CFTR contains an N-linked oligosaccharide. All N-linked oligosaccharides are formed from a common precursor, and the attachment of this precursor oligosaccharide to specific asparagine residues of proteins occurs in the lumen of rough ER [12]. Immediately after the oligosaccharide is transferred, all three glucose residues are removed and additional modifications take place in the Golgi complex where CFTR attains a mature, fully glycosylated form. Nonclathrin-coated vesicles transport proteins from the ER to the Golgi and from one Golgi vesicle to the next [13]. A number of proteins are involved in the budding, targeting and fusion of transport vesicles. Mature and fully functional CFTR is packaged into vesicles destined for the plasma membrane.

Calnexin is a type I integral membrane protein embedded in the membrane of ER. Since calnexin is present exclusively in the ER and it has a C-terminus that protrudes into the cytosol, its cytosolic domain can therefore be used as a marker for isolating ER. As a chaperone, it associates with and facilitates the folding of many proteins including CFTR.
Several studies have confirmed that calnexin binds selectively to glycoproteins with monoglucosylated N-linked glycans [14, 15]. The objective of this thesis is to develop and characterize a monoclonal antibody against the cytosolic domain of calnexin, which could be used to immunoisolate ER. A method of rapid purification of ER would allow identification of protein components that interact closely with CFTR.

1.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

1.3.1 Structure of CFTR

One of the landmarks in CF research history was the identification of the gene responsible for the disease in 1989. The CF gene is located on chromosome 7, it consists of 27 exons, and it spans over 250 kb of genomic DNA [16, 17]. The protein product of the gene was named the cystic fibrosis transmembrane conductance regulator (CFTR).

The CFTR is made up of 2 repeated elements, each incorporating six putative transmembrane α-helices followed by a nucleotide binding fold (NBF) (see Fig. 1). The 2 elements are connected by a large, highly charged domain called the regulatory or R domain [5]. The structure of CFTR is homologous to a group of proteins known as the ATP-binding cassette (ABC) superfamily of transport proteins. Other members of this family include mammalian multi-drug resistance glycoprotein, and several other eukaryotic and prokaryotic proteins [17]. The R domain is a unique feature of CFTR that is not present in any other member of the ABC transporter superfamily [5].
Fig. 1. Model of cystic fibrosis transmembrane conductance regulator (CFTR).

(Adapted from [18].)

An overwhelming body of evidence has proved that CFTR is a Cl⁻ channel whose gating is regulated by PKA phosphorylation and ATP hydrolysis. Site-directed mutagenesis [19] and peptide-sequencing [20] studies have identified five serine residues in the R-domain as the major targets for phosphorylation by PKA. When these sites are phosphorylated by PKA and ATP is bound to NBF1, the channel opens and chloride ions move through according to the electrochemical gradient [17]. ATP occupancy of NBF2 in more fully phosphorylated channels is thought to delay channel closing, and cause longer openings. In other words, hydrolysis of ATP at NBF1 is required for channel opening while hydrolysis of ATP at NBF2 prompts channel closing [21].
1.3.2 AF508 Mutation

Over 600 mutations have been identified in the CFTR gene. Among all of the mutations identified, AF508 accounts for the majority of CF cases. AF508 results in the deletion of the phenylalanine residue at amino acid position 508 in the first NBF. The allele frequency of AF508 is about 0.7, therefore, 91% of patients have at least one chromosome bearing this mutation. AF508 leads to improper folding of the CFTR protein. Instead of being transported to the cell surface as the wild-type protein, the AF508 CFTR is trapped and degraded in the ER. Although this mutant protein undergoes abnormal posttranslational modification and intracellular transport, it seems to be capable of functioning as a Cl⁻ channel. Electrophysiological studies have been done in plasma membranes of Spodoptera frugiperda (Sf9) insect cells infected with a AF508 CFTR-baculovirus construct where, due to the lower incubation temperature of Sf9 cells (27°C), some of the AF508 CFTR traffics normally to the cell surface [7]. Since defective intracellular processing of CFTR is the molecular basis of most CF cases, it is very important to understand the pathway of CFTR biosynthesis.

1.4 Intracellular trafficking of integral membrane proteins

Integral membrane proteins are synthesized on ribosomes bound to the rough ER. The signal sequence present at the N-terminus of the protein directs the ribosome to the ER membrane, and initiates the transport of the growing polypeptide across the ER membrane. As the polypeptide is being translated, N-glycosylation and folding occur.
Each integral membrane protein has a unique orientation, and this is determined by its topogenic sequence. These proteins move to their final destinations, such as the plasma membrane or the membranes of other organelles via transport vesicles. During this transport, membrane proteins retain the same orientation. Improperly folded proteins are unable to exit the ER, and are destined to be degraded.

1.4.1 Protein translocation across the ER membrane

Blobel and Sabatini first proposed the signal hypothesis in 1971 to account for the binding of polysomes to the ER. A model showing the possible roles of SRP, SRP receptor, translocating chain-associated membrane protein (TRAM), and signal peptidase is illustrated in Fig. 2. The emerging signal sequence binds to the SRP [22], the SRP then binds to its receptor, the docking protein, and targets the nascent polypeptide from the cytoplasm to the ER membrane [23]. After the signal sequence dissociates from the SRP, the amino-terminus of the signal sequence binds to TRAM, and later the signal sequence is inserted into the translocon channel. During or immediately after translocation, the signal sequence is often cleaved from the nascent polypeptide by the peptidase-containing signal peptidase complex (SPC) [24].
1.4.2 Topogenic sequences determine the orientation of the protein in the membrane

It is known that correct orientation of eukaryotic integral membrane proteins is dictated by the actions of topogenic determinants such as signal, stop transfer, and signal anchor sequences [26]. Polytopic topology is achieved cotranslationally as these topogenic sequences emerge from the ribosome. Since topogenic determinants direct membrane integration, initiation and termination of translocation, any transmembrane orientation can be generated by combinations of signal, stop transfer, and signal anchor sequences.
Fig. 3. Structure of a typical cleavable amino-terminal signal sequence. The numbers indicate the number of amino acids (aa) in each domain. (Adapted from [25].)

A signal sequence is normally found at the N-terminus of a protein. It contains 1 to 5 positively charged amino acids, a continuous stretch of 7 to 17 hydrophobic residues in the center, followed by a 3 to 7 amino acid sequence before the site of signal sequence cleavage [27] (see Fig. 3). Type I membrane proteins, such as insulin receptor, have their amino termini facing the cell exterior or the lumen of intracellular organelles and their carboxyl termini in the cytosol (see Fig 4). The signal sequence targets the polysome to the ER membrane, and the nascent polypeptide is translocated through the Sec 61 complex as translation proceeds. The signal sequence is cleaved while the polypeptide is still growing, and the new N-terminus is extruded across the ER membrane into the lumen. Translocation of the nascent chain is terminated by a sequence of approximately 22 hydrophobic residues in the middle of the protein, and this sequence remains anchored in the membrane. Hence, it is named the stop-transfer sequence [28]. The C-terminus of the insulin receptor is not transferred, and remains in the cytosol. Experiments showed that the membrane-spanning α-helix of simple type I membrane proteins is a stop-transfer sequence.
Type I  Type
Single-span membrane  Multi-span membrane
proteins          protein

Fig. 4. Different types of intrinsic membrane proteins. (Adapted from [25].)

Type II intrinsic membrane proteins such as the asialoglycoprotein receptors have a cytosolic N-terminus and an exoplasmic C-terminus; these proteins do not utilize a cleaved N-terminal signal sequence, but an uncleaved internal signal-anchor sequence that is also the hydrophobic stop-transfer membrane-anchor sequence. Similar to the cleaved signal sequences, the N-terminus of the internal uncleaved signal sequence faces the cytosol. Thus, the N-terminus of the protein is cytosolic, while the C-terminus of the growing chain extrudes into the ER lumen [29].

Many important proteins, such as ion channels and transporters, span the membrane multiple times. The first α-helix is an internal uncleaved signal-anchor sequence, and the second α-helix is a stop-transfer sequence. Engelman and Steitz proposed that insertion of a polypeptide into a membrane was accomplished by a hairpin structure composed of two α-helical segments [30]. Accordingly, the next two helices insert into the membrane as a hairpin just as the first two do [10].
Lu et al. have recently identified two topogenic determinants encoded within the first and second transmembrane regions of CFTR. Each sequence is able to independently direct the nascent CFTR chains to the ER, translocate appropriate flanking residues, and achieve its proper membrane-spanning orientation [31]. Experiments have shown that the signal sequence activity of the first transmembrane segment (TM1) was inefficient and was able to direct correct topology for less than half of nascent CFTR chains. On the other hand, the TM2 signal sequence activity is more efficient, and is able to direct CFTR N-terminal topology when the TM1 signal sequence is not functioning. This finding has shed some light on alternative pathways of polytopic protein biogenesis.

1.4.3 Intracellular degradation of wild-type and ΔF508 CFTR

All of the ΔF508 CFTR and approximately 75% of the wild-type precursor that fail to mature are rapidly degraded with a half-life of 30 min. Recent studies indicate that the proteasome is involved in the ER degradation of the wild-type and ΔF508 CFTR [32]. Treatment with inhibitors of the cytosolic proteasome, including lactacystin [11] and the peptide aldehyde N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) [33], induces the accumulation of immature forms of CFTR. Moreover, CFTR was shown to be ubiquitinated [34]. Proteins modified by polyubiquitination are destined to be degraded in an ATP-dependent cytoplasmic proteolytic process. Co-expression of wild-type and ΔF508 CFTR with a dominant-negative ubiquitin mutant K48R inhibited the
ubiquitin pathway, and thus resulted in a significant accumulation of insoluble forms of CFTR.

Evidence has suggested that polyubiquitination serves as a signal leading to the degradation of wild-type or mutant CFTR that have exited the proper folding pathway. It is important to note that inhibitors of the proteasome and inhibitors of the ubiquitin pathway can only induce the accumulation of detergent-insoluble CFTR that has entered the ‘off-pathway’ process, but not the maturation-competent CFTR intermediate. Hence, inhibition of proteasome-mediated ΔF508 CFTR degradation is not likely to be an effective therapy for treating CF due to the ΔF508 mutation.

1.4.4 Protein glycosylation

Modification of the carbohydrate chains is often essential for a protein to fold correctly. Some glycosylation reactions occur in the ER, and others occur in the Golgi apparatus. N- and O-linked oligosaccharides have distinct structures and are composed of different sugar residues. For instance, the N-acetylgalactosamine in O-linked oligosaccharides is either joined to serine or threonine, while that in N-linked sugars is joined to asparagine. O-linked oligosaccharides are generally shorter and contain less sugar residues than N-linked oligosaccharides. N-linked and O-linked oligosaccharides are also synthesized differently. O-linked sugars are added one at a time, and each residue transfer is catalyzed by a different enzyme [35]. On the other hand, the biosynthesis of all N-linked oligosaccharides begins with a large precursor oligosaccharide. This
oligosaccharide is preassembled on a long-chain polyisoprenoid lipid called dolichol, and is transferred \textit{en bloc} to an asparagine residue on the nascent polypeptide by an ER enzyme [12]. Once attached, 3 glucose residues and 1 mannose residue are removed. The final modifications to the N-linked oligosaccharides take place in the Golgi apparatus. Different enzymes in the cis-, medial, and trans-Golgi act sequentially on the protein to produce complex, high-mannose, or hybrid oligosaccharides [36] (see Fig. 5).

![Diagram](image)

**Fig. 5.** Structure of various classes of N-linked oligosaccharides. (Adapted from [13]).
All N-linked oligosaccharides are formed from a common precursor which consists of 3 mannose and 2 N-acetylglucosamine residues. Sequential removal and addition of certain sugar residues leads to the formation of various classes of N-linked oligosaccharides. *N-linked complex oligosaccharides* contain N-acetylglucosamine, mannose, fucose, galactose, and N-acetylneuraminic acid. A second class, the *N-linked high-mannose oligosaccharides*, contain only N-acetylglucosamine and mannose [37]. The third class comprises the *hybrid N-linked oligosaccharides*, in which one of the branches has a typical complex structure while the other two contain only mannose [38].

### 1.4.5 Two types of coated vesicles responsible for protein transfer between organelles

There are two types of coated vesicles that transport proteins from one organelle to another. One type has a coat of clathrin on its cytosolic surface, and is formed from the plasma membrane or the trans-Golgi. The other type has a coat made of a different set of proteins, and they transport proteins from the ER to the Golgi and from one set of membranes in the Golgi to another. The two types of protein coats serve two common purposes. First, polymerization of the coat proteins on the cytosolic face of a budding vesicle helps the vesicle to pinch off from the parent organelle. Second, the coat proteins assist in selecting which membrane proteins can enter the transport vesicles.

Purified clathrin has a three-limbed triskelion structure. Each limb contains one clathrin heavy chain of molecular weight 180,000 and one clathrin light chain of molecular weight
weight 35,000 to 40,000 [39, 40]. Clathrin polymerizes into a lattice along the cytosolic face of a membrane region to form a clathrin-coated pit, and eventually pinches off from the membrane. Between the clathrin coat and the vesicle membrane, there is a 20 nm space that contains assembly particles. These assembly particles bind to the globular domain of the clathrin heavy chain, and promote the polymerization of clathrin triskelions into cages [13]. Because the assembly particles also bind to the cytosolic face of membrane proteins, they determine which proteins are included in or excluded from the budding transport vesicle. A cytosolic chaperone protein hsc70 is found to catalyze the depolymerization of the clathrin coat into clathrin triskelions, thus allowing the recycling of clathrin [41]. The hydrolysis of ATP is required in this process.

Vesicles that were not coated with clathrin were found to contain a coat composed of α cytosolic coat protein (αCOP). αCOP forms a fibrous outer shell around membrane vesicles [42]. Like the clathrin coat, the coat made with αCOP also promotes budding of vesicles. Between the αCOP coat and the vesicle membrane lies a set of assembly particles. As in clathrin-coated vesicles, these assembly particles act as a bridge between the cytosolic ends of membrane proteins and the fibrous cage that surrounds the vesicles [13]. Specific incorporation of proteins into αCOP coated vesicles is also mediated by these assembly particles.
1.4.6 Vesicle Budding and Fusion

Several proteins are required for the formation of the transport vesicles. ADP-ribosylation factor (ARF) is a GTP-binding protein in the cytosol. Budding is initiated when ARF proteins are induced to exchange their bound GDP for GTP, and then bind to ARF receptors on Golgi membrane [43]. αCOP, βCOP, γCOP, together with four other proteins that coat nonclathrin-coated vesicles, are found in the cytosol of eukaryotic cells as a complex called a coatomer [44]. It is the binding of coatomer to the cytosolic face of the Golgi membrane that induces budding of transport vesicle. Fatty acyl CoA is essential for the final fission that creates a completed transport vesicle, however, its mechanism of action is unknown [45].

Several proteins are involved in the fusion of transport vesicles with acceptor Golgi membrane. In the first step, a vesicle associated membrane protein (VAMP)-like protein in the transport vesicles and a syntaxin-like protein in the acceptor Golgi vesicles bind to each other. N-ethylmaleimide Sensitive Factor (NSF) mediates the fusion of the transport vesicle membrane with the membrane of the acceptor medial-Golgi vesicle [43]. NSF is so named because it is uniquely sensitive to low concentrations of N-ethylmaleimide in the in vitro cis to medial Golgi transport assay [46]. Soluble NSF Attachment Protein (SNAP) acts in close conjunction with NSF in vesicle fusion [47, 48]. Each of the α-, β-, and γ-SNAP proteins alone is sufficient to bind NSF to Golgi membranes [49]. The binding and release of SNAP and NSF from membranes is coupled to a nucleotide hydrolysis cycle [50].
1.4.7 Rab proteins target transport vesicles to their correct destinations

Rab is a family of GTP-binding proteins that participate in vesicular trafficking [51]. Studies indicate that individual rab proteins localize exclusively to a different organelle in the secretory or endocytic pathways [52] (see Fig. 6). After a rab protein has exchanged its bound GDP for a GTP, it undergoes a conformational change. During this time, it binds to a surface protein on a particular transport vesicle just as it buds off from a donor vesicle. The rab-GTP complex then facilitates the binding of transport vesicle to its proper acceptor organelle [13]. Once vesicle fusion is initiated, hydrolysis of GTP occurs and triggers the release of the Rab protein from the membrane.

Several lines of evidence suggest that rab1 and rab2 are involved in transport between the ER and Golgi. The rab1 protein has been localized to ER and Golgi compartments in tissue culture cells by both immunofluorescence and cell fractionation studies [53]. Experimental findings indicate that rab1 acts prior to vesicle fusion, and is probably involved in vesicle formation or targeting. Rab5 is localized to early endosomes that are formed from clathrin-coated vesicles just after they bud from the plasma membrane. Much information about the important proteins required for vesicle-targeting reactions remains to be discovered.
Fig. 6. Subcellular location of Rab proteins in mammalian cells. Each rab protein is found in a discrete transport vesicle, and is thought to participate in directing that transport vesicle to the appropriate acceptor organelle. (Adapted from [13].)
1.5 Biosynthesis of Wild-type and ΔF508 CFTR

Newly synthesized proteins must undergo several changes in the rough ER before they can be transported to the Golgi vesicles. These changes include the formation of disulfide bonds, cis-trans prolyl isomerization, chaperone-mediated folding of the protein, addition of carbohydrates, and specific proteolytic cleavages. These modifications are essential to ensure that only correctly folded and assembled proteins are transported from the ER to the Golgi complex and to the cell surface.

As with other integral membrane proteins, CFTR is synthesized on the ribosomes bound to the rough ER, and the nascent polypeptide is inserted into the ER membrane by the well-defined SRP insertion mechanism [22]. Two N-linked oligosaccharide chains are added to both the wild-type and ΔF508 CFTR as they are synthesized. Molecular chaperones bind to the growing chain cotranslationally, and prevent denaturation, aggregation, or inappropriate folding of the polypeptide. Calnexin [54], a transmembrane ER chaperone, and heat shock protein 70 (hsc70) [54, 55], a cytosolic chaperone, have been observed to associate transiently with the immature CFTR. As part of the quality control mechanism, molecular chaperones exhibit prolonged binding to misfolded or incompletely folded proteins, thus preventing further trafficking of these proteins. Strickland et al. have demonstrated that hsc70 interacts with NBF1 of CFTR to increase the folding yield of the domain and inhibit the formation of aggregates. There is also evidence which shows that hsc70 may act on a later folding intermediate instead of proteins in their early stages of folding [56]. Only the properly folded wild-type CFTR
is packaged into transport vesicles, and is carried to the Golgi complex. The N-linked oligosaccharides on the protein are modified in the Golgi vesicles (see Fig. 7). The complete glycosylated CFTR is then transported to the plasma membrane where it functions as a regulated Cl⁻ channel.

Pathway of CFTR synthesis

Fig. 7. Both the wild-type and ΔF508 CFTR are partially glycosylated in the ER. However, only a fraction of the wild-type protein undergoes an ATP-dependent process and attain protease-resistance. All of the ΔF508 CFTR is degraded by proteases in the ER. The wild-type CFTR that has escaped from the ER is processed in the Golgi apparatus, and is then transported to the plasma membrane.

At their earliest stages of the biosynthetic pathway, the carbohydrate moieties of both the wild-type and ΔF508 CFTR are susceptible to endoglycosidase H cleavage.
Because the mutant protein is unable to attain a protease-resistant conformation, ΔF508 CFTR is rapidly degraded with a half-life of 0.5 h [6]. The undegraded ΔF508 CFTR migrates as a doublet of Mr 135 and 145 kDa on an immunoblot (see band B in Fig. 8). Both bands represent core glycosylated CFTR, but the difference in molecular weight is due to an alternate translational start site in the CFTR gene. On the other hand, about 25% of the wild-type protein undergoes an ATP-dependent process to attain an endo H-resistant conformation [6]. The wild-type CFTR is then transported to the Golgi apparatus where the carbohydrate is processed to the complex form. It then migrates with a Mr of about 165 kDa in SDS-PAGE, and is referred to as mature CFTR, or band C in some studies (see Fig. 8). The mature protein is more stable than its immature precursor, and has a half-life greater than 8 h.

**Wild-type and ΔF508 CFTR**

![Immunoblot](image.png)

**Fig. 8.** RIPA detergent extracts of BQ2 and BQΔF cells were analyzed by SDS-PAGE and immunoblotting and were probed with anti-CFTR mAb M3A7. Lane 1: RIPA extract of BQ2 cells; lane 2: RIPA extract of BQΔF cells. The positions of bands B and C are indicated on the right margin.
1.6 Chloride channel activity of ΔF508 CFTR

Kartner and colleagues have confirmed in situ biosynthetic arrest of ΔF508 CFTR in epithelial cells of patients homozygous for the ΔF508 mutation [5]. However, this mutation does not severely affect the function of CFTR. The ΔF508 CFTR expressed using baculovirus in Sf9 insect cells does not undergo biosynthetic arrest. This provides a unique opportunity to assess the functional activity of the mutant protein using patch clamp electrophysiology. Plasma membrane ΔF508 CFTR is expressed at high levels in Sf9 cells because the cells are grown at low temperature (27 °C). Li et al. have reported that ΔF508 CFTR is able to function as a Cl⁻ channel, and its activity is very similar to that of wild-type CFTR [7]. This indicates that the cause of CF due to ΔF508 mutation is not that the mutant CFTR is unable to function, but that it fails to arrive at its correct location. If ΔF508 CFTR can be made to localize to the plasma membrane, then over 90% of CF patients could be cured. It has been reported that the maturation and trafficking of ΔF508 CFTR to the plasma membrane can be promoted at reduced temperature [57], and by growing cells in the presence of glycerol [58]. Protein folding mechanism is temperature sensitive [59], while glycerol is known to stabilize protein conformation [60] and increase the rate of in vitro protein refolding [61]. It has been suggested that an intermediate in CFTR biosynthesis is stabilized under these conditions, and is maintained in a conformation that is competent for native folding [58].
Unfortunately, these two methods are not clinically useful, but they do suggest that overcoming the ΔF508 CFTR processing defect may be feasible.

1.7 Calnexin

1.7.1 Discovery of calnexin

Degen and Williams discovered that newly synthesized class I major histocompatibility (MHC) molecules transiently associated with an 88 kDa protein (termed p88) in murine lymphoma cell lines [62]. MHC class I molecules are cell surface glycoproteins that contain three subunits: a transmembrane heavy chain (45 kDa), a soluble polypeptide called β2-microglobulin (β2m) and a peptide of about 8 to 10 amino acids arising from the degradation of cellular or foreign proteins. Incompletely assembled MHC class I molecules in mutant cells that lack either β2m or peptide ligand exhibit prolonged association with p88. Transport of these incompletely assembled MHC class I molecules to the Golgi apparatus was also impaired [63]. These experimental findings implied that p88 is a molecular chaperone.

At the same time, Wada et al. identified four membrane proteins in the ER-enriched canine microsomes that could be phosphorylated in vitro using [γ-32P] GTP [64]. One of them was a phosphoprotein of 90 kDa. By immunofluorescence microscopy, this phosphoprotein was shown to be localized to the ER. Isolation and sequencing of the corresponding cDNA predicted a 573 amino acid type I integral membrane protein. It has a 460-residue luminal domain, a single transmembrane sequence, and a cytosolic domain of
about 90 amino acids (see Fig. 9) [64]. At the extreme carboxyl terminus lies the sequence RKPRRE. An arginine at position –3 and a lysine at position –5 from the C-terminus is similar to motifs that have been hypothesized to play a role in the ER localization of other type I membrane proteins [65]. The luminal domain closely resembles calreticulin, the major calcium binding protein of the ER. By carrying out calcium overlay experiments, Wada et al. proved that calnexin binds calcium. Due to its resemblance to calreticulin, this 90 kDa phosphoprotein was named calnexin [64].

Fig. 9. Topology of mammalian calnexins. The unfilled boxes represent segments that share high sequence similarity (42-78%) with calreticulin. The numbers 1 and 2 represent the repeated motifs IXDP(D/E)(A/D)XKP(E/D)DWD(D/E) and GXWXXPXIXNPXY, respectively. An ER-localization sequence (RKPRRE) is present at the carboxyl terminus.
A human protein, IP90, was found to associate with incompletely assembled forms of the T-cell receptor, the B-cell antigen receptor, and MHC class I molecules [66]. Immuno-electron microscopy localized IP90 to membranes of the ER and nuclear envelope. Subsequent cDNA cloning demonstrated that mouse p88 and human IP90 are homologues of dog calnexin [67].

1.7.2 Interaction between calnexin and newly synthesized proteins

Bergeron and colleagues first demonstrated that glycoproteins selectively associated with calnexin. Treating cells with tunicamycin (an N-linked glycosylation inhibitor) prior to immunoprecipitation with anti-calnexin antiserum results in a profound reduction in the level of complexes between calnexin and newly synthesized glycoproteins [68]. Other studies have shown that inhibitors of oligosaccharide-processing enzymes, glucosidases I and II (castanospermine and 1-deoxynojirimycin, respectively) prevent association of glycoproteins with calnexin [14]. These findings suggested that calnexin is a lectin and that it binds selectively to glycoproteins with monoglucosylated N-linked glycans. However, several studies indicated that proteins devoid of glycans also associate with calnexin. For example, the T-cell receptor ε subunit that does not possess any N-linked oligosaccharides [69], and the multidrug-resistance P glycoprotein with all N-linked glycosylation sites removed [70] were still able to form complexes with calnexin.
Ware et al. used a more direct approach to determine whether calnexin functions as a lectin. The ER luminal domain of calnexin was expressed in Drosophila cells. This soluble calnexin was purified and then incubated with a mixture of radiolabeled oligosaccharides consisting of Glc$_{0,3}$Man$_9$GlcNac$_2$. Calnexin was found to bind exclusively to the monoglucosylated oligosaccharide (Glc$_1$Man$_9$GlcNac$_2$) [71]. Calnexin appears to be a novel type of lectin because primary sequence comparisons indicate that calnexin does not have the carbohydrate-recognition domains normally present in the three major groups of animal lectins, that is the Ca$^{2+}$-dependent C-type lectins, the S-type or galectins, and the P-type or mannose-6-phosphate lectins [71]. Surprisingly, removal of oligosaccharides by endo H after the glycoproteins are bound to calnexin did not cause dissociation of the proteins from calnexin [15]. It was then speculated that the association of calnexin and carbohydrate yields little or no contribution to the binding interaction once stable complexes are formed.
Fig. 10. A model for the association of calnexin and calreticulin with monoglucosylated glycoproteins. After two glucose residues are removed, the monoglucosylated glycan is a substrate for calnexin and calreticulin. Newly synthesized glycoproteins contact calnexin initially via their monoglucosylated oligosaccharide chains. Once it has been placed in proximity to calnexin, the unfolded polypeptide associates directly with calnexin. Glucosidase II may cleave the remaining glucose residue, and produce a deglucosylated glycoprotein which is no longer a substrate for calnexin or calreticulin. Reglucosylation by UDP-glucose: glycoprotein glucosyltransferase facilitates rebinding of unfolded polypeptide to calnexin or calreticulin. (Adapted from [72])
It was proposed that the release of substrate from calnexin requires the removal of the final glucose by glucosidase II, and that the glycoproteins devoid of glucose can undergo reglucosylation by the UDP-glucose:glycoprotein glucosyltransferase (glucosyltransferase) [72, 71]. The preferred substrates of glucosyltransferase are deglucosylated glycoproteins that possess non-native conformations [73]. Reglucosylation may be important in allowing rebinding to calnexin if a glycoprotein dissociates from calnexin before it is completely folded. Interestingly, calreticulin was also shown to associate specifically with monoglucosylated glycoproteins [72]. Calreticulin is likely to play a role in the quality control pathway proposed for calnexin (see Fig. 10). Immunoprecipitation experiments revealed that populations of cellular proteins precipitated with calnexin or calreticulin were very similar but not identical. Although both calreticulin and calnexin associate with monoglucosylated glycoproteins, accessibility of oligosaccharides to either the membrane-bound calnexin or the luminal calreticulin plays a role in dictating substrate preference.
2 Materials and Methods

2.1 Generation of GST-calnexin fusion protein

2.1.1 Generation of cDNA for fusion protein expression

Much of the characterization of CFTR biogenesis and trafficking has been done in heterologous expression system, especially in the CHO system. Future work was intended to be done using the well characterized CHO cell lines BQ2 and BQΔF, and thus we were interested in developing an antibody specifically directed to Chinese hamster calnexin. Since the Chinese hamster calnexin sequence was not known prior to the start of these experiments, human primers were designed to bind to regions of highest homology among other calnexin species. cDNA corresponding to the region from the last 7 amino acids (aa) of the transmembrane (TM) domain to the end of the C-terminus was obtained by PCR using the primers shown in Fig. 11. The PCR product was expected to contain approximately 290 bp.

\[ \text{a) Eco RI} \quad 2 \text{ Gly} \quad \text{the last 7 aa of the TM region} \]
\[ \text{AAGAATTCAAGCGGTATCCTCTTCTGCTGTTCTGGA} \]

\[ \text{Eco RI} \quad \text{the last 6 aa of the C-terminus} \]
\[ \text{CGGAATTCACTCTCTCGTGGCTTTCT} \]

\[ \text{stop codon} \]

Fig. 11. Sequences of primers used to generate the calnexin cytoplasmic domain. a) Upstream primer  b) Reverse complement sequence of downstream primer.
An Eco RI site was engineered into both primers, and the bases encoding two glycine residues were also added to the upstream primer. Since the cDNA would be cloned into pGEX-3X, which is a GST gene fusion vector, the two glycine residues would function as a flexible spacer to separate GST from calnexin. The two adenosine bases in front of the Eco RI site would become part of the sticky end after the PCR product was cleaved with the enzyme. The adenosine base in between the Eco RI site and two glycine residues was added so that the DNA could be translated in the correct reading frame. As with the upstream primer, two extra bases were also added to the downstream primer to create a sticky end after cleavage with Eco RI. A stop codon was included in the downstream primer to ensure the termination of translation. The lengths of the primers were adjusted so that the melting temperatures of the region that binds the calnexin cDNA were approximately 60°C.

Total RNA was isolated from BQ2 cells (Chinese hamster ovary cells transfected with pNUT expression vector containing wild-type CFTR cDNA) [74] using Trizol reagent (GIBCO/BRL) according to instructions provided by the supplier. The RNA isolated was then treated with DNase I (FPLC pure, Pharmacia Biotech), and the concentration of total RNA was determined by measuring the absorbance at 260 nm.

cDNA was transcribed using 1 µg of total RNA, 1 µg of oligo(dT) 12-18 primer, and 200 units of Superscript™ II under conditions provided with the enzyme. The cDNA was amplified in a polymerase chain reaction (PCR) with a Perkin Elmer GeneAmp PCR system 2400. Twenty-five cycles of PCR were performed under the
following conditions: 94° C for 40 seconds, 55° C for 1 min., 72° C for 2 min. 1 µg of each of the primers, 3 mM MgCl₂, 0.4 mM dNTP mixture, 5 units Platinum Taq DNA polymerase were used in the reaction. All reagents used in reverse transcription and PCR were purchased from GIBCO/BRL. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen).

The PCR product was then cleaved with Eco RI, and purified by phenol-chloroform extraction [75]. The pGEX-3X vector was also cut with Eco RI, and the phosphate groups at both ends were removed using calf intestine phosphatase to prevent re-ligation of the vector [75]. The ligation reaction was carried out overnight at 16° C using T4 DNA ligase (GIBCO/BRL).

2.1.2 Expression of GST-calnexin fusion protein

The constructs were transformed into *E. coli* SURE™ cells via electroporation. The electro-competent cells were prepared as follows: 1 litre of LB medium (10 g/l bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0) was inoculated with 5 ml of fresh overnight culture. The cells were grown at 37° C in a shaker incubator at 225 rpm until the A₆₀₀ was 0.5 to 0.7. To harvest, the cells were chilled on ice for 15 to 30 minutes, and centrifuged at 4000g for 15 min. Cell pellets were resuspended in a total of 1 litre of ice-cold, sterile 10% glycerol, and then centrifuged as above. This step was repeated twice, but the cell pellets were resuspended in different volumes. First in 0.5 l, and then in 20 ml. In the final step, cell pellets were resuspended in 2 to 3 ml of ice-cold 10% glycerol.
The cell suspension was then frozen in 40 μl aliquots, and stored at -70°C. In the transformation process, an aliquot of electro-competent cells was incubated with the expression vector for 2 min on ice. Electroporation was conducted at 25 μF, 1.8 kV, 200 Ω in a 0.1 cm cuvette. Immediately after electroporation, 1 ml of SOC medium (20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM MgCl₂, 5% glucose, pH 7.0) was added to the cuvette. The cell suspension was transferred to a 17 x 100 mm polypropylene tube, and shaken at 225 rpm in a 37°C incubator for 1 h. The transformed cells were then spread on a LB plate (15 g/l agar) containing 50 μg/ml ampicillin.

Colonies on the agar plate were inoculated into 5 ml of LB containing 50 μg/ml ampicillin, and were allowed to grow overnight at 37°C at 225 rpm. Bacteria from the saturated culture were lysed, and plasmid DNA was isolated using a Qiagen miniprep 8 kit. Since the ends of both the insert and the vector were cut with only one enzyme, the insert could be ligated into the vector in two different directions. Restriction digestion was conducted to ensure that the bacterial colonies chosen contained construct with correctly oriented insert. The construct was then sequenced and the Chinese hamster sequence was aligned with calnexin sequences of other species using a computer program called Lasergene (DNASTar).

Before the fusion protein could be purified, it was important to determine the solubility of the protein. 5 ml of fresh overnight bacteria culture expressing the GST-calnexin fusion protein was inoculated into 50 ml of LB containing 50 μg/ml ampicillin.
After growing for 1 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM. Cells were harvested after a further growth of 3 h. The pelleted cells were resuspended in 1 ml of MTPBS (150 mM NaCl, 16 mM Na$_2$HPO$_4$, 4 mM NaH$_2$PO$_4$, pH 7.3), and sonicated for 1 min on ice. After adding Triton X-100 to 1%, the lysed cells were centrifuged at 10,000g for 5 min at 4°C. The supernatant and the pellet were analyzed by SDS-PAGE on a 10% polyacrylamide gel. In another experiment, IPTG concentration and induction time were varied to determine the optimal induction conditions.

The procedure for SDS-PAGE was based on the classic discontinuous gel system of Laemmli [76] with minor modifications. Gel electrophoresis was carried out in a Mini-Protean II gel apparatus (Bio-Rad) at 150 V constant voltage for approximately 1 h. The separated proteins were transferred to nitrocellulose filter paper by the method of Towbin et al. [77]. The nitrocellulose blot was then probed with the commercial polyclonal calnexin antibody (Stratagene) and processed following the instructions provided with the Amersham ECL system.

2.1.3 Purification of GST-calnexin fusion protein

Since the fusion protein was soluble, it was present in the supernatant of crude bacterial lysate. The GST-calnexin fusion protein was purified using glutathione-agarose beads (Sigma) according to the protocol described by Smith and Johnson [78]. Supernatant from 800 ml of bacterial culture was mixed with 1-2 ml of 50 % glutathione
beads for 2 min. The beads were then briefly centrifuged at 500 g and washed three times with 50 ml MTPBS. Fusion protein was eluted twice with 1 bed volume of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione (final pH 7.5). Glutathione-agarose beads were washed thoroughly after use, and recycled for purification of the same fusion protein. A rough estimate of the yield was calculated by measuring the absorbance at 280 nm. (1 AU280 = 0.5 mg/ml). Modified micro Lowry protein assays [79] were later conducted to determine the concentration of the protein. If the fusion protein was prepared for immunizing mice, then it was concentrated in Centricon 10 centrifugal ultrafiltration units (Amicon) so that the concentration was at least 2 mg/ml.

2.2 Immunization Schedule

Female BALB/c mice, 12 to 16 weeks old were immunized with GST-calnexin fusion protein according to the immunization schedule in Table 1. To prepare the inoculum, 2 mg/ml purified fusion protein was mixed with an equal volume of Freund’s adjuvant, and the mixture was emulsified by passage between syringes. All injections were i.p. except the last one. In the final injection, the fusion protein was diluted to 1 mg/ml using PBS, sterile filtered through a 0.22 μm microcentrifuge filter unit, and injected i.v. via the tail vein.
Table 1. Immunization Schedule

<table>
<thead>
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<th>Time (days)</th>
<th>Amount (µg)</th>
<th>Route of immunization</th>
<th>Freund's adjuvant / PBS</th>
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<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>i.p.</td>
<td>Complete adjuvant</td>
</tr>
<tr>
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<td>100</td>
<td>i.p.</td>
<td>Incomplete adjuvant</td>
</tr>
<tr>
<td>42</td>
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<td>i.p.</td>
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<tr>
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<td>i.p.</td>
<td>Incomplete adjuvant</td>
</tr>
<tr>
<td>63</td>
<td>100</td>
<td>i.v.</td>
<td>PBS</td>
</tr>
</tbody>
</table>

2.3 Hybridoma Production

The partner cells used in the fusion experiment were Sp2/0-Ag14 (Sp2) [80, 81] murine myeloma cells. To ensure that these cells were deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT), they were grown in 20 µg/ml 8-azaguanine (8-AG) before they were frozen for long-term storage. Sp2 cells were normally maintained in RPMI 1640 (GIBCO/BRL) plus 8% fetal bovine serum (FBS) (Sigma). Twenty-four hours before fusion, Sp2 cells were grown in complete medium. Composition of the complete medium was as follows: 250 ml Opti-MEM (GIBCO/BRL) + 250 ml RPMI 1640 (GIBCO/BRL) + 35 ml FBS (Sigma) + 35 ml CPSR-3 (Sigma) + 25 ml horse serum (Sigma) + 2 mM glutamine (Sigma) + 1X antibiotic/antimycotic mix (100 units penicillin + 100 µg streptomycin + 250 ng amphotericin B per ml, Sigma). On day 67, the immunized mice were killed by cervical dislocation and their spleens were removed.

The spleen cells were mixed 3:1 with Sp2/0-Ag14 cells, and fused according to the method described by Galfre [82] using 50% polyethylene glycol (PEG, molecular weight
1000) plus 10% DMSO. The splenocytes and the myeloma cells were washed twice by centrifugation at 400g in Opti-MEM. One ml of PEG/DMSO mixture was slowly added to the cell pellet over a period of 1 min while resuspending the cells by stirring with the end of a pipet. After stirring for an additional minute, 10 ml of Opti-MEM was slowly added to the cell suspension over 2 min. The cells were then centrifuged at 400g for 5 min. The supernatant was discarded and the fused cells were seeded in HT medium, and incubated for 12 to 18 h at 37°C and 8% CO₂. Composition of HT medium was as follows: complete medium + 1X HT mix (1 x 10⁻⁴ M hypoxanthine + 1.6 x 10⁻⁵ M thymidine, Sigma) supplemented with 10% hybridoma enhancing supplement (Sigma) and 1X OPI (0.15 g oxalacetic acid + 0.05 g sodium pyruvate + 0.0082 g bovine insulin per litre, Sigma). To select hybrid cells, aminopterin was added to 1.0 μM. There were approximately 2.5 x 10⁵ myeloma cells per well before selection, and typically four 96-well plates were seeded per spleen. Supernatants from wells containing hybridoma cells were collected and screened for specific antibody production.

2.4 Screening of hybridoma cells

2.4.1 Dot blot assay

An array of 0.5 cm by 2.5 cm rectangles was marked on sheets of nitrocellulose, and each rectangle was numbered according to the well to be screened. 1 μl of 0.5 mg/ml GST-calnexin fusion protein in 1% SDS solution was spotted near the left end of each rectangle, while the same amount of GST protein was spotted to the right of the fusion
protein. The spots were dried for 20 min, and the sheets of nitrocellulose were then soaked in transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol) for 15 min and blocked with 5% skim milk (Carnation) in PBST for 1 h. The nitrocellulose strips were cut out and inserted individually into wells of 96 deep-well microtiter plates (Beckman) each containing 900 μl of 5% skim milk/PBST and 100 μl of hybridoma supernatant. Incubation with the primary antibody was done on a nutator and it lasted for 2 h. The strips were then washed and incubated with horse radish peroxidase-labeled goat anti mouse secondary IgG, diluted 1:5000 in 5% skim milk/PBST, for 1 h. After washing out the secondary antibody, the nitrocellulose strips were arranged on a plastic sheet, secured with Scotch tape, and developed using ECL detection (Amersham).

2.4.2 Enzyme-linked immunosorbent assays (ELISA)

Supernatants from the hybridoma cells were screened again using ELISA. Each well of the ELISA plate (Immulon® 3, Dynatech Laboratories, Inc.) was coated with 2.5 μg of GST-calnexin, or GST protein, diluted in 50 μl of 50 mM sodium carbonate, pH 9.0. ELISA was performed as described [83], using ABTS (2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diaminonium) as the substrate.
2.4.3 Detection of calnexin in fusion protein and CHO cell lysate using hybridoma cell supernatant

To ensure that the hybridoma clone chosen was able to secrete antibody specific to calnexin, supernatant from that clone was used to probe for calnexin in the cleaved fusion protein and in CHO cells. 8 μg of GST-calnexin fusion protein was incubated with 0.8 μg of Factor Xa in 10 μl of buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM CaCl₂) for 2 h at room temperature.

BQ2 cell lysates were prepared by washing the monolayers twice with ice-cold PBS and solubilizing the membrane for 10 min on ice using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) supplemented with a protease inhibitor cocktail (1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprtinin, 3 μg/ml antipain, 1 μg/ml pepstatin, 0.4 mM PMSF). The cleaved fusion protein and BQ2 cell lysate were analyzed by SDS-PAGE electrophoresis followed by Western blotting as described in section 2.1.2.

2.5 Production and purification of ascites fluid

The hybridoma cell chosen was recloned once by limiting dilution to ensure that it was monoclonal, and to select for a clone that produced a higher antibody titer. Hybridoma cells were counted carefully using a hemacytometer after mixing cells 1:1 with 0.5% Nigrosin/PBS to stain non-viable cells. Viability by dye-exclusion was typically > 95%. 30, 10, 3, or 1 cell(s) were seeded per plate. Growth of cells was monitored on a
daily basis to ensure that clones came from a single cell. The clones obtained by limiting dilution were screened again using ELISA and dot blot assays. The best clone was named 1H82.

Female BALB/c mice, 12 weeks old were primed by an i.p. injection of 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane). Two weeks later, $5 \times 10^6$ hybridoma cells suspended in 0.5 ml PBS were injected i.p. The cells were in log-phase and had a viability > 95%. Ascites fluid was drained when the mouse had swollen to about the size of a pregnant female near term. The time taken for the ascites fluid to develop varied, and the animals were under constant observation for any sign of discomfort. Tapping of ascites fluid was performed on a mouse killed by cervical dislocation and disinfected with 70% ethanol. After exposing the peritoneal wall, a Pasteur pipette was inserted into the cavity and was used to collect ascites fluid.

The ascites fluid harvested often contained blood. The fluid was incubated at 37°C for 1 h, and then transferred to 4°C overnight. After centrifuging the ascites at 3000g for 10 min, the layer of pristane was removed and the cell pellet was discarded. The ascites fluid was then heat inactivated at 56°C for 30 min. To ensure that the pristane was completely removed, the ascites fluid was passed through a glass wool column in a Pasteur pipette.

The ascites fluid was passed onto a protein G column, and the bound fraction was eluted with 50 mM MES in 1% acetic acid. The eluate was then neutralized with 2 M Tris base, and exchanged into 20 mM Tris-HCl, pH 7.6 on a PD-10 desalting column.
(Pharmacia). Other contaminating IgG isotypes were removed by passage over a protein A column. The flow-through was collected and loaded onto a Mono Q column (Pharmacia). The bound fractions were eluted with a linear gradient starting in 20 mM Tris-HCl, pH 7.6 and ending in 0.3 M NaCl, 20 mM Tris-HCl, pH 7.6.

2.6 Characterization of antibody

2.6.1 Isotyping of antibody

Isotyping of the antibody was performed using the IsoDetect™ Mouse Monoclonal Antibody Isotyping kit (Stratagene). 5 µl of tissue culture supernatant was diluted into 495 µl of sample diluent, and the diluted supernatant was slowly added to the sample well on an IsoDetect test card. After several minutes, pink lines appeared in the reading window of the test card next to the kappa or lambda designation, and next to one of the antibody classes or subclasses.

2.6.2 Determination of antibody affinity

An ELISA based method was employed to determine the affinity of the antibody. The assay was conducted in 96 well plates with 0.45 µm mixed cellulose esters membrane filter bottoms (Millipore multiscreen assay system). Solutions in the wells were emptied through the filter bottom using a vacuum manifold. The GST-calnexin protein was diluted in TBS to various concentrations ranging from 1 ng in 50 µl to 64 µg in 50 µl, and was added to different wells. Like all incubation steps carried out in this assay, the incubation
was done at room temperature for 1 h. The plate was then washed twice with TBS, and excess binding sites were blocked with 2.5% skim milk. After washing, 1 µg/ml of purified antibody diluted in 2.5% skim milk was added to all wells, and allowed to incubate. The wells were then washed 4 times with TBS. Approximately 0.1 µCi of ¹²⁵I-labelled sheep anti mouse antibody was added per well. After incubation with the radiolabelled secondary antibody, the plate was washed five times using TBS. The membrane filters were then punched out, and counted in a gamma counter.

A concentration of GST-calnexin protein was selected from the above experiment such that its reading was about 50% of the maximum. An experiment similar to the one above was then performed. All wells were coated with the same concentration of GST-calnexin protein, but incubated with different concentrations of 1H82 antibody, ranging from 10 ng/ml to 64 µg/ml. To measure the non-specific binding, an equal number of wells was coated with 1 µg of GST, and were processed in parallel with the GST-calnexin coated wells. A saturation curve was fitted to the data that represented specific binding, and the data were also analyzed in a Scatchard plot. A $K_A$ value was derived. Three samples were done at each antibody concentration, and the experiment was repeated three times.

Models with one and two binding sites were fitted to data using a computer program called Prism. Prism fits equations by non-linear regression. It starts by making an estimate of the variables in the equation, and then calculates the sum of squares of the vertical distances between the data point and the curve. By changing the variables, it
minimizes the sum of squares. Prism repeats this process until the sum of squares changes by less than 0.01% in three consecutive iterations. To determine which equation is more appropriate for the data, Prism calculates the sums of squares of both equations and perform an F test. The F test calculates a P value that answers this question: If the one site model is correct, what is the chance of randomly obtaining data that fits the two site model so much better?

Affinity was also assessed by the IC$_{50}$ value in inhibition assays. In this assay, the wells were coated with the same concentration of GST-calnexin protein, and probed with a fixed amount of antibody that was pre-incubated with various concentrations of GST-calnexin fusion protein. The data were then plotted, and an IC$_{50}$ value was obtained. Again, three samples were done at each antibody concentration, and the experiment was repeated three times. Models with one and two competition sites were fitted to the data, and an F test was performed to decide which model was more appropriate. A K$_D$ was calculated from the IC$_{50}$. A two-tailed t-test was performed to determine if the K$_D$ values obtained from the saturation binding experiments were significantly different from those obtained from the competition experiments.

2.6.3 Epitope mapping

As mentioned in section 2.1.1, Chinese hamster calnexin cDNA corresponding to the region from the last 7 amino acids of the transmembrane domain to the C-terminus was generated. The cDNA was sequenced, and was found to contain 92 amino acids. The first 7 amino acids of the cDNA were part of the predicted transmembrane domain,
and the next 85 amino acids made up the cytosolic domain. Using the first amino acid of the N-terminal end of the cytosolic domain as a reference point, the amino acids were numbered accordingly. Four cDNAs of roughly the same length, corresponding to amino acids -7 to 26, 14 to 46, 27 to 66, and 54 to 85 were generated using primers listed in Table 2. The primers were designed as described in section 2.1.1.

<table>
<thead>
<tr>
<th>Fragment size (amino acids)</th>
<th>Sequences of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7 to 26</td>
<td>sense: AAGAATTCAGGCGGTATCCTCTTTCTGCTGTTCGGGA</td>
</tr>
<tr>
<td></td>
<td>anti-sense: CGGAATTCATTTCTCTTCCCTTCTTCCTTCCTT</td>
</tr>
<tr>
<td>14 to 46</td>
<td>sense: AAGAATTCAGGGCGTGCTGGCCAGGCCAGATG</td>
</tr>
<tr>
<td></td>
<td>anti-sense: CGGAATTCATTTCTTCTTTCTTTCAAGCTTCTC</td>
</tr>
<tr>
<td>27 to 66</td>
<td>sense: AAGAATTCAGGCCTGTAAGAGAACAGAAGGGGGG</td>
</tr>
<tr>
<td></td>
<td>anti-sense: CGGAATTCATGTTGTGTCTCTATCTTCTC</td>
</tr>
<tr>
<td>54 to 85</td>
<td>sense: AAGAATTCAGGCGGTGCTCGGACTGACCTCAAGAT</td>
</tr>
<tr>
<td></td>
<td>anti-sense: CGGAATTCATTTCTCTTCCGTGGCTTTCT</td>
</tr>
</tbody>
</table>

Table 2. Sequences of primers used to generate various cDNA.

Since epitopes are generally 6 to 10 amino acids long, an overlapping segment of at least 13 amino acids between every two fragments would ensure that the epitope would not be missed. Eco RI sites were engineered into the primers, and PCR was performed as previously described. After digestion with Eco RI, the insert was cloned into the Eco RI site of the expression vector. pGEX-3X was used in cloning the cDNA corresponding to amino acids 14 to 46, and pGEX-4T3 was employed in cloning all other fragments. DH5α bacteria transformed with the construct were grown on LB agar plates containing 50 µg/ml ampicillin. Each colony was inoculated into 5 ml of LB medium containing 50
µg/ml ampicillin, and the cells were allowed to grow overnight at 37°C at 225 rpm. PCR was performed using DNA extracted from the overnight cultures to determine if the insert was oriented in the correct direction. The 5' primer was located in the vector, upstream of the insert, and the 3' primer was the anti-sense primer used to generate the insert.

![Diagram of amino acid segments](image)

**Fig. 12.** Fragments of Chinese hamster calnexin cytoplasmic domain tested. The protein segment recognized by 1H82 monoclonal antibody is in the shaded rectangles. The numbers indicate the position of each fragment, according to amino acid (aa) number (the first amino acid of the N-terminal end of the cytosolic domain is aa 1).

10 µl of the overnight cultures was inoculated into 250 µl of 1X electrophoresis sample buffer (25 mM Tris-HCl pH 6.8, 0.05% glycerol, 1% SDS, 0.0025% β-mercaptoethanol, 0.0015% bromophenol blue), and denatured at 55°C for 5 min. 20 µl of
each of the samples was separated by SDS-PAGE, transferred to nitrocellulose filter paper, and probed with ascites fluid purified in section 2.5. Once the epitope could be localized to one of the cloned segments, that segment was further subdivided by selecting appropriate PCR primers and generating cDNA sub-fragments. Similar cloning and screening procedures were carried out until the protein segment containing the epitope was narrowed down to 20 amino acids (see Fig. 12).

2.6.4 Binding of antibody to ER in microsomal membranes

An experiment was performed to assess whether the monoclonal calnexin antibody was able to recognize calnexin in its native membrane-bound state. BQ2 cells were washed and resuspended at $1 \times 10^7$ cells/ml in cytosol buffer (36.5 mM Hepes pH 7.2, 2.5 mM Mg$^{2+}$, 77 mM K$^+$, 82 mM acetate, 18 mM Ca$^{2+}$/5 mM EGTA (100 nM free Ca$^{2+}$) ) supplemented with 100 µg/ml DNase I, 100 µg/ml RNase A, and 1X protease inhibitor cocktail (Boehringer Mannheim). Nitrogen cavitation (150 psi, 10 min, on ice) was employed to homogenize cells. The cell homogenate was first centrifuged at 300g for 10 min to pellet intact cells and nuclei, and then centrifuged at 4000g for 10 min to remove mitochondria. Supernatants obtained after the second centrifugation were enriched in ER microsomes, and were mixed with 1 µg antibody (and GST-calnexin/GST peptide) according to Table 3. Each sample also contained 0.1% skim milk as a blocking reagent. After incubating at 4°C for 2 h, each sample was passed through a 0.22 µm microcentrifuge filter (Sigma) that was pre-treated with 0.5% skim milk to prevent non-
specific binding. To remove any free antibody, the filter units were washed 3 times with 0.7 ml cytosol buffer. 30 μl of 1X electrophoresis sample buffer was used to elute protein from the filters. The eluted protein was then separated by SDS-PAGE, transferred onto nitrocellulose, and probed with goat anti mouse antibody.

<table>
<thead>
<tr>
<th>sample #</th>
<th>antibody / peptide mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 μg 1H82 antibody</td>
</tr>
<tr>
<td>2</td>
<td>1 μg 1H82 antibody + 40 μg (1 nmol) GST-calnexin fusion protein</td>
</tr>
<tr>
<td>3</td>
<td>1 μg 1H82 antibody + 28 μg (1 nmol) GST protein</td>
</tr>
<tr>
<td>4</td>
<td>1 μg non-specific murine IgG (Pharminogen)</td>
</tr>
<tr>
<td>5</td>
<td>1 μg non-specific murine IgG + 40 μg (1 nmol) GST-calnexin</td>
</tr>
<tr>
<td>6</td>
<td>1 μg non-specific murine IgG + 28 μg (1 nmol) GST</td>
</tr>
<tr>
<td>7</td>
<td>no antibody</td>
</tr>
</tbody>
</table>

**Table 3.** Supernatant obtained after the mitochondrial spin was incubated with 1 μg antibody or antibody/fusion protein mix.
3 Results

3.1 Generation of GST-calnexin fusion protein

3.1.1 Sequence of CHO calnexin

The region of Chinese hamster calnexin cDNA from the 3' end of the protein's transmembrane region to its C-terminus was sequenced for the first time. The enzyme used in PCR, Platinum Taq DNA polymerase (GIBCO/BRL), is a high fidelity enzyme with an error frequency of $(1.8 \pm 0.37) \times 10^{-6}$. Since the PCR product was only about 280 bp long, the chance of introducing an artifact was very small. The DNA and amino acid sequence alignments of Chinese hamster, human [84], mouse [84] and rat [84] calnexin are shown in Fig. 13 and Fig. 14, respectively. The DNA sequence of Chinese hamster calnexin is approximately 82% homologous to that of human calnexin, and 93% homologous to that of both mouse and rat calnexin. The amino acid sequence of Chinese hamster calnexin is approximately 85% homologous to that of human calnexin, and 95% homologous to that of both mouse and rat calnexin. Calnexin is a remarkably acidic protein, and therefore has a higher apparent molecular weight on SDS-PAGE than is predicted from its cDNA sequence.

```
1  T A T C C T C T T C T G C T G T T C T G A A A G A A A C A
1  T A T C C T C T T C T G C T G T T C T G A A A G A A A C A
1  G A T C C T C T T C T G C T G T T C T G A A A G A A A C A
1  G A T C C T C T T C T G C T G T T C T G A A A G A A A C A
1  C A T G C A T A G G A G T A C A A G A A G A C G G A
1  C A T G C A T A G G A G T A C A A G A A G A C G G A
1  C A T G C A T A G G A G T A C A A G A A G A C G G A
1  C A T G C A T A G G A G T A C A A G A A G A C G G A
```

Chinese hamster calnexin

human calnexin

mouse calnexin

rat calnexin

Chinese hamster calnexin

human calnexin

mouse calnexin

rat calnexin
<table>
<thead>
<tr>
<th>Residues</th>
<th>Chinese hamster calnexin</th>
<th>Human calnexin</th>
<th>Mouse calnexin</th>
<th>Rat calnexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>TGCTGCCAGATGTAACAGAAGAGATGA</td>
<td>TGCLCACTCGAAGATGAACAGAAGAGATGA</td>
<td>TGCTGCCAGATGTAACAGAAGAGATGA</td>
<td>TGCTGCCAGATGTAACAGAAGAGATGA</td>
</tr>
<tr>
<td>87</td>
<td>AGGGAAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
</tr>
<tr>
<td>91</td>
<td>AGGGAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
<td>AGGGAGGAACAGAAGAGATGAACAGAAGAGATGA</td>
</tr>
</tbody>
</table>

Fig. 13. DNA sequence alignment of Chinese hamster, human, mouse and rat calnexin.

Residues that differ from the Chinese hamster sequence are marked in boxes.
Fig. 14. Amino acid sequence alignment of Chinese hamster, human, mouse and rat calnexin. Amino acid residues that differ from the Chinese hamster sequence are marked in boxes.

3.1.2 Expression of GST-calnexin fusion protein

Before the fusion protein could be purified, it was essential to determine the solubility of the protein. Bacterial cells expressing the GST-calnexin fusion protein were mildly sonicated, and the lysed bacterial cells were centrifuged. The supernatant and the pellet were analyzed by electrophoresis followed by immunoblotting. A commercial polyclonal antibody (StressGen) to canine calnexin C-terminus was employed in the Western blotting analysis. The IPTG concentration and the induction time were varied to
determine the optimal expression parameters. Data shown in Fig. 15 and Fig. 16 indicate that the GST-calnexin fusion protein was present in both the supernatant and the pellet. Induction with any one of the three concentrations of IPTG resulted in the production of a significant amount of fusion protein. Since IPTG is a relatively expensive compound, and the amounts of protein expressed when induced with 1 mM or 0.1 mM IPTG were similar, 0.1 mM IPTG was chosen to be the optimal IPTG concentration. The optimal induction time in this experiment was 3 h. The apparent $M_r$ of the fusion protein is roughly 45 kDa. GST has a $M_r$ of 27 kDa. The segment of calnexin being synthesized contained only 92 amino acids, and is expected to have a $M_r$ of about 11 kDa. Thus, the expected $M_r$ of the entire fusion protein is approximately 38 kDa. The discrepancy between the apparent and the expected $M_r$ is likely due to the acidity of the calnexin part of the fusion protein.

![Graph with IPTG concentrations and time intervals]

$[\text{IPTG}]$ (mM): 0.05, 0.1, 1

Time (hr): 1, 2, 3, 0.5, 1, 2, 3

83 kDa
62 kDa
47.5 kDa
32 kDa
25 kDa

Fig. 15. The bacterial culture transformed with the GST-calnexin construct was induced for various lengths of time and with several IPTG concentrations. The supernatants obtained after centrifugation of the crude bacterial lysates were electrophoretically separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with commercial polyclonal calnexin antibody.
Fig. 16. The pellets obtained after centrifugation of the crude bacterial lysates were electrophoretically separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with commercial polyclonal calnexin antibody.

3.1.3 Purification of GST-calnexin fusion protein

The GST-calnexin fusion protein was purified according to the procedure described by Smith and Johnson (see Materials and Methods) [78]. The purified protein was separated on a polyacrylamide gel, and stained with Coomassie blue (see Fig. 17). The apparent $M_r$ of the fusion protein is approx. 45 kDa. The band at 32 kDa is probably a degraded calnexin protein. An 800 ml bacterial culture yielded about 1.6 mg of fusion protein.
Fig. 17. GST-calnexin and GST were run on a 10% polyacrylamide gel, the gel was subsequently stained with Coomassie blue. Lane 1: 2 µg GST-calnexin; lane 2: 1 µg GST.

3.2 Screening of hybridoma cells

3.2.1 Dot blot assay

Spleen cells from immunized mice were fused with Sp2/0-Ag14 [72, 73] murine myeloma cells accordingly to the procedure described in Section 2.3 (Materials and Methods). About 30 to 40% of the seeded wells were positive for hybridoma cell growth. Several fusion experiments were performed, but only the result of the experiment where positive clones were found is shown here in Fig. 18. 1 µl of 0.5 mg/ml GST-calnexin fusion protein was spotted on the right of the nitrocellulose strips, while the same amount of GST protein was spotted on the left. The strips were probed with tissue culture supernatants from individual hybridoma wells, and developed using the ECL system. Positive signals on both the left and the right of the strip indicate that the antibody is directed to GST while a positive signal on just the right indicates that the antibody is directed to calnexin.
Fig. 18. Each strip of nitrocellulose membrane (four columns shown) contains a dot of GST-calnexin on the right, and a dot of GST on the left as indicated by the paired arrows at the top of the figure. Clones 1H8, 2F5, 2G5, 2C10, 2A11, 3B1, 3F1, 4F4 and 5B4 secreted antibody specific for calnexin.
3.2.2 Enzyme-linked immunosorbent assays (ELISA)

The putatively positive hybridoma cell supernatants were screened twice using ELISA. The result of the secondary screening is shown in Fig. 19. Clones 1A12, 1H8, 2B1, 2A11 (indicated in bold) were positive for calnexin.

<table>
<thead>
<tr>
<th>No.</th>
<th>Clone Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>4C5</td>
<td>2H9</td>
</tr>
<tr>
<td>4G9</td>
<td>2G4</td>
</tr>
<tr>
<td>2A10</td>
<td>1H8</td>
</tr>
<tr>
<td>2A11</td>
<td>1G12</td>
</tr>
<tr>
<td>2A4</td>
<td>2H6</td>
</tr>
<tr>
<td>3F1</td>
<td>2B1</td>
</tr>
<tr>
<td>3F11</td>
<td>2E1</td>
</tr>
<tr>
<td>4A8</td>
<td>2G5</td>
</tr>
</tbody>
</table>

Fig.19. a) This table contains names of the hybridoma clones screened in the ELISA assay. Positive clones were highlighted. b) A picture of the ELISA plate. The wells were coated with 50 μg/ml GST. c) The wells were coated with 50 μg/ml GST-calnexin.

3.2.3 Detection of calnexin in fusion protein and CHO cells using hybridoma cell supernatant

There was a Factor Xa cleavage site between the GST and the calnexin part of the fusion protein. Cleavage was performed according to the procedure given in Materials and Methods. The cleaved protein was analyzed by immunoblotting using supernatants from various hybridoma clones (see Fig. 20). Lane 1 contained 50 ng of cleaved GST-
calnexin fusion protein while lane 2 contained 35 ng of GST protein. Both the 1H8 supernatant and the commercial polyclonal anti-calnexin antibody were able to recognize the same calnexin band.

![Image of a gel electrophoresis pattern with bands labeled at 50 kDa, 36 kDa, 30 kDa, and 16 kDa. The gel shows lanes 1 and 2 with bands for GST and GST-calnexin, respectively.]

**Fig. 20.** lane 1: 50 ng of cleaved GST-calnexin; lane 2: 35 ng of GST. Immunoblots performed using commercial calnexin antibody and supernatants from various hybridoma clones.

Clone 1H8 was chosen to pursue further study since it is the only clone that is positive in all the above screening experiments. HT29 (a human adenocarcinoma cell line) and BQ2 (a Chinese hamster ovary cell line transfected with pNUT-CFTR and which stably expresses human CFTR) cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the commercial anti-calnexin antibody and with culture supernatant from clone 1H8. The same amount of protein was loaded in each lane. The human and Chinese hamster calnexin bands detected by the monoclonal antibody 1H8 were of approx. the same intensity (see Fig. 21a); however, the human calnexin band detected by the commercial anti-calnexin antibody was much more intense than the Chinese hamster calnexin band (see Fig. 21b). From the results of
Western blotting analysis and ELISA, it was concluded that 1H8 was able to recognize denatured calnexin and native GST-calnexin fusion protein.

Fig. 21. Lane 1: BQ2 cell extract; lane 2: HT29 cell extract. a) The immunoblot was performed using 1H8 culture supernatant. b) The immunoblot was performed using the commercial calnexin antibody.

3.3 Purification of ascites fluid

1H8 was re-cloned by limiting dilution (see Materials and Methods), and the subclones obtained were screened again using the dot blot assay and ELISA as previously described. Out of 22 subclones, 6 were negative and 16 were positive. All positive subclones seemed to have a similar antibody titer. One clone with a slightly higher antibody titer was selected for further expansion. It was named 1H82.
Ascites fluid was produced by injecting 1H82 hybridoma cells into pristane primed mice. Cells and pristane were removed from the ascites fluid, and the clean ascites fluid was purified using HPLC. First, it was passed onto a protein G column, the bound fraction was eluted with 50 mM MES in 1% acetic acid (see Fig. 22).

Fig. 22. The ascites fluid was loaded onto a protein G column at 0 min, and the column was washed with 1X TBS. Elution of the bound antibodies with 50 mM MES in 1% acetic acid started at 4 min. A sharp peak was obtained at 6 min.
The eluate was then neutralized with 2M Tris base, and exchanged into 20 mM Tris-HCl, pH 7.6 on a PD-10 desalting column. Other contaminating IgG isotypes (i.e. IgG2a, IgG2b, IgG3) were removed by passing over a protein A column. The flow-through was collected and loaded onto a Mono Q column. The bound fractions were eluted with a linear gradient starting in 20 mM Tris-HCl, pH 7.6 and ending in 0.3 M NaCl, 20 mM Tris-HCl, pH 7.6 (see Fig 23).

Fig. 23. After passing over a protein A column, the flow-through was collected and loaded onto a Mono Q column. The bound fractions were eluted with a linear gradient starting in 20 mM Tris-HCl, pH 7.6 at 0 min, and ending in 0.3 M NaCl, 20 mM Tris-HCl, pH 7.6 at 10 min. A sharp peak was detected at 5 min.
The purified antibody was analyzed by electrophoresis, and the polyacrylamide gel was stained with Coomassie blue (see Fig. 24). Both the heavy and light chains were of appropriate M, according to SDS-PAGE analysis.

![Polyacrylamide gel stained with Coomassie blue](image)

Fig. 24. 1 ug of antibody was separated on a 10% polyacrylamide gel. The gel was subsequently stained with Coomassie blue.

3.4 Characterization of antibody

3.4.1 Isotyping of antibody

Isotyping of the antibody was performed using IsoDetect™ Mouse Monoclonal Antibody Isotyping kit (Stratagene). The 1H82 monoclonal antibody was found to be of isotype IgG1 with kappa light chains (see Fig. 25).
Fig. 25. 5 μl of tissue culture supernatant was diluted into 495 μl of sample diluent, and the diluted supernatant was slowly added to the sample well on an IsoDetect test card.

3.4.2 Determination of antibody affinity

In the preliminary experiment, varying amounts of GST-calnexin fusion protein were added to different wells of a filter-bottom plate. The wells were then incubated with a fixed concentration of 1H82 antibody and 125I-labeled sheep anti mouse secondary antibody. The excised filter membranes were counted and the results were plotted in Fig. 26. The concentration of GST-calnexin that resulted in a 50% saturation was about 1 μg.

In the next experiment, each well was coated with 1 μg of GST-calnexin, but probed with various concentrations of 1H82 antibody ranging from 10 ng/ml to 100 μg/ml. To measure the non-specific binding, an equal number of wells was coated with 1 μg of GST, and were processed in parallel with the GST-calnexin coated wells. The experiment was carried out in triplicate.
Fig. 26. The amount of GST-calnexin fusion protein was varied. The concentration of GST-calnexin that resulted in 50% saturation was about 1 μg.

Homogeneous and heterogeneous binding models were fitted to the data obtained from the saturation binding experiment using a computer program. Goodness of fit is quantified by the sum-of-squares of the vertical distances of the points from the curve. An F test was performed to determine which model was more appropriate for the data (see Table 4). Since the P value is less than 0.05, it was concluded that the heterogeneous binding model fitted significantly better than the homogeneous binding model.
Table 4. Homogeneous and heterogeneous binding models were fitted to the data. An F

test was performed, and the two site binding model was found to fit the data

t better.

A saturation curve using a heterogeneous binding model was fitted to the data,

and is shown in Fig. 27. These data were further analyzed in a Scatchard plot (see Fig.

28). Result from only one of three experiments is shown for clarity. The dissociation

constant (K_D) is determined at 50% saturation, and the affinity constant (K_A) is defined as

the reciprocal of K_D. The K_A values obtained from the different experiments were 1.5 ×

10^8 M^{-1}, 2.3 × 10^8 M^{-1} and 1.4 × 10^8 M^{-1}. The mean value with its standard deviation was

(1.7 ± 0.5) × 10^8 M^{-1}.

As observed in Fig. 28, the Scatchard plot had a concave shape. This suggested

that there was heterogeneous binding. The Scatchard transformation was then resolved

into two components. It was speculated that K_A1 = 1.5 × 10^8 M^{-1} was the measurement of

intrinsic affinity while K_A2 = 1.0 × 10^{10} M^{-1} was the measurement of avidity enhanced by

bivalent binding.
Fig. 27. Data for specific binding was fitted into the following equation:

\[ B = B_{\text{max}1} \times \frac{[\text{Ab}]}{(K_{D1} + [\text{Ab}])} + B_{\text{max}2} \times \frac{[\text{Ab}]}{(K_{D2} + [\text{Ab}] so\)} \]

The following values were obtained: \( K_{D1} = 6.6 \times 10^{-9} \) M, \( K_{D2} = 9.8 \times 10^{-11} \) M, \( B_{\text{max}1} = 4499, B_{\text{max}2} = 856 \). \( B_{\text{max}1} \) or \( B_{\text{max}2} \) is the maximum reading owing to saturation of all class 1 or 2 sites, respectively.

In the inhibition assay, the wells were each coated with 1 µg of GST-calnexin protein, and probed with 1 µg/ml of antibody that was pre-incubated with various concentrations of GST-calnexin fusion protein. The assay was carried out in triplicate, and the experiment was repeated three times. Homogeneous and heterogeneous competition models were fitted to the data obtained from the inhibition experiment using a computer program. Again, an F test was performed to determine which model was
more appropriate for the data (see Table 5). Since the P value is greater than 0.05, it was concluded that the homogeneous competition model fitted significantly better than the heterogeneous model.

![Scatchard plot](image)

**Fig. 28.** The Scatchard transformation was resolved into two straight lines $y = 1.5 \times 10^8 x + 6.7 \times 10^{11}$ and $y = 1.0 \times 10^{10} x + 8.5 \times 10^{12}$ by a computer program based on the equation $B = \frac{B_{max1} \times [Ab]}{(K_D1 + [Ab])} + \frac{B_{max2} \times [Ab]}{(K_D2 + [Ab])}$.

<table>
<thead>
<tr>
<th>Comparison of Fits</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of squares (one site)</td>
<td>32380</td>
</tr>
<tr>
<td>Sum of squares (two site)</td>
<td>43310</td>
</tr>
<tr>
<td>F</td>
<td>1.948</td>
</tr>
<tr>
<td>P value</td>
<td>0.1930</td>
</tr>
<tr>
<td>Best fit model</td>
<td>one site model</td>
</tr>
</tbody>
</table>

**Table 5.** Homogeneous and heterogeneous competition models were fitted to the data. An F test was performed, and the homogeneous competition model was found to fit the data significantly better.
The data was fitted to a one site competition model, and is shown in Fig. 29. An IC_{50} value was obtained. The IC_{50} values obtained from three different experiments were 8.2 nM, 9.1 nM and 6.8 nM. The mean IC_{50} value was (8.0 ± 1.2) nM. A K_D (dissociation constant of the antibody (Ab) and competing peptide) was calculated from the IC_{50} using the following formula: K_D = IC_{50} / (1 + [Ab] / K_D^*) where [Ab] = 1 μg/ml and K_D^* is the dissociation constant of the antibody and antigen. K_A was then derived from the relationship K_A = 1 / K_D. The results are summarized in Table 6. The mean value was (2.4 ± 0.4) × 10^8 M^{-1}. A two-tailed t-test was performed to determine if the K_A values obtained from the saturation binding experiments were significantly different from those generated from the competition experiments. Since the P value (0.1605) > 0.05, it was concluded that there was no significant difference between the K_A values generated from both experiments.

**Inhibition assay**

![Inhibition assay graph](image)

**Fig. 29.** The data were best fitted into a competition curve. The IC_{50} value was 8.2 nM.
<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (nM)</th>
<th>$K_D = IC_{50} / (1 + [Ab] / K_D^*)$ (nM)</th>
<th>$K_A \times 10^6 \text{ M}^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>experiment #1</td>
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<td>4.3</td>
<td>2.3</td>
</tr>
<tr>
<td>experiment #2</td>
<td>9.1</td>
<td>3.6</td>
<td>2.8</td>
</tr>
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<td>experiment #3</td>
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<td>2.0</td>
</tr>
<tr>
<td>mean</td>
<td>8.0</td>
<td>4.2</td>
<td>2.4</td>
</tr>
<tr>
<td>standard deviation</td>
<td>1.2</td>
<td>0.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 6. Summary of IC$_{50}$, $K_D$ and $K_A$ values obtained from inhibition assays.

3.4.3 Epitope mapping

Chinese hamster calnexin cDNA corresponding to the region from the last 7 amino acids of the transmembrane domain to the C-terminus was generated. The cDNA was found to contain 92 amino acids, and the last 85 amino acids made up the cytosolic domain. Using the first amino acid of the 5' end of the cytosolic domain as a reference point, the amino acids were numbered accordingly. Four cDNAs of roughly the same length, corresponding to amino acids -7 to 26, 14 to 46, 27 to 66, and 54 to 85 were generated. The cDNAs were cloned into the Eco RI site of the expression vector in a manner similar to that used to generate the GST-calnexin fusion protein. pGEX-3X was used in cloning the cDNA corresponding to amino acids 14 to 46, and pGEX-4T3 was employed in cloning all other fragments.

Colonies transformed with the construct containing amino acids 14 to 46, or 27 to 66, yielded positive signals (see Fig. 30 & 31). Smaller fragments containing amino acids 27 to 46, 27 to 60, 46 to 66 were then tested. Seven out of 13 colonies transformed with the construct containing amino acids 27 to 60 (see Fig. 32), and 3 out of 12 colonies transformed with the construct containing amino acids 27 to 46 were positive (see Fig. 33). Plasmid DNA was extracted from colonies transformed with the construct containing amino acids 27 to 46 using a Qiagen miniprep kit (Qiagen). PCR was then
performed using the purified DNA to determine the orientation of the insert. As expected, PCR product was obtained from reactions done using DNA extracted from colonies 1, 6 and 12, which were the same colonies that yielded positive signals in immunoblotting (see Fig. 34). The construct was then sequenced to verify that the DNA sequence was correct. Approximately 30 colonies were screened before a fragment was confirmed to be negative. Eco RI digestion was also performed to ensure that the negative colonies did contain inserts.

Fig. 30. Bacterial colonies transformed with construct containing amino acids 14 to 46. They were lysed using 1X Laemmli sample buffer, and separated by SDS-PAGE on a 10% polyacrylamide gel. The blot was probed with 1H82 antibody.
Fig. 31. Bacterial colonies transformed with construct containing amino acids 27 to 66. They were lysed using 1X Laemmli sample buffer, and separated by SDS-PAGE on a 10% polyacrylamide gel. The blot was probed with 1H82 antibody.

Fig. 32. Bacterial colonies transformed with construct containing amino acids 27 to 60. They were lysed using 1X Laemmli sample buffer, and separated by SDS-PAGE on a 10% polyacrylamide gel. The blot was probed with 1H82 antibody.
Fig. 33. Bacterial colonies transformed with construct containing amino acids 27 to 46. They were lysed using 1X Laemmli sample buffer, and separated by SDS-PAGE on a 12% polyacrylamide gel. The blot was probed with 1H82 antibody.

Fig. 34. The 5' PCR primer was located in the vector, upstream of the insert, and the 3' primer was the one used to generate the insert. Plasmid DNA was extracted from colonies transformed with the construct containing amino acids 27 to 46. PCR product was obtained from reactions done using DNA extracted from colonies 1, 6 and 12. This indicated that colonies 1, 6 and 12 contained insert ligated in the correct orientation.
3.4.4 Binding of antibody to ER in microsomal membranes

ER microsomes were prepared from BQ2 cells as described in section 2.6.4 (Materials and Methods), and were incubated with an antibody or an antibody/fusion protein mixture. After 2 h of incubation, the antibody-microsome complexes were passed through 0.22 μm nylon microcentrifuge filter units. Excess free antibody was removed by washing the centrifuge unit with cytosol buffer. The antibody-microsome complexes were eluted from the filter using 1X Laemmli sample buffer, and were then separated by SDS-PAGE. Goat anti mouse antibody was used to detect any mouse antibody that was present in the samples.

![Diagram](image)

**Fig. 35.** Proteins eluted from the filter units were separated on a 12 % polyacrylamide gel. Lane 1, ER microsomes incubated with 1H82; lane 2, with 1H82 and excess GST-calnexin; lane 3, with 1H82 and excess GST; lane 4, with non-specific antibody; lane 5, with non-specific antibody and excess GST-calnexin; lane 6, with non-specific antibody and excess GST; lane 7, ER microsomes were not incubated with any antibody; lane 8, 10 ng of 1H82; lane 9, 10 ng of non-specific antibody.
1H82 was able to bind to ER microsomes (see lane 1 of Fig. 35), but this binding was not observed in the presence of excess GST-calnexin fusion protein (see lane 2). 1H8 was binding specifically to calnexin, since the presence of excess GST protein was not able to compete with ER microsomal membranes for binding sites (see lane 3). The non-specific mouse IgG1 was used as a negative control, and it was not able to recognize ER microsomal membranes (see lanes 4 – 6). It was concluded from this experiment that 1H82 was able to recognize calnexin while it was in its native membrane-bound state.
4 Discussion

4.1 Rationale for developing antibody against cytoplasmic domain of calnexin

Calnexin is a type I integral membrane protein embedded in the membrane of the ER. It has a 460-residue luminal domain, a single transmembrane sequence, and a cytosolic domain of about 90 amino acids (see Fig. 9) [71]. At the carboxyl terminus lies the sequence RKPRRE. An arginine at position -3 and a lysine at position -5 from the C-terminus is similar to motifs that have been hypothesized to play a role in the ER localization of other type I membrane proteins [65]. In mammals, deletion of the segment RKPRRE was shown to cause redistribution of calnexin to Golgi membranes and the cell surface [69]. Immunofluorescence microscopy of cultured cells revealed that both canine [64] and human calnexin [67] are localized to the ER. This evidence confirms that calnexin is exclusively an ER constituent. Since calnexin is present only in the ER and has a C-terminal domain that protrudes into the cytosol, it can therefore be used as a marker for immunoaffinity isolation of ER.

Like calnexin, signal recognition particle α (SRPα), UDP-glucuronosyltransferase and HMG-CoA reductase are ER resident transmembrane proteins. Antibodies towards the cytoplasmic domain of any of these proteins could in theory be used for immunoisolating the ER, but calnexin is present in relatively high abundance. Also, the long-term goal of this project was to identify protein components that interact with CFTR. Since it was known that calnexin associates with CFTR, it was perceived that it might be convenient to have an indefinite supply of anti-calnexin antibody, not only for immunoisolation of ER, but also for coprecipitation of calnexin-
bound CFTR along with other factors that might be associated with CFTR in its early stages of folding. Moreover, calnexin is one of the better characterized proteins among all ER resident transmembrane proteins. Therefore, we chose to develop antibody to calnexin.

Serum contains many different types of antibodies. Even in hyperimmune animals, often less than one-tenth of the circulating antibodies are specific for one antigen. The commercially available antiserum raised against canine calnexin C-terminus is not affinity purified, and probably contains other contaminating antibodies. Using this antiserum to purify ER by affinity immunoisolation may result in the co-isolation of other proteins. Because hybridomas are cloned prior to use, antibodies produced by hybridomas are monospecific. Another disadvantage of polyclonal antiserum is that antiserum produced in one animal varies from that produced in another animal, and there is significant variation from bleed to bleed, and consequently from batch to batch. On the other hand, all antibodies produced by descendants of one hybridoma cell are identical, and hybridoma cell lines provide an unlimited supply of antibodies. In view of the shortcomings of polyclonal sera, it was decided that a monoclonal antibody against cytoplasmic domain of calnexin would be developed.

4.2 Rationale for purifying ER by immunoisolation

Immunoisolation of organelles using paramagnetic beads offers several advantages over the conventional method of sucrose gradient fractionation. First, the process of immunoisolation is much faster. A major portion of the time required in the procedure is used in incubating antibody coated beads with ER-enriched microsomes,
which is approximately 2 h. On the other hand, sucrose gradient fractionation often requires overnight centrifugation. This is a long time under non-viable condition, so association between CFTR and other components might not be retained as they were inside the viable cell. Performing chemical cross-linking on ER microsomes prepared after an overnight centrifugation might not allow us to observe the protein-protein interactions that were originally present in a viable cell. Second, the use of an antibody can provide excellent specificity. Since monoclonal antibody recognizes a specific epitope, ER microsomes affinity immunosolated using an anti-calnexin antibody should be very pure. On the other hand, sucrose gradient fractionation separates subcellular organelles by the difference in densities. Organelles with a similar density cannot be separated. Successful immunosolation of ER using paramagnetic beads would represent a novel method of ER purification.

4.3 Characterization of 1H82 antibody

4.3.1 Determination of specificity

Commercial polyclonal anti-calnexin antibody (StressGen) had been raised against a synthetic peptide containing the last 19 amino acids at the C-terminus (AEEDEILNRSNPRKPRRE) of canine calnexin. Since this sequence is 100% homologous in canine, human and Chinese hamster, the antibody should have the same affinity for the three species of calnexin; however, the human calnexin band (apparent Mr, approx. 90 kDa) detected by the polyclonal anti-calnexin antibody was more intense than the Chinese hamster calnexin band (apparent Mr, approx. 88 kDa; see Fig. 21b). Since the same amount of protein was loaded in each lane, the above observation implied that the
quantity of calnexin present in CHO cells was less than that in human cells. On the other hand, Chinese hamster and human calnexin bands detected by 1H82 were of similar intensities. This suggested that 1H82 had a higher affinity for Chinese hamster calnexin than its human homologue.

4.3.2 Determination of antibody affinity

Affinity is a measure of the strength of the binding of an antibody to an epitope. The affinity of the 1H82 antibody is an indication of how strongly 1H82 binds to calnexin, and learning about the affinity allows the speculation of whether this antibody is useful for immunoisolating ER.

In most cases, the following criteria must be met if an accurate measurement of the affinity of an interaction is to be obtained: 1) In a binding experiment, the concentration of one binding partner (e.g. antigen) must be kept constant while the other (e.g. antibody) is varied. Enough time must be allowed at each point for an equilibrium to be achieved. 2) Both binding partners should be in solution for them to diffuse freely. 3) For each titration point, the concentration of bound ligand and/or free ligand must be measured under conditions which do not perturb the established equilibrium. Techniques such as equilibrium dialysis, fluorescence quenching, and surface plasmon resonance meet the third criterion. On the other hand, any type of assay that involves a washing or gel filtration step to separate the free from the bound ligand does not meet the third criterion. However, applying techniques such as fluorescence quenching and surface plasmon resonance necessitate the use of equipment that is not easily accessible. Since
our intention was to obtain an estimate of the antibody affinity, an ELISA-based method that is more convenient and simple was employed.

Homogeneous and heterogeneous binding models were fitted to the data obtained from the saturation binding experiment using a computer program. The goodness of fit was determined by the sum of squares of the vertical distances between the data point and the curve. From the result of the F test, the heterogeneous binding model was found to be more appropriate for the data (see Table 4). The data were also transformed in a Scatchard plot. As observed in Fig. 28, the Scatchard transformation had a concave shape. The simplest situation where this can occur is where there is either heterogeneous binding or negative cooperativity. Negative cooperativity means that when one binding site is occupied, the occupancy of additional sites is disfavored. Since an antibody molecule contains two identical antigen combining sites, negative cooperativity is unlikely. It was thus concluded that heterogeneous binding occurred during the saturation binding experiment. The Scatchard transformation was resolved into two components. The following data were obtained: $B_{\text{max}1} = 4499$, $B_{\text{max}2} = 856$, $K_A1 = 1.5 \times 10^8 \text{M}^{-1}$ and $K_A2 = 1.0 \times 10^{10} \text{M}^{-1}$. The dissociation constant ($K_D$) for the antibody and antigen is the concentration of antibody at which 50% of the antigen is bound. The affinity constant ($K_A$) is defined as the reciprocal of $K_D$. $B_{\text{max}}$ is the maximum $^{125}\text{I}$ count obtained when the antibody and antigen binding is saturated. It should be noted that the smaller $K_A$ occurred at higher antibody concentration. This suggests that the antibody binds monovalently at high concentration, possibly due to steric hindrance (see Fig. 36a). At lower concentration, the antibody has room to bind bivalently (see Fig. 36b).
Fig. 36. a) At low concentration, antibodies have room to bind bivalently when the antigen density is sufficiently high. b) At high concentration, antibodies may bind monovalently due to steric hindrance. Note: Antibody representations are diagramatic only.

The binding of an antibody to antigen is dynamic. If an antibody is binding monovalently to an antigen, once the antibody dissociates, it may diffuse away (see Fig. 37a). However, in the case of bivalent binding, even if one arm of the antibody is dissociated from the antigen, the other arm is still bound. Because the antibody is still in close proximity to antigen molecules, the free arm may quickly reassociate (see Fig. 37b). Therefore, it is expected to observe a higher $K_A$ for a bivalent antibody-antigen interaction. It was speculated that $K_{A1} = 1.5 \times 10^8 \text{ M}^{-1}$ was a measurement of the intrinsic affinity while $K_{A2} = 1.0 \times 10^{10} \text{ M}^{-1}$ was a measurement of avidity enhanced by bivalent binding. Also, 19% ($B_{\text{max}2}$/$B_{\text{max}1}$) of the binding was bivalent while 81% was monovalent.
Fig. 37.  a) If it were bound monovalently, once the antibody dissociates, it diffuses away. b) If it were bound bivalently, even if one arm is dissociated, the antibody is still in close proximity to antigen molecules. The free arm may quickly reassociate.

The affinity of an antibody can also be assessed by the IC₅₀ values in inhibition assays. Models with homogeneous and heterogeneous competition sites were fitted to the data obtained from the inhibition experiment using a computer program. It was concluded from the result of the F test that the homogeneous competition site model is more appropriate for the data (see Table 5). IC₅₀ is the concentration of free antigen that produces a 50% decrease in the maximum response. A Kᵥ (dissociation constant of the antibody for the competing fusion protein antigen) was calculated from the IC₅₀ using the following formula: \[ Kᵥ = IC₅₀ / \left( 1 + [\text{Ab}] / Kᵥ^* \right) \] where [Ab] = 1 µg/ml and Kᵥ^* is the dissociation constant of the antibody and antigen. The result of a two-tailed t test indicated that the Kᵥ values obtained in the inhibition assays [(2.4 ± 0.4) × 10⁸ M⁻¹] were not significantly different from those derived from the Scatchard analysis [(1.7 ± 0.5) × 10⁸ M⁻¹].
A homogeneous competition site model was fitted to the data obtained from the inhibition assays. However, heterogeneous binding was observed from the Scatchard analysis. The concentration of antibody used in the inhibition assays was $6.67 \times 10^{-9}$ M ($= 1$ µg/ml), which was very close to the $K_D$ value ($6.6 \times 10^{-9}$ M, see Fig. 27) at which presumptive monovalent binding occurred. At $6.67 \times 10^{-9}$ M, it was proposed that the antibody molecules could only bind monovalently because they were sterically hindered. Incubation of antibody with GST-calnexin further inhibited any bivalent binding. Since there was only monovalent binding, it was not surprising to observe homogeneous binding in the competitive binding experiment; however, the antibody bound bivalently at low concentration and monovalently at high concentration in the saturation binding experiment, and therefore, heterogeneous binding was observed.

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Affinity ($M^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>Cell staining</td>
<td>weak signal $10^6$</td>
</tr>
<tr>
<td></td>
<td>strong signal $10^4$</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>weak signal $10^7$</td>
</tr>
<tr>
<td></td>
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<td>Immunoblotting</td>
<td>weak signal $10^6$</td>
</tr>
<tr>
<td></td>
<td>strong signal $10^8$</td>
</tr>
</tbody>
</table>

**Table 7.** Factors affecting the strength of antibody binding.

Table 7 lists the required affinities for several common immunochemical techniques [83]. The affinity of 1H82 is in the order of $10^8$ M$^{-1}$, and the antibody did yield a strong signal in immunoblotting. It is speculated that the 1H82 monoclonal antibody should be able to bind ER vesicles with good affinity.
4.3.3 Epitope mapping

Inserts containing various regions of calnexin cytoplasmic domain were all cloned into pGEX-4T3 expression vector except the one containing amino acids 14 to 46. After the attempt to clone the cDNA encoding amino acids 14 to 46 into pGEX-4T3 failed, we tried cloning it into another vector, pGEX-3X. Like pGEX-4T3, pGEX-3X is also a GST gene fusion expression vector. However, pGEX-3X contains a factor Xa instead of a thrombin recognition site downstream of the GST gene. Since the cDNA encoding amino acids 14 to 46 was successfully cloned into pGEX-3X but not pGEX-4T3, it was speculated that the combination of DNA sequence beginning from the thrombin recognition site in the vector to somewhere in the insert was toxic to bacterial cells (see Fig. 38).

Fig. 38. pGEX-4T3 expression vector ligated with amino acids 14 to 46 of the cytoplasmic domain of Chinese hamster calnexin. It was speculated that the combination of DNA sequence beginning from the thrombin recognition site in the vector to somewhere in the insert was toxic to bacterial cells.

Gregory et al. identified a cryptic bacterial promoter within the CFTR coding sequence [85]. Initial attempts to assemble a full-length CFTR coding sequence resulted in extensive rearrangements in the DNA. Construction of a full-length CFTR cDNA in a
low-copy-number plasmid avoided the deleterious effects of CFTR expression on *E. coli.* Therefore, it was conceivable that the combination of DNA sequence beginning from the thrombin recognition site in the vector to somewhere in the insert was harmful to *E. coli.*

The epitope of the 1H82 antibody was localized between amino acids 27 to 46 of the cytoplasmic domain of Chinese hamster calnexin. The amino acid sequence alignment of residues 28 to 48 of human calnexin and residues 27 to 46 of Chinese hamster calnexin is shown in Fig. 39. An asparagine at position 30 of Chinese hamster calnexin is altered from an aspartic acid in the human homologue and there is a deletion of glycine at position 38 in Chinese hamster calnexin. These variations presumably reduce the affinity of 1H82 for human calnexin. Nonetheless, 1H82 was able to detect both human and Chinese hamster calnexin (see Fig. 21a). Since it is not known how calnexin is folded in the ER membrane, it is difficult to predict whether the epitope on the native membrane-bound calnexin is accessible. We therefore sought to determine this empirically.

<table>
<thead>
<tr>
<th>Position</th>
<th>Calnexin</th>
<th>Human Calnexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 30 37 38 46</td>
<td>EEKNKDDEEEE - EEKLEEKQK</td>
<td>EEKDNGDEEEE - GEEKLEEKQK</td>
</tr>
</tbody>
</table>

**Fig. 39.** Amino acid sequence alignment of residues 28 to 48 of human calnexin cytoplasmic domain and residues 27 to 46 of Chinese hamster calnexin cytoplasmic domain.
4.3.4 Binding of antibody to ER in microsomal membranes

The results of this experiment indicated that 1H82 was able to bind native calnexin embedded in the membrane of ER. The binding of 1H82 to calnexin was specific, since it could be competed by an excess of GST-calnexin fusion protein. A murine immunoglobulin with the same subclass as 1H82 (IgG1·κ) was used as a negative control. The fact that the non-specific antibody did not bind ER microsomes provided further evidence that 1H82 bound ER microsomal membrane specifically by interacting with calnexin.

4.4 Summary of experimental findings

The development and characterization of a monoclonal antibody, 1H82, to the cytoplasmic domain of Chinese hamster calnexin were described in this thesis. 1H82 is of isotype IgG1 with kappa light chains. It recognizes both human and Chinese hamster calnexin in immunoblots. By comparing the intensity of the calnexin bands on immunoblots, 1H82 appears to have a higher affinity for Chinese hamster calnexin than the commercial polyclonal calnexin antibody. The affinity of 1H82 for Chinese hamster calnexin is in the order of $10^8$ M$^{-1}$. The epitope of 1H82 lies between amino acids 27 to 46 of the cytoplasmic domain of Chinese hamster calnexin. 1H82 was able to bind native calnexin embedded in the membrane of ER. Therefore, it was concluded that 1H82 could be useful in the immunoisolation of ER microsomes.
5 Future studies

Since 1H82 is proved to recognize native membrane-bound calnexin with good affinity, it can then be used for immunoisolating ER microsomes. Saucan et al. has successfully immunoisolated vesicular carriers operating between the Golgi complex and the basolateral plasmalemma using paramagnetic beads coated with an antibody against the last 11 amino acids of the poly IgA receptor tail [1]. It is worthwhile to try immunoisolating ER microsomes using different types of paramagnetic beads. Another method of accomplishing this goal would be to use protein G beads, since this method has been widely used for immunoprecipitation and the protocol is well established. If the immunoisolation procedure were successful, it would then be possible to investigate the protein components that associate closely with the immature ΔF508 CFTR and wild-type CFTR. Components that interact differentially with the mutant or the wild-type CFTR would be of particular interest. As mentioned before, chemical cross-linking can be employed to study protein-protein interactions. The long-term goal of this research is to identify and characterize proteins that interact closely with immature CFTR. The findings will hopefully enhance our understanding of the biosynthetic arrest of the ΔF508 CFTR.

Purification of ER microsomes may be useful in other areas of research. The basis of diseases such as marfan syndrome, α₁-antitrypsin deficiency and Tay-Sachs disease also involves misfolding or improper trafficking of proteins [86]. Therefore, a method of rapidly purifying ER can be a valuable tool in studying a number of diseases, and the basic biology of membrane protein biogenesis.
6 References


80. Accession no. CRL 1581; American Type Culture Collection, Rockville, MD.


