**UNIVERSITY OF SOUTHAMPTON**

**FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES**

**School of Biological Sciences**

# Targeting the Calcium ATPase to the Endoplasmic Reticulum

**by**

**Helen Rachel Watson**

**Thesis for the degree of Doctor of Philosophy**

**December 2009**

**UNIVERSITY OF SOUTHAMPTON**

## ABSTRACT

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# TARGETING THE CALCIUM ATPASE TO THE ENDOPLASMIC RETICULUM

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The sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps calcium from the cytoplasm into the lumen of the endoplasmic or sarcoplasmic reticulum (ER/SR), removing excess $Ca^{2+}$ from the cytoplasm and replenishing ER/SR $Ca^{2+}$ stores. SERCA is located in both the ER and the ER-Golgi intermediate compartment, and so is likely maintained in the ER by retrieval. To locate the ER retrieval signal(s) in SERCA, a series of chimeric calcium pumps have been constructed. Sections of SERCA were replaced with corresponding sequence from its plasma membrane counterpart; plasma membrane calcium ATPase (PMCA). Replacing the C-terminus of SERCA with corresponding PMCA sequence results in mistargeting of the protein to the plasma membrane. The opposite construct (consisting of PMCA with the C-terminus replaced by that of SERCA) is located in the ER, suggesting that the ER retrieval signal lies towards the C-terminus of the protein. Many of the chimeras built were located in the ER. This is likely to be due to protein misfolding in some cases. Attempts were made to detect the unfolded protein response in cells expressing chimeras by measuring levels of the chaperone protein BiP. BiP upregulation was only seen when the unfolded protein response was induced pharmacologically, and not in cells expressing chimeras. More subtle mutagenesis was then carried out to assess the role of the tenth transmembrane domain of SERCA in ER retrieval and CD8 reporter constructs were used to study the tenth transmembrane domains of SERCA and PMCA. The study then focussed on determining the mechanism by which SERCA is retrieved to the ER. Rer1p and BAP31 are both candidate receptors for the retrieval of SERCA. An antibody to two epitopes in human Rer1p was raised and characterised. Immunoprecipitation and cross-linking showed that although Rer1p appears not to interact with SERCA, BAP31 shows a potential interaction and therefore could be involved in the retrieval of the calcium pump to the ER.

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# Declaration of authorship

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declare that the thesis entitled:

Targeting the Calcium ATPase to the Endoplasmic Reticulum

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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# Acknowledgements

Thank you Malcolm for being a fantastic supervisor over the last few years. Your unwavering support and general cheerfulness has made working with you a real pleasure. Thanks for helping me out whenever I got stuck and for taking the time to explain so many things to me.

Thank you to all members of the AGL/JME lab, past and present. It's been an honour to work with you all. Thanks especially to John, Juan and Nik for helping me out when I needed you to and making me laugh the rest of the time.

Thank you to those with whom I have had useful discussions and who have given me helpful advice over the years, especially Tony and Vincent.

Thank you Hans for guiding me through the intricacies of confocal microscopy. Your patience and advice has been hugely appreciated.

Thank you to Lorraine and Diane for general reassurance and help with non-science related issues.

Thank you to everyone who has worked in office 5093 for good humour and distracting me from writing this thesis. I've enjoyed having you guys around. Thanks also to the postgraduate cohort of '06 – it's been fun!

Finally, thank you to all the friends, family members and housemates who have been around for me and have made the last three years so memorable.

# List of abbreviations

APS – ammonium persulfate

ATP – adenosine triphosphate

BAP – B cell antigen receptor associated protein (29 or 31 kDa)

BFA – brefeldin A

CaM – calmodulin

CART – cytoskeleton-associated recycling or transport

CFTR – cystic fibrosis transmembrane conductance regulator

ConA – concanavalin A

COP – coat protein (I or II)

DAPI – 4',6-diamidino-2-phenylindole

DMSO – dimethyl sulfoxide

dNTP – deoxyribonucleotide

DTBP – dimethyl-3,3'-dithiobispropionimidate

EGFP – enhanced green fluorescent protein

ER – endoplasmic reticulum

ERAD – ER-associated degradation

ERES – ER exit sites

ERGIC – ER-Golgi intermediate compartment

ERGIC-53 – ER-Golgi intermediate protein of 53 kDa

ESCRT – endosomal sorting complex required for transport

FITC – fluorescein isothiocyanate

GAP – GTPase-activating protein

GEF – guanine nucleotide exchange factor

GPI – glycosylphosphatidylinositol

GTP – guanosine triphosphate

HRP – horseradish peroxidase

IgG – immunoglobulin G

kDa – kilodalton

LB – Luria-Bertani

MHC – major histocompatibility complex

OD – optical density

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PDI – protein disulfide isomerase

PKA – protein kinase A

PLB – phospholamban

PMCA – plasma membrane calcium ATPase

Rer1p – retention of ER proteins

SDS – sodium dodecyl sulfate

SDS-PAGE – SDS polyacrylamide gel electrophoresis

SERCA – sarco/endoplasmic reticulum calcium ATPase

SLN – sarcolipin

SNARE – soluble N-ethylmaleimide sensitive factor association protein receptor

SNX – sorting nexin

SPCA – secretory pathway calcium ATPase

SR – sarcoplasmic reticulum

SRP – signal recognition particle

TEMED – tetramethylethylenediamine

TGN46 – trans-Golgi network protein of 46 kDa

TPC – trans-Golgi to plasma membrane carrier

TRAM – translocating chain-associated membrane protein

UPR – unfolded protein response

YFP – yellow fluorescent protein

# 1. Introduction

## 1.1 Eukaryotic cells, organelles and membrane proteins

Within the plasma membrane surrounding eukaryotic cells lie distinct membrane bound compartments: organelles. Each organelle contains a specific complement of proteins, equipping it to carry out its particular role within the cell. Cell viability depends on the correct functioning of its proteins and organelles and so the billions of protein molecules inside each eukaryotic cell[1] must be delivered to the appropriate compartment and maintained there. With the exception of a handful of proteins encoded by the mitochondrial genome, all proteins begin synthesis on cytosolic ribosomes. Signals within the proteins then direct them from the cytoplasm to their target destination within the cell. Each organelle employs specific machinery to recognise these sequences and in some cases, recognise additional sorting signals which determine whether a protein resides there or is passed to another compartment. The external (plasma) membrane of the cell and the organellar membranes within it each contain a specific compendium of membrane proteins. Membrane proteins play many and diverse roles in eukaryotic cells. Channels and transporters move substances across membranes, receptors receive and propagate signals from outside and inside the cell, and certain proteins in the plasma membrane play a role in cell adhesion. In addition, the display of some membrane proteins on the surface of cells or organelles functions as an identity tag which can be recognised by proteins involved in intracellular protein traffic[1,2].

## 1.2 Overview of the secretory pathway

The secretory pathway consists of a series of membrane bound organelles linked by membrane traffic in forwards (anterograde) and backwards (retrograde) directions. Approximately 30% of all cellular proteins enter the secretory pathway by insertion into the endoplasmic reticulum (ER)[3]. Some remain there, while others are transported by anterograde transport to the ER-Golgi intermediate compartment (ERGIC) then the Golgi (through the cis, medial and trans cisternae) and into secretory vesicles destined for the plasma membrane. Proteins arrive at endosomes predominantly by retrograde traffic from the cell surface, and the route to

lysosomes is via endosomes. This incredibly complex pathway is simplified in figure 1.1. Proteins can also be maintained in any compartment in the secretory pathway either by mechanisms of retention or retrieval from downstream compartments. Although some trafficking pathways within the secretory pathway are understood, many of the protein components and mechanisms involved are still yet to be elucidated[4,5]. In this review I will discuss in detail the initial targeting events that cause protein delivery to the ER and how ER proteins are then maintained in that compartment, focussing on the targeting and trafficking of an ER calcium pump.

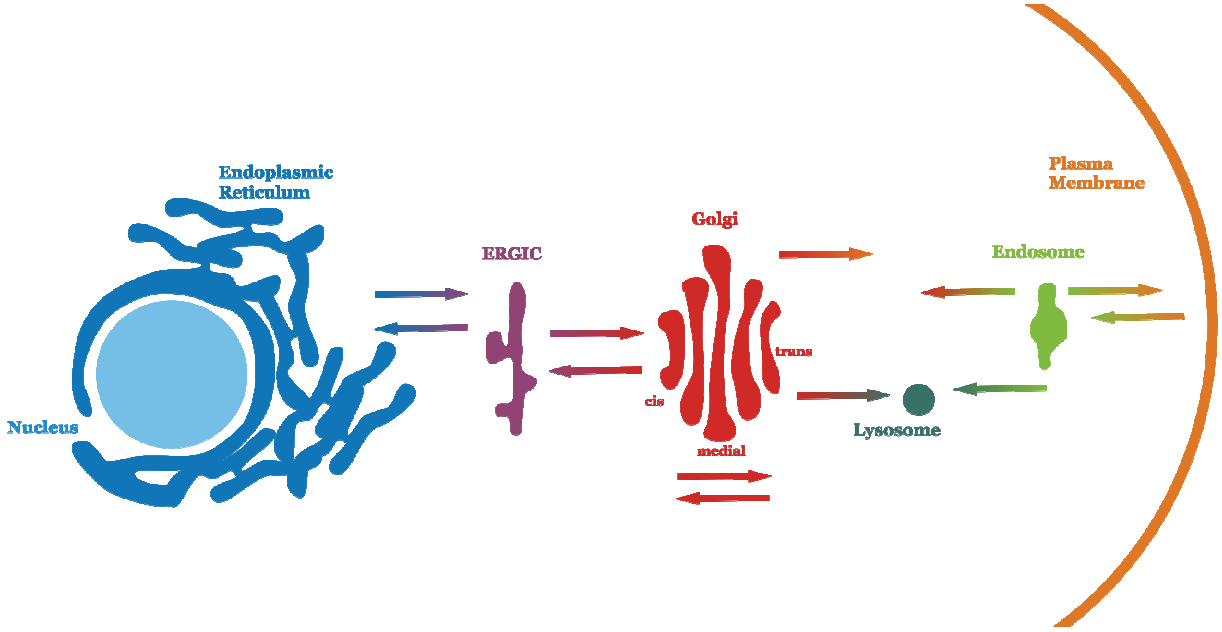



**Figure 1.1 The secretory pathway**

A simplified diagram of the secretory pathway showing forward (anterograde) and backward (retrograde) traffic. Arrows are coloured according to the donor and acceptor compartments of the transport complex. Based on Bonifacino, J.S. and Glick, B.S., 2004[4].

## 1.3 Calcium signaling and calcium ATPases

Calcium ($Ca^{2+}$) is a vital intracellular messenger involved in many different cellular signalling pathways including those causing muscle contraction, gene transcription and cell death[6]. In order to function as such a ubiquitous messenger, effective organisation of calcium concentrations within the cell is critical. At rest, the cytoplasmic calcium concentration is approximately 100 nM, with the cellular calcium pool concentrated in internal stores.

Extracellular calcium concentrations are around 20,000 times higher than intracellular concentrations[7]. The cell devotes significant amounts of ATP to maintaining these large gradients between the cytosol and external milieu. Any calcium released into the cytoplasm for signalling purposes must be quickly removed, either into internal stores or out of the cell entirely, as prolonged increases in cytoplasmic calcium can result in aberrant signalling and cell death[6]. Calcium was selected early in evolution over other abundant cations (magnesium, sodium and potassium) for use as a biological messenger, capable of triggering a vast array of cellular events. Due to their size, calcium ions have the ability to coordinate to oxygen atoms in binding sites with varying bond lengths, as opposed to smaller magnesium ions which favour a perfect octahedral site. This flexibility suits biological systems, and allows calcium to bind to a range of often irregularly shaped binding sites found in target proteins[8].

## Calcium signaling

The question of how calcium is able to exert specific effects, given its large variety of binding partners, is an important one. Certainly the varying affinities of different proteins for calcium allow some discrimination between calcium signals of different amplitudes, but other factors are also involved. Calcium enters the cytoplasm, through influx channels, from the internal stores (endoplasmic or sarcoplasmic reticulum and mitochondria) or from outside the cell. Spatially isolated calcium ‘sparks’ or ‘puffs’ can cause a local effect (termed an elementary event) in the immediate vicinity, giving spatial control of the signal. An example of an effect caused by such a local signal is the release of secretory vesicles, such as in the case of neurotransmitter release at synapses which is triggered by a local influx of calcium upon the arrival of an action potential[6,7]. These initial calcium sparks can also induce global calcium waves which proliferate throughout the cell. Skeletal muscle contraction is triggered by release of calcium from the sarcoplasmic reticulum (SR; specialised ER found in muscle cells) upon membrane depolarisation. Voltage dependent calcium channels in T-tubules (invaginated sections of the plasma membrane close to the SR) let calcium in as the muscle cell is depolarised, causing further release of calcium from the SR through ryanodine receptors. This elevation in cytoplasmic calcium concentration causes shortening of the sarcomeres

(contractile units of muscle) by allowing myosin and actin filaments to slide together, having the overall effect of shortening the muscle filaments and causing contraction[9,10].

Oscillations in cytoplasmic calcium concentrations can be detected by some proteins. Calmodulin (CaM) is a conserved calcium binding protein containing four calcium binding sites in EF-hand motifs. Upon calcium binding, CaM transforms into a more elongated, active conformation. Active CaM has a wide variety of target proteins including calcium-calmodulin-dependent protein kinase II (CaMKII). CaMKII is able to detect the frequency of calcium spikes in the cytoplasm and translate that information into differing levels of kinase activity, in order to phosphorylate target proteins. Upon CaM binding, CaMKII is autophosphorylated causing activation of the enzyme. This autophosphorylation can maintain the activity of the enzyme as the cytosolic calcium decreases, giving the protein an ability to 'remember' calcium spikes. Repeated calcium spikes can therefore increase the activity of the enzyme in a stepwise fashion, allowing specific interpretation of the frequency of calcium signals[11].

## Calcium ATPases

Regulation of cytoplasmic calcium and refilling of internal stores of calcium are both crucial for the effectiveness of calcium signalling, upon which so many processes rely. The sarco/endoplasmic reticulum calcium ATPases (SERCAs), secretory pathway calcium ATPases (SPCAs) and plasma membrane calcium ATPases (PMCAs) work in concert to maintain calcium concentrations within internal calcium stores, compartments of the secretory pathway and in the cytoplasm[12-14]. These enzymes all belong to the P-type ATPase family (named after the phosphorylated intermediate formed during the catalytic cycle) and are similar to each other in both structure and function[13].

## SERCAs

SERCA is, at least structurally, the most well characterised enzyme of this group of P-type ATPases. The SERCA pumps were initially described as a 'relaxing factor' which could allow muscle cells to relax following contraction, but were later shown to be involved in calcium signalling events in all cells[15,16]. SERCA uses the energy generated by ATP hydrolysis to

drive calcium ions against their concentration gradient across the ER or SR membrane, into the lumen. By virtue of its high abundance in skeletal muscle tissue, SERCA can be purified in relatively large amounts. This has allowed extensive structural analysis by X-ray crystallography; something that still eludes many membrane proteins, including PMCA. The first crystal structure of SERCA1 was published by Toyoshima *et al.* in 2000 and paved the way for subsequent structural studies, which followed in the next few years[15,17,18]. The first structure provided a snapshot of the calcium pump in a calcium bound E1 conformation, and confirmed structural features previously suggested, including ten transmembrane segments, two calcium ion binding sites and three distinct cytoplasmic domains[17]. As a result of this, and the subsequently elucidated structures, we now have a collection of freeze-frames of SERCA in different conformations, which have hugely increased our understanding of the conformational changes that occur during the catalytic cycle of the enzyme[18].

The sarcoplasmic reticulum calcium ATPase (SERCA1a) contains ten transmembrane helices, small lumenal loops, and a large cytoplasmic domain which can be subdivided into three functionally and spatially distinct domains (figure 1.2). The P (phosphorylation) domain contains the conserved residue Asp351, which is phosphorylated upon ATP binding. The ATP binding site is located in the N (nucleotide binding) domain of the enzyme. The structure of the N domain showed Phe487, Lys515 and Lys492 positioned close to the bound ATP analogue, TNP-AMP, all of which are residues previously shown by mutagenesis to be important for nucleotide binding. The smallest of the cytoplasmic domains, the A (actuator) domain, undergoes significant movement during calcium transport and is believed to assist in dephosphorylation of the enzyme following calcium release[17,18]. The ten transmembrane helices (M1-M10) of SERCA differ in length and orientation with respect to the membrane surface. The two calcium ion binding sites are located within the transmembrane domain, and binding of calcium is sequential. The first calcium ion binds between M5, M6 and M8 and the second binds very close to (almost 'on') M4[18]. Prior to the availability of the crystal structure of the ATPase, site directed mutagenesis of residues in M4, M5, M6 and M8 had implicated these helices in calcium binding[19]. The bound calcium ions are stabilised in their binding sites by residues Asn 768, Glu771 (M5), Asn796, Thr799, Asp800 (M6) and Glu908 (M8) which contribute to a hydrogen bonding network, coordinating the ions[18].

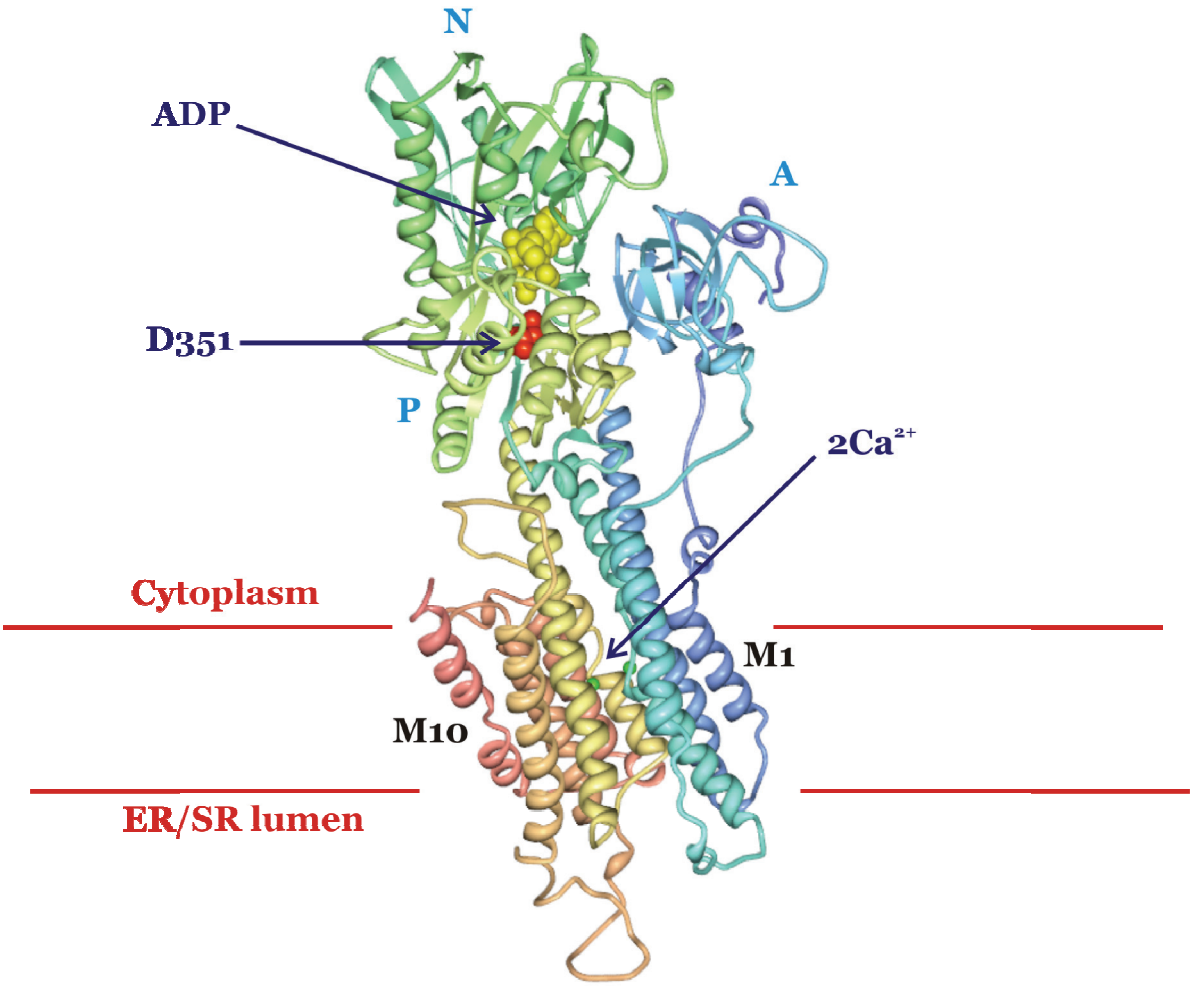



**Figure 1.2 X-ray crystal structure of SERCA in the E1 $Ca^{2+}$ ADP bound state**

This structure of SERCA in the E1 conformation is bound to two $Ca^{2+}$ (shown in green) and ADP (shown in yellow space fill). The three cytoplasmic domains; actuator (A), phosphorylation (P) and nucleotide binding (N) domains are labelled. The phosphorylated residue, D351, is shown in red space fill. The protein is coloured from blue at the N-terminus to red at the C-terminus. Based on Toyoshima 2008[18]. PDB code 2ZBD, rendered using PDB Protein Workshop.

Our understanding of the mechanistic details of the catalytic cycle of SERCA has been dramatically improved by the availability of over 20 crystal structures of the enzyme in different conformations. The E1-E2 mechanism is the generally accepted model for the transport of calcium into the ER or SR lumen by SERCA (figure 1.3)[18]. This model is based upon the transition of high affinity calcium binding sites facing the cytosol to low affinity calcium binding sites facing the ER or SR lumen. Two calcium ions enter SERCA from the cytoplasmic side and bind to the high affinity sites of the E1 state. This causes the P and A domains to separate, allowing ATP to reach and phosphorylate Asp351, following nucleotide binding to the N domain. Transfer of the γ-phosphate of ATP to Asp351 causes occlusion of the bound calcium ions in their binding sites by the M1 helix, preventing exit into the cytoplasm. Phosphorylation causes transport of calcium ions into the lumen by breaking the salt bridge between Lys684 and Asp351. This moves two small helices (P1 and P2) close to the loop between M6 and M7, causing movement of the M6-M7 loop and subsequent changes in the packing of the M6 and M7 helices and affinity of the calcium binding sites. The A domain rotates, moving M4, M5 and M6, and removing the calcium binding sites. ADP is released upon phosphorylation, and the E2 state is formed. The destruction of the calcium binding sites, by movement of the M6-M7 loop and A domain rotation, results in release of the calcium ions into the lumen, gated by movement in M1 and M2. Further rotation of the A domain allows dephosphorylation and closure of the luminal calcium release gate, returning the pump to the E1 state in preparation for the next cycle. These large conformational changes that SERCA undergoes enable the protein to couple phosphorylation and calcium transport; two processes that are spatially separated by relatively large distances within the protein. Evidence from the numerous snapshots of the protein we have from crystal structures shows that the C-terminal region of SERCA undergoes the least structural changes during the catalytic cycle[12,18,20].

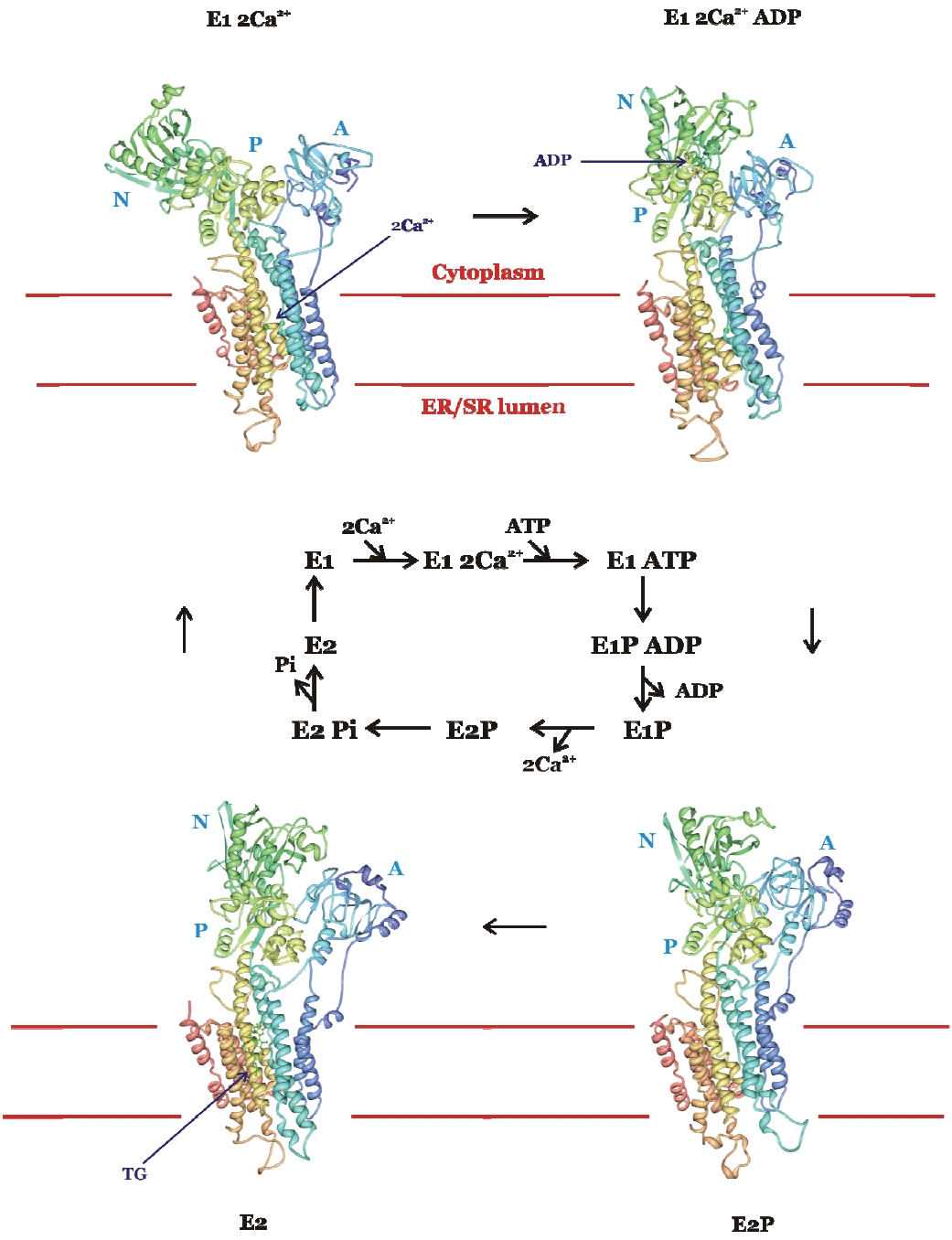



**Figure 1.3 Reaction scheme of SERCA**

Structures are coloured from blue to red from the N- to C-termini. All putative intermediates are shown in the centre, with four shown in structural detail. The binding site of the inhibitor thapsigargin (TG) is shown. Based Toyoshima, C. 2008[18]. PDB codes are 1SU4 (E1 $2Ca^{2+}$), 2ZBD (E1 $2Ca^{2+}$ ADP), 2ZBE (E2P) and 2AGV (E2), rendered in PDB Protein Workshop.

There are three human SERCA pump isoforms; SERCA1, SERCA2 and SERCA3, encoded by three separate genes; *ATP2A1*, *ATP2A2* and *ATP2A3* respectively. RNA splicing of the transcripts of these genes produces SERCA1a/b, SERCA2a/b/c and SERCA3a/b/c/d/e/f isoforms. Different SERCA isoforms are adapted to different functions and vary in the cell types in which they are expressed. SERCA1a is the predominant isoform in fast-twitch skeletal muscle and functions as the 'relaxing factor' to reduce calcium concentrations in the cytoplasm of myocytes and bring about relaxation[16]. SERCA1b plays the same role in neonatal muscle[15,21]. SERCA2a is found in cardiac muscle and slow-twitch skeletal muscle. The ubiquitous SERCA2b is expressed in all tissues and functions as a house-keeping ER calcium pump. The RNA splicing of SERCA2b creates an eleventh transmembrane domain, resulting in a luminal C-terminus in contrast to the cytoplasmic C-termini of the other SERCA isoforms. It has been proposed that this extended C-terminus may allow regulation of the activity of the pump[15,21-23]. SERCA3 is present in several cell types including platelets, lymphocytes, Purkinje neurons, intestinal epithelial cells and endothelial cells. SERCA3 has a lower affinity for calcium than either SERCA1 or SERCA2, and its unusual distribution in specialised cell types may reflect a specific function in cells with high cytoplasmic calcium concentrations[15,24]. SERCA3a, b and c, produced by differential splicing of the ATP2A3 gene, show variations in their C-termini and in their affinities for calcium[25]. The more recently described SERCA3d is expressed in many more tissues than the other SERCA3 isoforms, suggesting that like SERCA2b, SERCA3d has a house-keeping function in many tissues[26].

Why are so many isoforms of SERCA required? Different cell types have specific requirements for calcium pumping into the ER or SR, and this corresponds to the SERCA isoform(s) present in each cell type. For example, the fast turnover number of SERCA1a renders it suitable for fast-twitch muscle cells which require rapid removal of calcium from the cytoplasm and replenishment of SR stores, in preparation for the next contractile event. In contrast, SERCA2b is used in non-muscle cells as a house-keeping pump with a slower turnover but higher affinity for calcium ions, allowing it to function at low cytoplasmic calcium concentrations[15]. SERCA2a and SERCA2b are co-expressed in pancreatic cells, but are physically separated within the cell, possibly indicating that different calcium pools are

spatially separated and maintained by different SERCAs, allowing precise control of different calcium signalling events[27].

Phospholamban (PLB) is a small (52 amino acids), C-terminally anchored, membrane protein located in the SR of cardiac, slow-twitch and smooth muscle cells[15,28,29]. PLB mediates the 'fight or flight' response in cardiac muscle as a result of β-adrenergic stimulation. The interaction of PLB with SERCA2 has an inhibitory effect on the pump, which is reversed upon phosphorylation of PLB by protein kinase A (PKA) or calmodulin kinase. cAMP produced as a result of β-adrenergic stimulation activates PKA causing phosphorylation of PLB and subsequent dissociation of PLB from SERCA2, resulting in an increased affinity of the calcium pump for calcium ions and a more rapid relaxation of the muscle[30]. PLB binds to a groove in the transmembrane region of SERCA formed by M2, M4, M6 and M9, interfering with calcium binding by M4 and M6 in order to inhibit the pump. The cytosolic portion of PLB interacts with that of SERCA, compromising the large conformational changes that SERCA requires for activity[29].

Sarcolipin (SLN) is another short (31 amino acids) membrane spanning protein which can be considered a functional PLB homologue due to conservation of sequence and function between the two proteins. SLN modulates SERCA1 activity in fast-twitch muscle and cardiac muscle[31]. SLN expression in slow-twitch muscle is much lower, mirroring the distribution of SERCA1 in this cell type[32]. SLN has the same effect on SERCA as PLB and is thought to interact with the same site in SERCA[31]. Unlike PLB, SLN is not regulated by phosphorylation, but instead its effect on SERCA is modulated by levels of SLN in the SR membrane[15]. Heterodimers of PLB and SLN have superinhibitory effects on SERCA[31]. The increase in protein interactions in a ternary complex of PLB, SLN and SERCA renders it more stable than complexes of the calcium pump with only one modulator protein, making the inhibition stronger and longer lived. This superinhibitory effect is also attributed to the disruption of PLB pentamers in the membrane and increased PLB/SLN dimers. PLB pentamers are most probably unable to inhibit SERCA due to their size, whereas the heterodimer with SLN is superinhibitory, causing increased inhibition of SERCA[31].

## Calcium in the secretory pathway

Aside from the obvious requirement for calcium in the ER and SR, the cell also needs to maintain high concentrations of calcium in the ERGIC and Golgi[13,33]. The ERGIC contains SERCA pumps and has the capacity for both calcium intake from the cytosol and storage of calcium by binding proteins. Furthermore, specific inhibition of SERCA by thapsigargin results in the secretion of KDEL (Lys-Asp-Glu-Leu) containing proteins that are normally retrieved from the ERGIC by the KDEL receptor (discussed in detail later in this review), indicating that calcium is required for protein traffic between the ERGIC and ER[33]. Calcium concentrations in the Golgi are high, and are maintained by both SERCAs and the secretory pathway calcium ATPases, SPCAs. Like SERCAs, the SPCAs are P-type ATPases with very similar overall domain architecture to the SERCA pumps[13]. SERCA is located in the cis-Golgi, whereas SPCA is the dominant calcium pump in the trans-Golgi. How this differential targeting of these similar proteins to different parts of the Golgi is achieved is not understood, but one possibility is that they are able to sense the increasing levels of cholesterol present from the proximal to distal sides of the Golgi, and distribute themselves accordingly[13]. Similarities in symptoms of the diseases caused by mutations in SERCAs or SPCAs show that regulation of calcium both in the cytoplasm and throughout the secretory pathway is critical. Loss of one copy of the human SERCA2 (*ATP2A2*) gene results in Darier disease, characterised by loss of skin cell adhesion and skin lesions[34]. Patients with Hailey-Hailey disease, caused by a loss-of-function mutation in one copy of the human *ATP2C1* gene (encoding the SPCA1 pump), exhibit very similar symptoms to those seen in Darier disease[13]. The symptoms of these diseases and the similarities between them have been, in part, attributed to the decreased abundance in intracellular calcium stores (either in the ER or Golgi), and the resulting disruption of glycosylation and folding of cell surface proteins on their journey through the secretory pathway[13]. Mutation of the human *ATP2A1* gene (corresponding to the SERCA1 isoform) causes Brody disease which has entirely different symptoms to the two diseases described above. Brody disease patients show muscle cramping, consistent with the role of SERCA1 as a 'relaxing factor' in skeletal muscle[9]. The similarities and differences in the physiological roles of ER/SR and secretory pathway calcium pumps are elegantly illustrated by the study of their associated diseases, which show the diverse consequences of mutations in the genes encoding them.

## PMCAs

The plasma membrane calcium ATPases (PMCAs) are also members of the P-type ATPase family, and their role is to transport calcium, against its concentration gradient, out of the cytoplasm and into the extracellular milieu[35]. Although the crystal structure of PMCA is yet to be solved, its sequence and functional similarity to SERCA allows structural predictions to be made (see figure 1.4). The C-terminus of PMCA is longer than that of SERCA and contains a calmodulin binding domain, which permits regulation of the pump as calcium concentrations in the cytoplasm fluctuate. A rise in calcium in the cytoplasm increases the proportion of calcium bound calmodulin. This activated calmodulin binds to PMCA, causing displacement of an autoinhibitory domain of the pump and increasing the affinity of PMCA for calcium by about 10 to 20 fold[35].

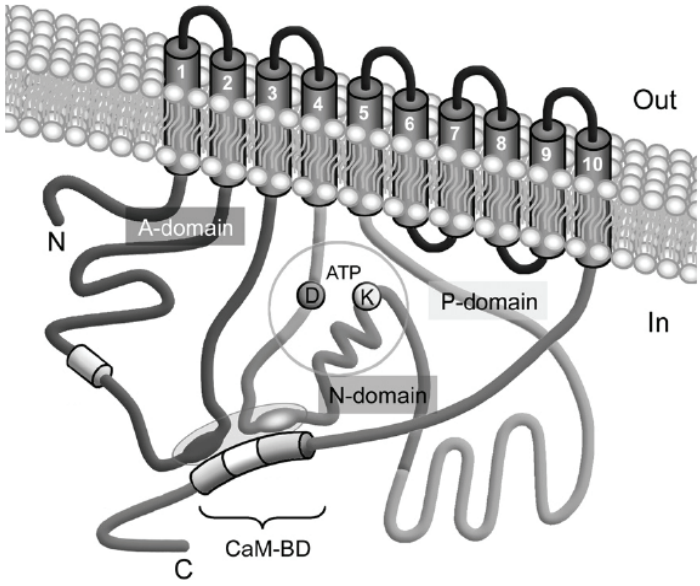



**Figure 1.4 Predicted architecture of PMCA**

PMCA contains three cytoplasmic domains (A, P and N) and ten transmembrane domains. This figure shows the calmodulin unbound state of the pump, in which the calmodulin binding domain (CaM-BD) binds PMCA and acts to inhibit the pump. Upon activation by calcium, calmodulin is able to bind PMCA at the CaM-BD, displacing the domain from its inhibitory position and activating the pump. Adapted from Di Leva, F. *et al.* (2008)[35].

In humans and rats, and most probably other mammals, PMCA RNA undergoes alternative splicing, resulting in production of various isoforms from four different genes[36]. It is assumed that, like SERCA, the varying isoforms are required to fulfil different functions, as they differ in characteristics including affinity for calcium and sensitivity to activation by calmodulin[36]. PMCA1 and 4 are most likely the house-keeping isoforms, as they are expressed in all tissues. Loss of both copies of the PMCA1 gene causes embryolethality in mice, supporting the hypothesis that PMCA1 is the crucial house-keeping pump. PMCA2 and 3 are more specifically expressed in certain tissue types and are considered to be involved in specific signalling events rather than functioning as house-keeping enzymes. For example, PMCA2 plays a role in signalling within the hair cells of the auditory system, loss of which is manifested as deafness in PMCA2 null mice[37]. The same question arises here as when discussing the Golgi and ER calcium pumps; what are the differences between the proteins that cause SERCA to locate primarily to the ER, SPCA to the Golgi and PMCA to the plasma membrane? This question will be addressed later in this review when I focus on how the SERCAs are maintained in the ER.

## 1.4 Endoplasmic reticulum protein targeting

ER resident proteins such as SERCA, as well as proteins destined for other compartments of the secretory pathway or plasma membrane such as SPCA and PMCA, all undergo a common targeting step to the ER[3,38]. All ER targeted proteins begin synthesis on cytosolic ribosomes. The majority are targeted to the ER during translation and complete their synthesis at the ER membrane. A small subset of proteins, including the SERCA modulator proteins PLB and SLN, are targeted following complete synthesis in the cytoplasm. Information contained within sequences of ER targeted proteins is recognised by protein and RNA machinery in the cytoplasm and brings about their targeting to the ER membrane[28,39,40].

The signal hypothesis proposed by Blobel, Sabatini and Dobberstein in the 1970s was an important landmark in our understanding of protein targeting to the ER[41,42]. The initial hypothesis suggested that a ‘unique sequence of codons’ at the very beginning of a translating mRNA causes a translating ribosome in the cytoplasm to associate with the ER membrane,

targeting the nascent protein to the ER[42]. This was thought to occur via a 'binding factor' able to bind both the ribosome and a 'factor on the membrane' thus allowing attachment of the translating ribosome to the ER membrane[41]. This requirement for a factor located exclusively on the ER membrane provides a simple answer as to how ribosomes and their nascent chains are specifically targeted to the ER. The N-terminal targeting sequence (or signal sequence) is now known to be recognised by the signal recognition particle (SRP) which binds to the signal sequence, the ribosome and the SRP receptor at the ER membrane. Binding of the SRP causes a pause in translation as the complex is targeted to the SRP receptor in the ER membrane. The ribosome then docks onto the translocon (a pore in the ER membrane) and following dissociation of the SRP and the SRP receptor, the majority of the protein is then synthesised at the ER membrane[43,44]. This mode of targeting is known as co-translational targeting, as opposed to post-translational targeting in which the full length protein is made before its delivery to the ER membrane[40]. In this discussion, I will focus on co-translational ER targeting, and will review post-translational targeting at the end of the section.

## Signal sequences

The signal hypothesis has held up remarkably well against the onslaught of 35 years of research by biochemists and cell biologists in the field. Detailed analysis of the signal sequence (the 'unique sequence of codons' initially suggested) has shown that relatively diverse sequences can be recognised and cause ER targeting[42,45,46]. An analysis of random sequences by Kaiser *et al.* in 1987 demonstrated that approximately one-fifth of random peptides fused to yeast invertase resulted in secretion of the protein, highlighting the low sequence specificity with which signal sequences are recognised[45]. Although no specific sequence motifs seem to be required for recognition, signal sequences do share these common characteristics: a sufficiently hydrophobic stretch (20-30 amino acids), a basic region at the N-terminus and a polar domain at the C-terminal end of the hydrophobic section[44,47]. In the 1975 paper which expanded on the signal hypothesis, Blobel and Dobberstein suggested that the signal sequence can be cleaved from the new protein following synthesis, or in some cases is left attached if it is required for the activity of the protein[42]. We now know that there are two possible fates for the N-terminal signal sequence following targeting of the protein to the ER

and the completion of protein synthesis. It can be removed by signal sequence peptidase, or can be retained and form a transmembrane domain in the mature protein[47]. Cleaved signal sequences are present in all soluble and secreted proteins targeted to the ER as well as many (but not all) membrane proteins[48]. The amino acids on the N-terminal side of the signal peptidase cleavage site (-1 and -3 with respect to the cleavage site) as well as the length of the hydrophobic stretch and the hydrophilicity of the domain N-terminal to the hydrophobic domain can all influence the likelihood of signal sequence cleavage[47,48]. Although it is generally understood that signal sequences are degraded by the proteasome following their detachment from the mature protein, there is evidence that they fulfil post-cleavage roles. For example, signal sequence derived peptides can be presented at the plasma membrane by MHC (major histocompatibility complex) class I molecules as part of a reporting system whereby surface expression of self-peptides prevents attack by cells of the immune system[49].

An uncleaved signal sequence that forms a transmembrane domain in the mature protein is termed a 'signal-anchor' sequence as it fulfils both signalling and anchoring roles during targeting and insertion into the membrane[50]. These signal sequences bring about targeting to the ER in the same way and by using the same machinery as cleaved signal sequences[51]. In order to remain attached to the protein and insert into the membrane, signal-anchor sequences must be void of signal peptidase cleavage sites and the hydrophobic stretch of amino acids must be of the correct length to span the lipid bilayer. Signal-anchor sequences can insert into the bilayer with either their N- or C-termini in the ER lumen to form type I and type II membrane proteins respectively. Charged residues on the N-terminal side of the hydrophobic region and a shorter hydrophobic region increase the chance of type II topology, whereas type I topology is favoured by a longer hydrophobic region and few or no charges at the N-terminus[47].

## The signal recognition particle (SRP)

The 'binding factor' that recognises the signal sequence of a nascent protein was discovered by Walter and Blobel almost ten years after the signal hypothesis was published[41,52]. They purified six proteins required for the co-translational translocation of ER targeted secretory

proteins, a complex which would be coined the 'signal recognition protein' (SRP)[52]. Further work revealed that the SRP was in fact made of both proteins and RNA, and was renamed the 'signal recognition particle'[53].

We now have a much clearer view of the structure and function of the SRP. The mammalian SRP comprises six proteins and a RNA molecule of 300 nucleotides. The SRP can be split into two domains: the Alu domain (containing the SRP9 and SRP14 proteins) and the S domain (the remaining four proteins SRP19, SRP54 and the heterodimer SRP68/72). The M domain (contained within the SRP54 subunit of the S domain) is the site of signal sequence binding. The M domain contains a hydrophobic pocket lined with methionine residues which, due to their flexible side chains, allow the binding of varied hydrophobic signal sequences. Interactions between basic residues at the N-terminus of the signal sequence and the negatively charged SRP RNA strengthen the binding of the signal sequence to the SRP[39,43]. There are two main contact sites between the ribosome and the SRP. One is between the SRP9/14 heterodimer in the Alu domain and small ribosome subunit rRNA and the other is an interaction between the SRP RNA in the Alu domain and protein and rRNA in the large ribosomal subunit. There is also an interaction between the S domain of SRP and proteins near the exit tunnel of the ribosome[43].

As a signal sequence begins to protrude from the ribosomal exit tunnel (after synthesis of around 70 amino acids if the signal sequence is at the very N-terminus of the protein), the SRP binds to the nascent chain and to the ribosome and causes a pause in translation[39,53]. It is thought that the SRP can bind any ribosome and any nascent chain with a low-affinity in order to 'sample' for a signal sequence protruding from the ribosome[39,54]. Recent work suggests that the SRP can detect the presence of signal anchor sequences hidden within the ribosome exit tunnel, causing tighter binding of the SRP to the ribosome than the low affinity sampling interaction, in preparation for the exit of the hydrophobic sequence[54]. Once the signal sequence has left the exit tunnel of the ribosome and has bound to the M domain of the SRP, the SRP-ribosome-nascent chain complex is competent for targeting to the ER (figure 1.5)[55]. This targeting event is mediated by the SRP receptor, an ER membrane associated heterodimeric protein consisting of one α- and one β-subunit. The β-subunit has one

membrane spanning helix, anchoring the receptor in the membrane and the α-subunit is cytoplasmic. Three G-proteins regulate the cycle of SRP-ribosome-nascent chain binding to the SRP receptor. Two of these are in the SRP receptor (one in each of the α- and β-subunits) and one is in the SRP itself (within SRP54). In order for the SRP to bind the SRP receptor, all three G-proteins must be in the GTP bound form. The GTP bound SRP54 subunit binds to the GTP bound SRP receptor α-subunit, which is anchored to the ER via interaction with the GTP bound β-subunit of the receptor. The nascent chain is transferred to the translocon (a pore in the ER membrane close to the SRP receptor), GTP hydrolysis causes dissociation of the ribosome-SRP-SRP receptor complex, and translation is resumed through the translocon[56].

Signal sequence mediated targeting, SRPs and SRP receptors are extremely well conserved in evolution, and exist in eukaryotes and prokaryotes[39]. In bacteria, N-terminal signal sequences can result in protein delivery to the plasma membrane (as opposed to the ER in eukaryotes)[44]. The structure of the SRP and SRP receptor are similar in prokaryotes and eukaryotes, the main differences being that the prokaryotic SRP receptor is only peripherally associated with the membrane (rather than containing a membrane spanning subunit as in eukaryotes)[39]. In higher eukaryotes, co-translational, SRP dependent targeting is the dominant mechanism for protein delivery to the ER, whereas yeast and prokaryotes appear to favour post-translational targeting, requiring ATP dependent chaperones and not SRP[40,57,58]. Tail-anchored proteins are a class of eukaryotic proteins (including the SERCA modulator peptides PLB and SLN)[28] which are post-translationally delivered to the ER membrane, due to the proximity of the only hydrophobic sequence to the C-terminus of the protein. This sequence (which acts as the signal sequence and membrane anchor) does not leave the ribosome exit tunnel until synthesis is complete, and so cannot be recognised by targeting factors until this point. Targeting of tail-anchored proteins to the ER appears to require ATP dependent chaperone proteins, and the involvement of the SRP in some cases, although the pathway (or pathways) by which this occurs is not well understood[40,59,60].

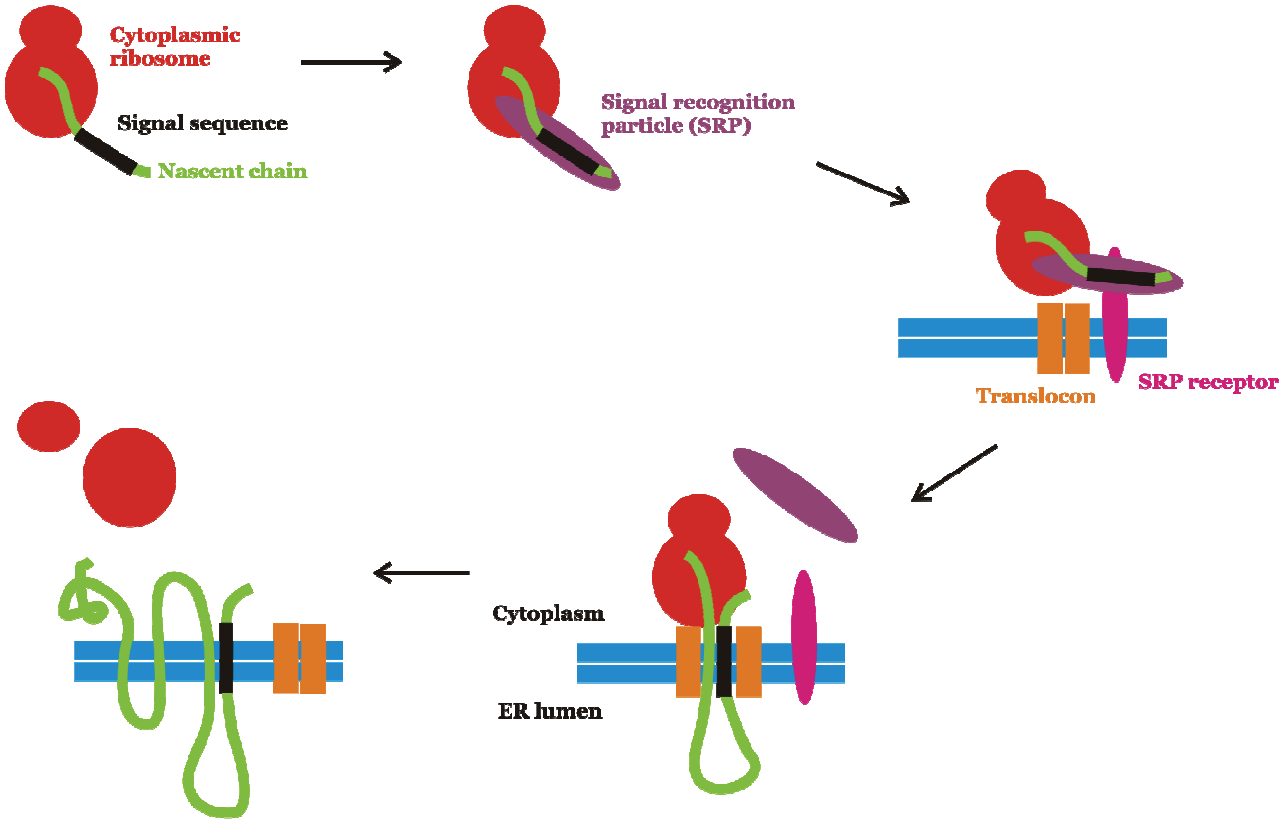



**Figure 1.5 SRP dependent ER protein targeting**

The main events in SRP dependent protein targeting are summarised. Protrusion of a signal sequence from a cytoplasmic ribosome allows binding of the SRP, resulting in a pause in protein synthesis. The SRP then binds the SRP receptor, targeting the ribosome and nascent chain to the ER. Once at the ER membrane, the SRP and SRP receptor dissociate from the ribosome and nascent chain, and translation is resumed through the translocon into the ER lumen. Following completion of synthesis, soluble proteins are released into the lumen, and membrane proteins (as shown in this figure) move laterally into the ER membrane. Based on Egea, P.F. *et al.* (2005)[39].

## The translocon

The eukaryotic Sec61 translocon (SecYEG in prokaryotes) is a heterotrimer consisting of the large Sec61 subunit (SecY in prokaryotes) with ten transmembrane domains and the two smaller Sec61β and Sec61γ subunits (prokaryotic SecG and SecE respectively)[61]. Obtaining the X-ray crystal structure of the SecYEβ translocon from the archaea *Methanococcus jannaschii* was an important step forward in understanding this protein complex, and how it is able to translocate proteins across the ER membrane in eukaryotes, or plasma membrane in prokaryotes[62]. The channel was shown to consist of two linked transmembrane sections (transmembrane domains 1-5 and 6-10 of the large Y subunit), a single transmembrane domain not essential for function (β subunit) and a transmembrane domain linked to an amphipathic helix on the cytoplasmic face of the membrane, clamping together the two halves of the large Y subunit (the E subunit). Comparison of this structure with the structure of the bacterial SecY translocon from *E. coli* (obtained with electron microscopy) shows these two protein complexes to be very similar. The *M. jannaschii* crystal structure provides a snapshot of the channel in its closed conformation. A short helix functions as a 'plug' and sits in the channel, preventing unwanted traffic of peptides and small molecules through the membrane. The channel itself forms an hourglass like structure which, at its narrowest point, forms a pore ring made up of hydrophobic amino acids. This is thought to provide a mechanism by which a seal can be maintained even when the channel is translocating a nascent chain. The division of the large membrane spanning Y subunit and the hinge between transmembrane domains 1-5 and 6-10 offer a solution as to how membrane proteins can exit the translocon into the bilayer. A lateral gating mechanism can be envisaged in which the two halves of the pore open and allow hydrophobic segments to move into the lipid bilyer, without contacting the hydrophobic environment of the cytosol[62].

The translocon itself is a passive complex, so the driving force for translocation of proteins must come from elsewhere. In eukaryotic cells, the luminal chaperone BiP works as a ratchet, holding onto the elongating peptide as it enters the lumen of the ER, preventing it slipping back towards the ribosome and resulting in a net movement of the protein into the ER. Bacterial translocons appear to use a pushing rather than pulling mechanism. The cytosolic

ATPase SecA associates with the translocon on the cytoplasmic side and pushes the nascent chain through the plasma membrane[58]. In 2008, Zimmer *et al.* published the crystal structure of a bacterial SecYEG channel in complex with SecA, giving important insight into how ATP hydrolysis by SecA is coupled to peptide translocation through the translocon[63]. Comparison of this structure with the closed *M. jannaschii* structure shows it to be in more of an open conformation, which the authors propose is a 'pre-active' state. Interaction of SecA with the channel causes loosening of the plug helix in preparation for translocation, and a rearrangement of the transmembrane domain to allow insertion of a domain of SecA termed the two helix finger. The model of peptide transport proposed in this paper is one in which SecA clamps the translocating chain and the two helix finger contacts the peptide and pushes it into the channel[63]. This hypothesis was tested by Erlandson *et al.* who used mutagenesis studies to show that residues at the tip of the two helix finger of SecA are important for protein translocation. A 'molecular endoscope' technique was then employed to probe the environment surrounding a translocating chain. Crosslinking of different positions of this pre-protein endoscope to SecA demonstrated that the two helix finger pushes the translocating chain directly into the translocon channel[64].

For single-pass membrane proteins, exit from the translocon by the lateral gating mechanism is easy to envisage, but for complex polytopic membrane proteins, exit from the translocon and assembly in the membrane is more difficult[65]. Transmembrane domains of polytopic membrane proteins interact with the translocon, TRAM (translocating chain-associated membrane protein), and other accessory components, and are held at the translocon to prevent them from diffusing away from the rest of the protein with which they need to assemble. Transmembrane domains of polytopic membrane proteins contain more charged and polar residues than those of single-pass proteins. These residues may cause a requirement for the protein to assemble, at least partially, before its release into the bilayer. The process by which membrane proteins assemble at the translocon and move into the bilayer is complex and it appears that different proteins assemble in different ways, with some transmembrane domains showing a greater affinity for the translocon than others[66-68].

## 1.5 Traffic through the secretory pathway

Following arrival at the ER, the entrance to the secretory pathway, proteins can be targeted to several different destinations, or leave the cell entirely. As mentioned above, much of the detail of the complex secretory pathway still remains unsolved, but here I will attempt to review what is known about the major compartments and trafficking routes, before looking more closely at transport between the ER and the Golgi with respect to the ER protein SERCA.

### The endoplasmic reticulum

Upon synthesis into the ER through the translocon, it is crucial that proteins are folded into the correct conformation. As the nascent chain enters the ER, BiP functions as a ratchet, preventing the growing peptide from sliding back into the cytosol, and following translation, it also enables newly synthesised proteins to fold into their mature conformation, along with other chaperones such as calnexin and calreticulin. Some chaperones, including protein disulphide isomerase (PDI), catalyse disulphide bond formation in proteins entering the ER, assisting in their proper folding. Oligosaccharyl transferase catalyses N-linked glycosylation of proteins containing the appropriate motif (Asn-X-Ser/Thr) as they enter the ER lumen[3]. In addition to assisting correct folding of proteins, ER chaperones also detect and provide solutions to aberrant folding of proteins. The ER employs two mechanisms to regulate protein folding. The unfolded protein response is elicited in the ER as a result of increasing amounts of unfolded proteins, and causes upregulation of ER chaperones in order to increase the capacity of the organelle to fold proteins. ER-associated degradation describes retrotranslocation and destruction of terminally misfolded proteins by the proteasome[69]. Protein misfolding and the ER will be discussed in more detail in chapter 4.

### ER exit

Following synthesis, correct folding and any relevant modification, proteins destined for downstream compartments of the secretory pathway must then leave the ER. ER exit sites (ERES) are punctuate structures on the ER membrane characterised by the presence of

components of the COPII (coat protein II) complex which mediate budding and transport of vesicles from the ER towards the Golgi[70]. Activation of Sar1, a small GTPase by its GEF (guanine nucleotide exchange factor), Sec12, causes the GTPase to insert an amphipathic tail into the bilayer, initiating the formation of the COPII complex. Heterodimeric proteins Sec23/24 and Sec13/31 are then recruited and coated vesicles are formed with the assistance of Sec16, which binds to the other components and stabilises the complex[70,71]. Cargo selection by COPII coats is controlled by Sec24 which binds ER export motifs on cargo proteins (such as LxxLE and DxE) and incorporates them into transport vesicles[72]. Other factors, aside from specific motifs, are thought to contribute to ER export of proteins. Transmembrane domain length is thought to be a determinant of ER export. Shortening of transmembrane domains (by 8 or more amino acids) of the exported (plasma membrane) protein VSVG results in exclusion from ERES and retention in the ER[73]. It has been suggested that as the membrane thickness through the secretory pathway increases, ERES should have slightly thicker bilayers than the rest of the ER membrane, favouring incorporation of longer transmembrane domains[73]. However, this difference, if there is indeed a difference, is subtle, and the changes in transmembrane domain length required to cause mislocalisation of VSVG to the ER are large. Elongation of transmembrane domains has been shown to mislocalise ER proteins to the plasma membrane[28,74], but again, the changes required are large in comparison to the subtle differences between thickness of the ER and plasma membranes. Bulk flow is also thought to account for protein exit from the ER, as cargo is incorporated into COPII vesicles in a non-selective manner independent of export motifs or cargo receptors[75].

Between the ER and the Golgi lies the ER-Golgi intermediate compartment (ERGIC), also known as the vesicular tubular clusters. The nature and dynamics of the ERGIC has been the subject of much debate in organelle biology. Many scientists are reluctant to describe it as a stable membrane bound organelle comparable to the ER or Golgi, but acknowledge it as a transport intermediate. Two models exist to explain the dynamics of the ERGIC. The stable compartment model describes an ERGIC which receives COPII vesicles from the ER and sends cargo towards the cis-Golgi in budding vesicles, all the time maintaining its integrity as a stable compartment[76]. The maturation, or transport complex, model proposes that the ERGIC is a collection of fused vesicles which move forwards as one complex towards the Golgi

where they become a new cis-Golgi. Recent evidence has pointed towards the stable compartment model[76,77], although there is also evidence to suggest that the ERGIC functions as a transport complex[78]. Regardless of whether the ERGIC is stable or a transient transport complex, it is generally agreed that it plays a role in protein sorting, and may also be involved in protein folding and quality control[33,76]. Retrograde transport from the ERGIC or early Golgi back to the ER will be discussed in a later section of this review.

## The Golgi

For proteins that leave the ER or ERGIC and continue in an anterograde direction without retrieval, the Golgi is the next destination. The mammalian Golgi is usually perinuclear and is composed of cisternae arranged in stacks[79]. Proteins enter the Golgi at the cis face (closest to the ER), travel through the medial-Golgi and leave the organelle at the trans-Golgi[80]. Proteins travelling through, or residing permanently in, the Golgi, may be subject to modifications including N-linked glycosylation, modification of existing N-linked glycans and O-linked glycosylation[81].

COPI coated vesicles are associated with the Golgi and are thought to play a role in the traffic of proteins through the Golgi in retrograde and possibly also anterograde directions. The extent to which COPI vesicular transport accounts for anterograde movement through the Golgi is unclear. Anterograde cargo has been shown to enter COPI vesicles, and vesicular transport has been proposed to provide a fast route through the Golgi for certain cargo proteins. However, the prevailing mechanism for anterograde transport from the cis- to trans-Golgi is thought to be cisternal maturation, in which proteins are moved across the Golgi simply by a constant forward motion of cisternae through the stack. This would explain how large structures such as collagen aggregates are able to progress through the Golgi, as the size of these complexes disallows packaging into vesicles. In the cisternal maturation model, Golgi resident proteins are maintained in the correct part of the Golgi by retrograde vesicular transport in COPI vesicles. It seems likely that both vesicular transport and cisternal maturation play a role in transport through the Golgi to differing extents for different proteins[79].

The question of how Golgi resident proteins are maintained in the correct region of the Golgi is an important one and has, so far, only partially been answered. In some cases, transmembrane regions of Golgi enzymes appear to be key to their localisation, whereas certain trans-Golgi proteins are dependent on short cytoplasmic sequences which cause their retrieval from the cell surface to the trans-Golgi[82]. How transmembrane domains confer localisation of Golgi proteins is not entirely understood. One explanation is that they cause the proteins to oligomerise and form large complexes. Termed the 'kin recognition' mechanism, this model proposes that proteins can detect their arrival at the correct compartment, at which point they oligomerise into large complexes which are too large to fit into transport vesicles. This explanation is incongruous with the cisternal maturation model, in which large protein complexes would be able to travel forwards without the necessity for transport vesicles[5,82]. Another explanation raises the notion of transmembrane domain length dictating localisation, as mentioned above with respect to ER export. It is feasible that the average difference between shorter Golgi transmembrane domains and longer plasma membrane transmembrane domains cause the Golgi proteins to be excluded from budding membrane at the trans-Golgi, and inclusion of proteins destined for the plasma membrane[82].

## Exit from the Golgi

The mechanism of sorting within, and exit from, the trans-Golgi is not as well characterised as some other pathways in the secretory pathway. Trans-Golgi to plasma membrane carriers (TPCs) is a term used to describe the vesicles into which cargo destined for the plasma membrane is packaged, although what these carriers are coated with is unknown. In polarised cells, sorting from the trans-Golgi into TPCs allows differentiation between proteins destined for apical and basolateral membranes. Glycosylphosphatidylinositol (GPI) anchored proteins and proteins containing N- or O-linked glycans show a preference for apical targeting, whereas basolateral targeting is dependent on short, cytosolic motifs[83]. In nonpolarised cells, plasma membrane targeting motifs have proved elusive, and transport of proteins may be, at least in part, attributable to bulk flow[84]. The carriers that transport proteins from the trans-Golgi to plasma membrane have been proposed to be pleiomorphic bodies, larger in size than

secretory vesicles coated by COPI, COPII or clathrin which would allow transport and secretion of large protein complexes[83-85].

## The endocytic pathway

Proteins present at the plasma membrane can be internalised by endocytosis. Endocytosis begins with invagination of the plasma membrane, mediated by clathrin or other coat proteins. This invagination undergoes scission from the plasma membrane by protein machinery, and the resulting vesicle fuses with an endosome; a sorting compartment with access to several onward destinations[86,87]. Numerous proteins have been shown to traffic from the endosomes to the trans-Golgi, facilitated by the retromer complex. The retromer complex, conserved from yeast to mammals, is a collection of proteins that causes vesicles to bud from the endosomal membrane. Sorting nexins (SNXs) also play a role in the process, causing tubulation of membranes. Short, hydrophobic sequences in cytoplasmic tails of proteins have been shown to cause retromer mediated endosome to trans-Golgi retrieval[87,88]. Ubiquitinated proteins destined for degradation in lysosomes travel through endosomes, and are recognised by the ESCRT (endosomal sorting complex required for transport) complex[88]. Lysosomal enzymes are transported from the trans-Golgi to the lysosomes by recognition of their mannose 6-phosphate tags by mannose 6-phosphate receptors[89]. There is also a recycling route from the endosomes to the plasma membrane for cell surface proteins such as receptors. This route is considered a 'fast' recycling route and requires the CART (cytoskeleton-associated recycling or transport) complex, which recognises the cytoplasmic tails of its cargo[88]. Sorting at the trans-Golgi and traffic to the endosomal pathway is complex, and many gaps in our understanding remain. I have not discussed this pathway in any detail, but simply highlight the importance of sorting signals and vesicular traffic here; something which seems to be a universal phenomenon throughout the secretory pathway[4].

## Vesicular transport

The events of vesicle budding and fusion are key to the traffic of proteins through the secretory pathway. Although the protein machinery that causes these events varies from compartment to compartment, the overall mechanism is similar in most cases (figure 1.6). The

main events occurring during vesicular transport are vesicle budding from the donor membrane (including the mechanisms of concentrating and excluding cargo), vesicle targeting, and vesicle fusion[4]. Selection of cargo for incorporation into vesicles is carried out by receptors (such as the KDEL receptor discussed below), or by direct interaction of targeting motifs with coat proteins (in the case of di-lysine proteins and the COPI coat)[90,91]. Coat protein complexes share common features. Initiation of COPII formation requires the small GTPase Sar1 and the equivalent event in COPI formation is dependent upon Arf; another small GTPase. Both COPI and COPII require various GEFs and GAPs (GTPase-activating proteins) in order to form vesicle coats. Clathrin coats, which form endocytic vesicles at the plasma membrane, are similar to COPI and COPII coats and also use the small GTPase Arf for coat formation. Clathrin coats are more complex, as they require adaptor proteins which form a layer between the membrane and the clathrin itself, unlike the COPI and COPII components which interact directly with the donor membrane[4]. Coat complexes serve to distort the shape of the membrane so as to form vesicles. In order to release the vesicle from its donor membrane, a scission event must occur, mediated by dynamin at endocytic vesicles or likely by polymerisation of coat proteins in COPI and COPII vesicles[4,92].

Following release from the donor membrane, transport vesicles arrive at their destination either by diffusion, or by 'walking' along the cytoskeleton aided by motor proteins kinesin, dynein and myosin. Tethering is the first interaction that the vesicle forms upon arrival at its destination, mediated by recognition of coated vesicles by tethering factors. Different transport routes are specified by interaction of tethering factors on the acceptor membrane, with coat proteins on the vesicle. The small GTPases Rabs aid this process by recruiting tethers to specific locations on the acceptor membrane, and may also be involved in the uncoating of vesicles[93]. After the interaction of the vesicle and target membrane tethering factors, a closer interaction is formed by SNAREs (soluble N-ethylmaleimide sensitive factor association protein receptors). SNAREs are present on the vesicle and target membranes, referred to as v-SNAREs and t-SNAREs respectively. SNAREs also confer specificity to the targeting process as only certain v- and t-SNAREs can interact with each other. As well as targeting vesicles, SNAREs also promote fusion between vesicles and their acceptor membranes. The coming together of the four α-helices involved in SNARE association (one from the v-SNARE and

three from the t-SNARE) results in a trans-SNARE complex which pulls together the two membranes. This closeness of the membranes brought about by the interaction of SNAREs, enables the energy barrier of membrane fusion to be lowered and fusion to occur[4].

The complexity of the secretory pathway and transport within it has only been touched upon here. Common mechanisms are at work in many cases to ensure specific delivery of proteins to the correct compartment. Some of the protein machinery and sequences involved in protein transport through the secretory and endocytic pathways are shown in figure 1.7. I will now focus on the transport between the ER, ERGIC and Golgi with respect to maintaining SERCA and other proteins in the ER.

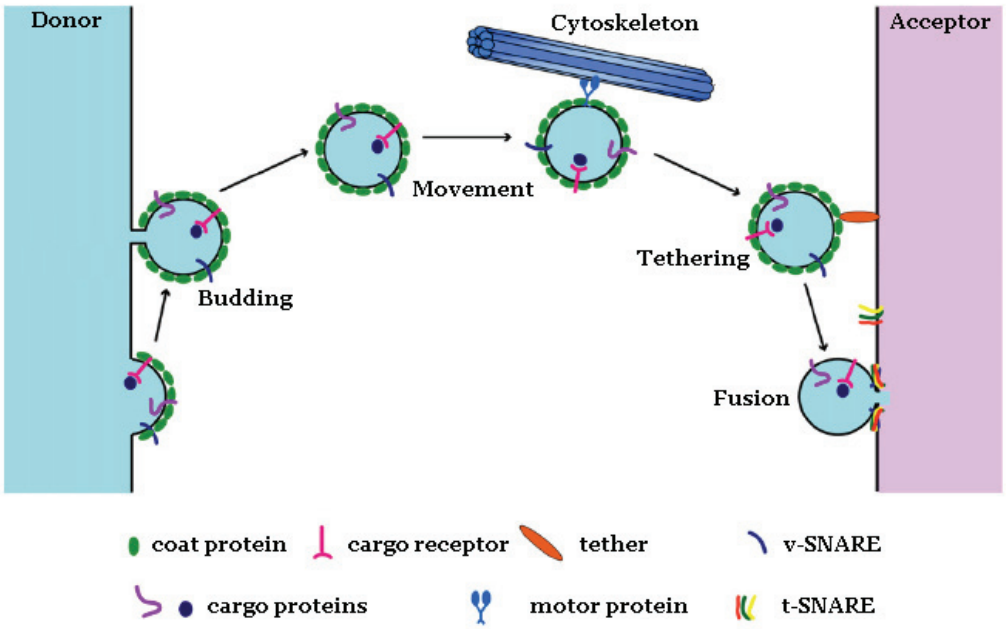



**Figure 1.6 Vesicular protein transport**

A summary of the main events in vesicular protein transport; budding, movement, tethering and fusion. The key protein components are shown. Adapted from Cai, H. *et al.* (2007)[93].

## 1.6 Maintaining proteins in the endoplasmic reticulum

SERCA has been shown to be present in both the ER and ERGIC and so is presumably retrieved from the ERGIC to the ER[33,94]. Several mechanisms for protein retrieval have been characterised to different extents. Some may be applicable to SERCA and some are not. I will now review known mechanisms for the maintenance of proteins in the ER.

### The KDEL motif

The discovery by Munro and Pelham in 1987 of the four amino acid KDEL (lysine-aspartate-glutamate-leucine) motif at the C-termini of soluble ER luminal proteins was an important breakthrough in understanding how proteins are maintained in the ER[90]. By mutating the ER luminal proteins BiP, grp94 and protein disulphide isomerase (PDI) which all have a conserved C-terminal KDEL motif, they demonstrated that the KDEL sequence is necessary and sufficient for correct localisation of these proteins in the ER. Not only does removal of KDEL from ER proteins cause their secretion, but adding KDEL to normally secreted proteins results in their retention in the ER. The authors point out that retention in the ER is most likely a selective and active process whereas export to the plasma membrane is non-selective. The reasoning behind such a suggestion is that truncated grp78 (lacking KDEL) is able to travel through the secretory pathway and be secreted. Grp78 however has no requirement for a specific secretion motif (as under normal circumstances is located in the ER) implying that travel to the plasma membrane is a default, signal independent process. They suggest that their "data could be explained if the proteins were not held in the ER, but instead were continually retrieved" from some downstream compartment; a crucial hypothesis and one which has remained prominent in the field since its suggestion in this paper[90]. The KDEL system of protein retrieval to the ER appears to be almost entirely conserved in yeast. Shortly after the publication of the KDEL sequence, Pelham and co-workers showed that in yeast, a C-terminal HDEL (histidine in place of lysine) motif plays the role of the KDEL sequence[95].

When the KDEL and HDEL motifs were characterised, it was unclear whether they functioned by anchoring proteins in the ER (direct retention) or by causing receptor mediated retrieval from the early Golgi. Evidence and logical reasoning was in favour of the latter theory. In

order to hold the entire ER pool of KDEL containing proteins in the ER, a binding protein must be in very high abundance, whereas a retrieval receptor would only bind a small subset of proteins whilst they are being returned, leading to a more efficient and less easily saturable mechanism[90,96]. Studies looking at diffusion of luminal ER proteins in oocytes show that KDEL proteins move freely through the ER, suggesting that a retrieval (rather than static retention) mechanism is responsible for their localisation[97]. In 1990, the ERD2 gene (ER retention defective) was characterised and found to encode the receptor for HDEL motifs in yeast[98,99]. ERD2 encodes a membrane spanning Golgi protein, mutants of which secrete the ER luminal (HDEL containing) protein BiP[99]. The use of yeast genetics then enabled the same group to test their hypothesis that the ERD2 gene did indeed code for the HDEL receptor[98]. The yeast *K. lactis* utilises a DDEL not HDEL (as in *S. cerevisiae*) retrieval motif. Transferring the DDEL recognising ERD2 gene from *K. lactis* to *S. cerevisiae* resulted in retrieval of both HDEL and DDEL compared to wild type *S. cerevisiae* which only weakly retained DDEL proteins. This highlighted the role of the product of the ERD2 gene in specific recognition of ER retrieval motifs[98]. Since the characterisation of the yeast HDEL receptor, three human homologues of the ERD2 gene have been identified that each appear to have different specificities for different variations of the KDEL motif in soluble ER proteins[100-102].

How does recognition of KDEL (or KDEL-like) motifs by the KDEL receptor bring about the retrieval of proteins from the early Golgi to the ER? The KDEL receptor has been detected in purified rat COPI vesicles[103] and the requirement of COPI for retrieval of HDEL and KDEL proteins has been demonstrated both *in vitro*[104] and *in vivo*[105]. The binding of the KDEL (or HDEL) ligand to the KDEL receptor (or Erd2) induces oligomerisation of the receptor. FRET studies indicate that the oligomeric receptor then interacts with an Arf1 GTPase activating protein (GAP) and components of the COPI machinery[106]. Phosphorylation of the C-terminus of the KDEL receptor is required for it to interact with Arf-GAP and COPI components and bring about retrieval of the bound ligand[107]. The KDEL receptor must have a means of releasing its cargo into the ER after binding it in the early Golgi. The decrease in pH from the ER to Golgi is an attractive theory, as this change could be detected by the KDEL receptor, causing it to release its cargo into the ER. This has been shown to be the case, as the KDEL

receptor preferentially binds ligand at an acidic pH and releases it in the neutral pH of the ER[108].

## Di-lysine and di-arginine retrieval signals

The di-lysine motif is a cytosolic, C-terminal signal on type I membrane proteins which causes retrieval from the ERGIC or early Golgi to the ER[109-111]. One lysine must be positioned at -3 (from the C-terminus) and another at -4, although Jackson and colleagues showed that moving the second lysine to the -5 position still resulted in ER localisation[109]. The amino acids surrounding the di-lysine motif (especially those in -2 and -1 positions) contribute to the strength of the signal, for example KKAA is a stronger signal than KKYF[112]. This shows that different proteins can be maintained in the ER at different levels, and simply having a di-lysine retrieval motif does not dictate exactly how the protein will be distributed at steady state. Post-translational modifications of di-lysine proteins have been shown to occur in post-ER compartment(s), resulting in the proposal that these proteins cycle between the ER and the ERGIC or Golgi. This implies that retrieval (rather than retention) is the mechanism that causes their maintenance in the ER, presumably analogous to the way in which KDEL proteins are maintained[111]. Di-lysine proteins have been shown to interact with components of the COPI machinery which appears to function as the receptor for this signal[91,113]. There is some controversy as to which subunit(s) of COPI di-lysine motifs bind. Photocrosslinking showed an exclusive interaction between di-lysine containing peptides and γ-COP (although as the COPI complex dissociated, the di-lysine motif was also able to interact with α- and β`-COP)[114,115]. Eugster *et al.* demonstrated the importance of the WD40 domains in α- and β`-COP for binding di-lysine motifs and causing ER retrieval of di-lysine containing proteins[116], whereas Zerangue *et al.* argue that only α-COP (through its WD40 domain) is a specific receptor for strong di-lysine motifs[112]. Controversy remains over exactly how COPI binds di-lysine motifs, but it is clear from genetic screens that perturbation of the complex prevents retrieval of di-lysine containing proteins, and that one or more of the subunits bind di-lysine directly[91,112,113,116].

N-terminal di-arginine signals on type II membrane proteins have also been shown to cause COPI mediated transport back to the ER[80,117]. As with the di-lysine signal at the C-terminus, it appears that the position of the di-arginine signal with respect to the N-terminus is crucial for its function; the arginines must be located at positions 2 and 3, 3 and 4, 4 and 5, 2 and 4 or 3 and 5[117]. Initially it was proposed that the similarity of the di-arginine motif to the di-lysine motif pointed to a common retrieval mechanism[117]. This is likely to be the case, as di-arginine proteins also seem to bind directly to components of the COPI machinery, although different parts of the COP protein complex are involved[118].

## Rer1p – a retrieval receptor for membrane proteins

A possible candidate for the ER retrieval of SERCA is the Golgi-resident protein Rer1p. Rer1p (retention of ER proteins) was identified by analysing yeast strains which lacked the ability to retain Sec12p (an ER protein involved in COPII vesicle formation) in the ER[119-122]. Initial studies in yeast demonstrated that mutations in the RER1 gene cause localisation of Sec12p in the late Golgi and at the cell surface but do not affect the localisation of the soluble ER protein BiP[119]. Following the identification of Rer1p as a receptor for Sec12p, other yeast proteins were shown to be dependent upon Rer1p for ER localisation. α1,2-mannosidase (Mns1p)[123], Fet3p (a yeast iron transporter)[124], Sec71p and Sec63p (both involved in translocation of proteins into the ER)[125] and Sed4p (involved in COPII vesicle formation)[126,127] have all been shown to be dependent upon Rer1p for ER localisation. Interestingly Sed4p contains a C-terminal HDEL motif (the yeast KDEL equivalent), but seems to require Rer1p for correct localisation. In fact, the transmembrane domain of Sed4p is sufficient for Rer1p mediated ER retrieval and shows significant homology to the transmembrane domain of Sec12p. The same study illustrated the Rer1p independent ER localisation of Sec20p, showing that not all ER membrane proteins need Rer1p for maintenance in the ER[126].

A human homologue of Rer1p (44% identical and 65% similar to yeast Rer1p) was identified and cloned from HeLa cells in 1997[122]. Human Rer1p has the same predicted W-shaped topology and orientation (cytosolic N- and C-termini) as the yeast protein[122]. The role of Rer1p in human cells appears to be the same as in yeast. Overexpression of human Rer1p

complemented the absent yeast Rer1p in deficient strains and rescued the phenotype, highlighting the extent of conservation in at least this trafficking pathway (and presumably others) between yeast and man[122]. Since its characterisation, human Rer1p has been implicated in the ER retrieval of mammalian proteins. Nicastrin (a component of the intramembrane γ-secretase protease complex) has been shown to interact with Rer1p via polar residues in the transmembrane domain of nicastrin[128]. Knockdown of Rer1p using RNA interference in HeLa cells resulted in an increase in nicastrin at the cell surface, but studies of the kinetics of glycosylation in the Golgi suggested the presence of another mechanism for ER retention of nicastrin in addition to the recognition of the transmembrane domain by Rer1p[128]. The authors of this study propose that Rer1p and another ER retention mechanism control the residence time of nicastrin in the ER in order to allow it to interact with the other components of the γ-secretase before leaving the Golgi and travelling to the cell surface[128]. A different group have shown that Rer1p mediates ER maintenance of Pen2, another component of the γ-secretase. They illustrated using coimmunoprecipitation and reporter constructs containing different domains of Pen2, that Rer1p interacts with the C-terminal portion of the first transmembrane domain (of two transmembrane domains) of Pen2[129]. A reporter construct containing the C-terminal portion of the first transmembrane domain of Pen2 was localised to the ER, and treatment of HEK293 cells with Rer1p siRNA resulted in redistribution of this construct to the plasma membrane[129].

The mechanism by which Rer1p returns proteins to the ER is not yet understood. Rer1p does not appear to recognise a single motif like KDEL, but seems able to bind to a variety of membrane proteins, the common feature of which is the presence of polar residues within a transmembrane domain[128,130]. Mutation of polar residues to leucine residues within the transmembrane domain of yeast Sec12p leads to a reduction in Rer1p binding[131]. It has been proposed that the oligomeric state of Rer1p substrates may be important for their recognition. Yeast Sec71p and Sec63p are part of the hetero-oligomeric translocon, and may be recognised as monomers by Rer1p in the early Golgi, causing their return to the ER and formation of the translocon complex[130]. Fet3p must form a complex with Ftr1p (the other subunit of the iron transporter) before it can travel to the plasma membrane, and whilst unassembled, it remains in the ER as a result of Rer1p mediated retrieval[124]. Similarly, both nicastrin and Pen2 are part

of a protein complex which must be formed in the ER before it can move to the plasma membrane where it fulfils its role as an intramembrane protease[128,129]. In these cases, one can imagine that the subunits of the protein complex display Rer1p recognition domains and are retrieved to the ER until they are oligomerised and travel through the Golgi to the plasma membrane. This theory could also explain how proteins dissociate from Rer1p upon their return to the ER, as their integration into protein complexes may involve higher affinity interactions than those between the proteins in question and Rer1p[130]. It also points to a quality control function of Rer1p, preventing unassembled protein subunits from prematurely escaping to the plasma membrane[124].

Sato *et al.* have demonstrated that Rer1p mediated ER retrieval is a COPI dependent process[131]. In yeast mutants lacking COPI components, Rer1p dependent ER proteins (Sec12p, Sec71p and Sec63p) travel to the late Golgi, and Rer1p itself is mislocalised to the vacuole. In the same study, it was also shown that the cytoplasmic C-terminal 25 amino acids of yeast Rer1p are required for its localisation in the Golgi, and for its activity as a retrieval receptor, as Rer1p lacking its C-terminus is unable to complement a Rer1p deficient yeast strain. *In vitro* binding assays demonstrated that the interactions between Rer1p and COPI components take place via the C-terminus of Rer1p[131]. Presumably the binding of the C-terminus of Rer1p to COPI components causes the integration of both Rer1p and its bound cargo into retrograde transport vesicles which travel to the ER, but the precise mechanistic details of this process are unclear[131].

Although the exact mechanism by which Rer1p returns proteins to the ER is not entirely understood, and other than polar residues in transmembrane domains there is no consensus sequence for recognition by Rer1p, it seems clear that it plays an important role in maintaining some membrane proteins in the ER. Rer1p is conserved between yeast and man, and the complementation study by Füllekrug *et al.* illustrated that mammalian Rer1p fulfils the same role as yeast Rer1p in returning proteins from the early Golgi to the ER[122]. More research in this field is needed to understand the exact mechanism of Rer1p mediated return of proteins to the ER, and to further characterise the role and targets of Rer1p in mammalian systems.

## BAP31 – a possible retrieval receptor for retrograde transport

BAP31 (B cell antigen receptor associated protein of 31 kDa) was initially discovered as a binding partner of the immunoglobulin D B cell antigen receptor[132,133]. BAP31 is a membrane protein with three putative membrane spanning regions and a C-terminal cytoplasmic dilysine motif[132-134]. BAP31 can form a heterodimer with the related (47% identical), 29 kDa protein BAP29 which is important for the function of BAP31[132,133,135,136]. BAP31 exhibits the typical reticular distribution of an ER protein both when detected by an antibody in immunofluorescence and when fused to a fluorescent protein tag[137,138]. Some studies have shown colocalisation of BAP31 with the ER-Golgi intermediate compartment marker ERGIC-53[136,138]. Others claim it to be absent from this compartment and present in a juxtanuclear compartment that is not the Golgi or an ERGIC-53 positive intermediate compartment, but a quality control compartment containing proteins involved in retrotranslocation[134]. The involvement of BAP31 in the sorting and vesicular transport of membrane proteins out of the ER has been demonstrated for several proteins including cellubrevin[137], MHC class I molecules[139] and members of the tetraspanin family of plasma membrane proteins[140].

MHC class I molecules are synthesised at the ER and travel to the cell surface upon loading with a high affinity antigenic peptide. BAP31 interacts with MHC class I molecules and has been shown to play a role in export of the protein out of the ER (towards the plasma membrane); increasing BAP31 levels (by overexpression in HeLa cells) leads to an increase in the amount of MHC class I at the cell surface. Increasing peptide loading of MHC class I molecules results in an increase of BAP31 in the ERGIC, as presumably a greater proportion of it is involved in forward traffic of MHC towards the cell surface[136]. In addition, overexpression of BAP31 increases the stability of MHC class I at the cell surface, suggesting that it may play a role in quality control of the protein in the ER, possibly by retrieval of MHC proteins which have dissociated from their antigenic peptide in a post-ER compartment[138]. Evidence from these studies on MHC class I suggests that BAP31 can be involved in both anterograde and retrograde protein transport from and to the ER. Cytochrome P450 2C2 (an ER membrane protein) is directly retained in the ER (rather than maintained there by retrieval from the ERGIC or Golgi) and has been shown to interact with BAP31[141]. Knockdown of BAP31 by RNA interference causes a redistribution of cytochrome P450 2C2 from the ER to

the nuclear membrane, perinuclear structures (containing the Golgi marker GM130) and plasma membrane[141]. How BAP31 causes retention of cytochrome P450 2C2 is unclear, but a transient interaction between the two proteins has been proposed to occur during synthesis and or folding of P450 2C2, followed by targeting of the cytochrome away from ER exit sites, or interaction with another protein that prevents ER exit by bulk flow[141].

BAP31 is thought to retain membrane bound immunoglobulin D in the ER[142] and assist with the folding and quality control of the ER protein tyrosine phosphatase-like B (PTPLB)[143]. The plasma membrane chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), also seems to require BAP31 for maintenance in the ER during protein synthesis and folding[144]. Reduction of BAP31 expression results in an increase of CFTR at the cell surface, suggesting a role for BAP31 in retention or retrieval of this protein in the early secretory pathway. This effect is dependent on the dilysine motif at the C-terminus of BAP31, indicating that its role in the trafficking of CFTR involves cycling of the protein between the ER and early Golgi[144].

The proteins that have so far been shown to interact with BAP31 (mentioned above) are all membrane proteins. In at least some cases, BAP31 interacts with transmembrane domains of its cargo[132,137,141]. For example, the interaction of BAP31 with membrane bound immunoglobulin D is heavily dependent on a threonine residue in the transmembrane sequence of immunoglobulin D[132]. How BAP31 works mechanistically to control trafficking and quality control of proteins is not understood. The next challenge will be to elucidate how BAP31 is seemingly able to carry proteins in both anterograde and retrograde directions in the early secretory pathway.

## How far have we come in understanding ER retrieval?

Since 1987, and the proposal that the KDEL motif mediates protein retrieval to the ER from a downstream compartment[90], we have made significant progress in understanding the various mechanisms of protein retrieval to the ER. KDEL and HDEL signal mediated retrieval of soluble proteins is arguably the most well studied and characterised mechanism for retrograde

transport to the ER, and this knowledge is useful in understanding other mechanisms. For example, the pH dependent association and dissociation of KDEL receptor and ligand[108] may be a mechanism shared with other receptors and their ligands. SERCA does not contain a KDEL, di-lysine or di-arginine motif. Interestingly, no sarcoplasmic reticulum proteins contain KDEL sequences, which may indicate that the retention or retrieval of proteins in this compartment may be different to the ER at large[145].

It is possible that Rer1p or BAP31 may play a role in SERCA trafficking. BAP31 has been shown to retrieve membrane proteins to the ER, but how it works is not understood. Rer1p interacts with polar residues in transmembrane domains[128,130]. SERCA has many polar residues within the membrane spanning regions (including those involved in calcium binding and transport), many of which face inwards or are totally inaccessible from the bilayer. Even with crystal structures of the protein[18], it is hard to predict which polar residues would be accessible to a receptor like Rer1p, but at present Rer1p is the most likely candidate for retrieval of SERCA to the ER. Studies using chimeric proteins have been carried out to attempt to identify the regions of SERCA that are important for ER retrieval. By replacing sections of SERCA sequence with corresponding sequence from PMCA, it is possible to identify key regions of SERCA. These studies indicate that the N-terminus of SERCA plays a role in its retrieval to the ER[94,146,147], but how this region is recognised remains unknown. The limitations and the value of studies of this type will be discussed in greater detail in chapter 3. It is possible that the slight differences in the transmembrane segment lengths between SERCA and PMCA could cause differential targeting of these proteins to the ER and plasma membrane respectively. However, in such a complex polytopic protein that undergoes such large conformational changes and has helices of different lengths within it, it seems hard to envisage this being the only factor contributing to ER localisation of the protein.

There is still much to learn about retrieval of proteins to the ER. Although motifs such as KDEL and di-lysine seem relatively specific and clear cut, there are many proteins (including SERCA) that do not contain any canonical signals. In addition, Rer1p and BAP31 appear not to recognise specific motifs, more certain characteristics of protein domains. This renders sequence analysis based searches for binding partners difficult, if not impossible.

## 1.7 Conclusion

Although biochemists and cell biologists have come a long way in understanding the intricacies of the secretory pathway (summarised in figure 1.7), many of the mechanisms and protein machinery which regulate protein transport remain elusive. The last two decades have seen a dramatic increase in our ability to solve the complex maze of intracellular protein traffic, accelerated by the power of yeast genetics, combined with recent advances in high resolution microscopy and structural studies. Some of the mechanisms that retrieve proteins from the ERGIC or Golgi to the ER, including KDEL and di-lysine signal mediated retrieval, are well characterised. However, many proteins located in the ER, including SERCA and its modulator peptides phospholamban and sarcolipin, do not contain these signals and are maintained there by as yet unknown mechanism(s)[5,28,33,90].

It is known that SERCA is located in the ER, ERGIC and early Golgi[13,33] and is presumably maintained in the ER by a process of retrieval. SERCA contains none of the canonical retrieval motifs such as KDEL or di-lysine, and it is unknown what sequence(s) within SERCA cause it to be maintained in the ER. Furthermore, no existing mechanisms or protein machinery have yet been associated with this process. The aim of this investigation is to define what sequence(s) within SERCA are responsible for this retrieval to the ER, and what protein machinery is involved.

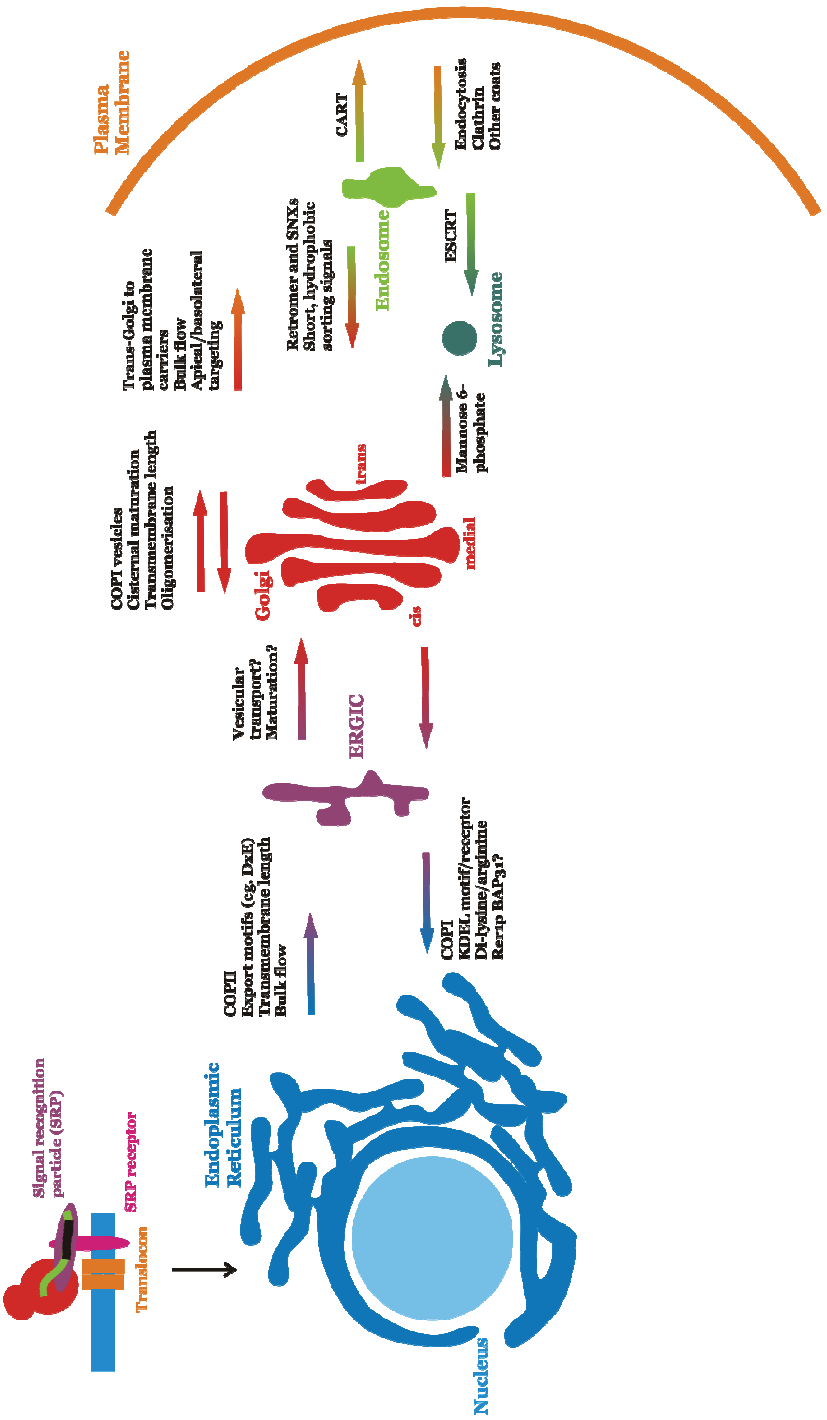



**Figure 1.7 The secretory pathway - sorting motifs and protein machinery**

Some sequence motifs and protein machinery involved in transport through the secretory and endocytic pathways are shown. Based on figures from[4,39,88].

# 2. Materials and Methods

## 2.1 Molecular biology

### The vector – pcDNA3.1 (+)

The vector used throughout the cloning procedures described below is pcDNA3.1 (+) from Invitrogen (see figure 2.1).

### LB broth

Bacterial cultures were grown in LB (Luria-Bertani) broth made from 6.2 g LB EZMix (Sigma-Aldrich) and 300 ml distilled water, sterilised by autoclaving for 20 minutes at 15 $lb/in^2$ and 121 °C. The broth was supplemented with ampicillin (final concentration 60 μg/ml) or kanamycin sulphate (final concentration 25 μg/ml) once the media had cooled to 50 °C or below.

### LB agar plates

4.5 g agar (Melford) and 6.2 g LB were added to 300 ml distilled water and the mixture sterilised by autoclaving. Ampicillin (60 μg/ml) or kanamycin sulphate (25 μg/ml) were added as described above, and the medium was poured into 9 cm petri dishes and left to set. The plates were used immediately or stored at 4 °C until required.

### DH5α *E. coli* electrocompetent cell preparation

The bacterial strain used throughout this investigation was DH5α *E. coli*. To prepare competent cells, 50 ml of antibiotic free LB media was inoculated with a single colony of DH5α *E. coli* electrocompetent cells. This was grown overnight in a shaking incubator at 37 °C. 1 l of LB media was inoculated with the entire 50 ml overnight culture and shaken for approximately two hours at 37 °C until an OD of 0.6 (at 600 nm) was achieved. The cells were then pelleted at 3000 g for 10 minutes. The cell pellets were washed twice by resuspending in sterile water, and were combined in two 50 ml tubes and centrifuged again at 3000 g. The cells

were resuspended in sterile 10% glycerol and 70 µl aliquots were frozen in liquid nitrogen. The cells were stored at -80 °C until required.

## Small scale DNA purification (Miniprep)

Overnight cultures were grown in 5 ml LB media (supplemented with appropriate antibiotic) by picking a single colony of DH5α *E. coli* containing the desired construct using a sterile pipette tip, and placing the tip into the media. The cultures were grown in a shaking incubator at 37 °C overnight. Plasmid DNA was obtained using the Wizard™ Miniprep kit (Promega) as detailed in the manufacturer's instructions.

## PCR

The polymerase chain reaction (PCR) was used to generate fragments for DNA constructs built in this investigation. Primers were designed using the Oligo software (Molecular Biology Insights). *Pfu* DNA polymerase, buffer and dNTPs were obtained from Promega (UK).
PCRs of 100 µl total volume were set up in 0.5 ml tubes as follows:

| | |
|---|---|
| Sterile distilled water | 81.2 µl |
| *Pfu* DNA polymerase buffer | 10.0 µl |
| dNTPs (25 mM each dNTP) | 0.8 µl |
| DNA template (100 ng/µl) | 1.0 µl |
| Primer 1 (100 ng/µl) | 2.5 µl |
| Primer 2 (100 ng/µl) | 2.5 µl |
| *Pfu* DNA polymerase (2.5 U/µl) | 2.0 µl |

The tubes were placed in a Peltier thermocycler (MJ Research) and reactions carried out according to the cycle in table 2.1. 30 cycles were used for amplification unless stated otherwise. The products of the reactions were cleaned to remove primers, buffer and unused dNTPs using the Qiagen QIAquick PCR purification kit (following manufacturer's instructions).
PCR was also used to verify the successful ligation of inserts directly from bacterial cells. This was carried out by picking a colony from agar plate following a ligation and dipping it into the PCR mixture. Colonies were also grown in known positions on a second agar plate so those

containing inserts could be grown and DNA purified. Ligated DNA verified in this way was also sequenced.

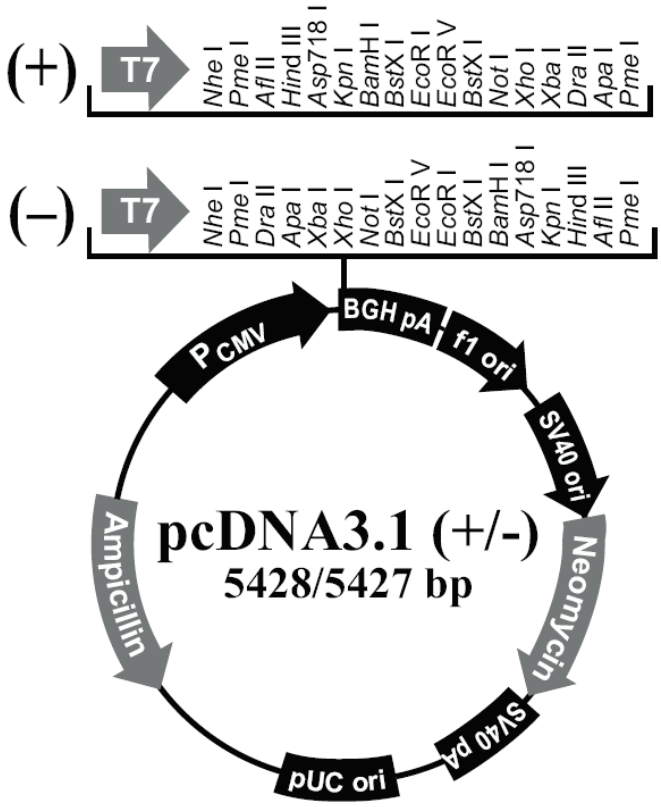



**Figure 2.1 pcDNA3.1 vector**

The pcDNA3.1 (+) vector was used throughout the cloning and expression of the chimeric SERCA/PMCA constructs. It contains a CMV promoter for high levels of expression in mammalian cells, as well as a bacterial origin of replication and ampicillin resistance gene for selection of positive *E. coli* colonies. The SV40 origin of replication allows high levels of protein expression in cell lines containing the large T-antigen.

From http://www.invitrogen.com/content/sfs/manuals/pcdna3.1_man.pdf

| Step | Process | Temperature (°C) | Duration |
|---|---|---|---|
| 1 | Denaturation | 95 | 45 seconds |
| 2 | Denaturation<br>Annealing<br>Elongation | 95<br>Primer $T_m$ -5<br>72 | 45 seconds<br>45 seconds<br>2 minutes per kb of target |
| 3 | Final elongation | 72 | 10 minutes |

**Table 2.1 PCR cycle**

This shows the general reaction cycle used for PCR in this investigation. Annealing temperatures and elongation times were adjusted according to the primer $T_m$ and the length of target DNA respectively.

## Restriction digests

DNA digests are used for diagnostic purposes or to prepare DNA for a ligation. Restriction enzymes (NEB, Promega) are used to cut DNA at specific sequences. One unit of restriction enzyme is defined as the amount required to cut 1 µg of the 35 937 base pair adenovirus-2 DNA in one hour (see NEB catalogue). NheI and KpnI cut adenovirus-2 DNA 4 and 8 times respectively. Therefore, in the case of a NheI/KpnI double digest on the SERCA-EGFP construct (9133 base pairs containing one site each of NheI and KpnI), approximately one unit of NheI and half a unit of KpnI will be required to digest 1 µg of this DNA in one hour. An example of a 20 µl digest is shown here:

| | |
|---|---|
| DNA | 1 µg |
| NheI | 1 unit |
| KpnI | 0.5 unit |
| Bovine serum albumin (BSA) | 1 µl |
| Buffer (10x) | 2 µl |
| Sterile distilled water | to make volume up to 20 µl |

Restriction digests were incubated at 37 °C for 1 hour. Agarose gel electrophoresis was then used to determine the sizes of the fragments produced. These digests were scaled up to allow cutting of large amounts of vector and insert for ligation reactions.

## Agarose gel electrophoresis

1% agarose gels were made from 1 g agarose and 100 ml 1x TAE buffer (50x TAE: 242 g Tris, 57.1 ml glacial acetic acid, 1.9 g NaEDTA and 1 l water, pH 8.0) and heated in a microwave until molten. Ethidium bromide was added to a final concentration of 0.3 µg/ml before the gel was poured into a mould with combs for the wells. After setting, the gel was placed into a tank containing TAE buffer and the gel loaded with DNA samples in gel loading buffer (5 ml glycerol, 30 mg bromophenol blue and 1 ml TAE, made up to 10 ml with distilled water). The samples were separated at 125 V and 200 mA for approximately 30 minutes and visualised and photographed with a UV transilluminator and camera.

## Extraction of DNA from agarose gel by 'freeze-squeeze'

This technique was used to purify vector DNA after restriction digest in preparation for a ligation reaction. Vector DNA was digested with restriction enzymes as described above. The entire digest volume was loaded into one large well on a 1% agarose gel, and the cut vector separated from the excised fragment by electrophoresis. The gel was visualised on a UV transilluminator and the vector band cut out from the gel with a scalpel blade. A plug of siliconised glass wool was placed at the bottom of a 0.5 ml tube and the gel band was added to the tube. The tube was frozen in liquid nitrogen for 5 minutes and then a hole was pierced in the top and bottom of the tube after the gel had been allowed to thaw slightly. The 0.5 ml tube was placed in a 1.9 ml tube and centrifuged for 4 minutes at 8000 g. The eluted liquid was removed and the centrifugation step repeated in a new 1.9 ml tube until no more liquid was eluted. This DNA was then cleaned using the Qiagen QIAquick PCR purification kit (following manufacturer's instructions) and eluted into 40 µl sterile distilled water.

## Ligation

Ligation reactions were carried out to insert fragments of DNA into vectors. Insert DNA was produced by PCR, digested with appropriate restriction enzymes (as detailed above) and cleaned using the Qiagen QIAquick PCR purification kit. Vector DNA was prepared by the 'freeze-squeeze' method (described above) following restriction digest. Two or three separate

ligation reactions were generally set up, each with a different ratio of vector to insert to maximise the chance of a successful ligation. Ratios were generally between 1:10 and 1:3 (vector:insert), but in some cases, it was necessary to use higher ratios (up to 1:30). Vector and insert DNA were added to 2 µl T4 DNA ligase (Promega) and 2 µl of ligase buffer, making the total volume up to 20 µl with sterile distilled water. The reactions were incubated at 16 °C for 24 hours.

### SOC solution

20 g tryptone, 5 g yeast extract and 0.5 g sodium chloride (NaCl) were added to 1 l of distilled water and autoclaved to produce SOB solution. Glucose was added, after autoclaving, through a sterile 0.2 µm filter to a final concentration of 20 mM to make SOC solution. This can be stored at -20 °C until required.

### Dialysis of DNA and transformation of DH5α *E. coli* by electroporation

After ligation, the entire 20 µl volume of a single ligation reaction (containing DNA, buffer and enzyme) was placed on a nitrocellulose filter and floated on 10% glycerol which was stirred slowly with a magnetic stirrer for 20 minutes. Ligation products were removed from the filters with a micropipette and added to DH5α *E. coli* cells. The bacteria and DNA were placed in a Gene Pulser cuvette and a high voltage pulse was applied using an electroporator. The cuvette was quickly filled with 1 ml SOC solution following electroporation and the bacteria were placed in a sterile 20 ml tube and shaken at 37 °C for 30 minutes. The bacteria were then plated onto LB agar plates and grown overnight at 37 °C in LB. Both the LB agar plates and LB media used were supplemented with the appropriate antibiotic to select for positive transformants.

Products from QuikChange reactions (described in chapter 5) were also dialysed and used to transform *E. coli* in this way. When new constructs were supplied, 100-200 ng of DNA (in water) were used to transform bacteria. In these cases dialysis was not required as such small volumes of DNA were used.

## Ethanol precipitation of DNA for sequencing

DNA was purified from bacteria as described above. 20 µl sodium acetate (3 M, pH 5.1) and 200 µl ice-cold 100% ethanol was added to 5-7 µg DNA in a microcentrifuge tube and the mixture was incubated on ice for 30 minutes. The sample was then centrifuged in a microcentrifuge at 16,000 g for 20 minutes. The supernatant was carefully removed so as not to disrupt the pellet, and 1 ml ice-cold 70% ethanol was gently added. The sample was centrifuged again at 16,000 g for 10 minutes and the supernatant discarded. The precipitated DNA was left to air dry and then sent to MWG Biotech (London, UK) with the appropriate primers for sequencing.

## Glycerol stocks

For long term storage of transformed bacteria, 1 ml of bacterial culture was added to 0.5 ml sterile glycerol and mixed by inverting. Glycerol stocks are then stored at -80 °C.

## 2.2 Cell culture, fluorescence microscopy and immunofluorescence

### Phosphate buffered saline (PBS)

For a 10x PBS solution 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4$ and 2 g $KH_2PO_4$ were diluted in distilled water to a total volume of 1 l, and the pH adjusted to 7.2.

### Mammalian cell culture and transfection

COS-7 and HeLa cells were grown in DMEM (Gibco) media with additives as follows (per 500 ml DMEM); 50 ml foetal bovine serum (Gibco), 5 ml fungizone (Gibco) and 2 ml gentamicin (Gibco). Caco-2 cells were grown in low glucose (1 g/L) DMEM (Gibco) supplemented with 10% foetal calf serum, 50 iu/ml penicillin, 50 mg/ml streptomycin and 1% non-essential amino acids (all from Invitrogen, UK). All cell lines were grown in Iwaki tissue culture treated flasks (150 $cm^2$) (Lennox). When cells reached 60-80% confluency, they were removed from the flask using trypsin diluted in HBSS (Gibco) and seeded onto coverslips in 24-well plates or larger plates for transfection (all Nunclon coated, from Thermo Fisher Scientific).

For transfection of cells, FuGENE-6 (Roche) was mixed with DMEM and added to DNA (see table 2.2). This mixture was incubated for 20 minutes at room temperature with occasional gentle mixing before it was added to cells. Cells were incubated for 48 hours before coverslips were mounted and viewed or cells harvested for use in western blotting, immunoprecipitation or cross-linking (as detailed in results chapters).

### Mammalian cell storage

COS-7, HeLa and Caco-2 cells were harvested at 90% confluency and collected by centrifugation. 6 ml cryogenic medium (10% DMSO, 25% foetal calf serum and 65% DMEM) was used to resuspend the cells and 1 ml aliquots were gradually frozen to -80 °C overnight, then placed in liquid nitrogen storage.

## Mowiol mountant

2.4 g mowiol (Calbiochem) was stirred for 3 hours in 6 ml glycerol and 6 ml water. 12 ml (0.2 M) Tris HCl was then added and the pH adjusted to 8.5. The mountant was then stirred at 50 °C for 10 minutes and centrifuged at 5000 g for 15 minutes to remove any insoluble material. Citifluor was added to a final concentration of 0.1%. The mowiol can then be stored at -20 °C until required.

| Culture Dish | DMEM (µl) | FuGENE-6 (µl) | DNA (µg) |
|---|---|---|---|
| 24-well plate (for 4 wells) | 100 | 3 | 1 |
| 10 cm dish | 600 | 20 | 7 |
| 15 cm dish | 1400 | 46 | 15 |

**Table 2.2 Transfection of mammalian cells**

Quantities of DNA, DMEM and transfection reagent (FuGENE-6) are shown for the various sized transfections used in this investigation. DMEM and FuGENE-6 were mixed together before DNA was added.

## Concanavalin A conjugate plasma membrane labelling

Concanavalin A (conA) binds glycoproteins on the cell surface and can be used to locate the plasma membrane of non-permeabilised cells when conjugated to a fluorophore[28]. ConA conjugated to Alexa Fluor 594 (Molecular Probes) was used to visualise the plasma membrane. The conA conjugate was diluted in PBS (1% BSA) to a concentration of 250 µg/ml and transfected COS-7 cells were incubated with this for 10 minutes. The conA conjugate was removed and the coverslips washed twice with PBS. The coverslips were then mounted onto glass slides in mowiol (CalBiochem) with 0.1% citifluor (Agar Scientific).

## TGN46 antibody labelling of the trans-Golgi network

Live transfected COS-7 cells were treated with brefeldin A (Sigma-Aldrich) at a concentration of 5 µg/ml for 1 hour, to improve separation and visualisation of the trans-Golgi network[94,148]. The cells were fixed with ice-cold methanol for 15 minutes and then washed twice with PBS.

Cells were blocked with a solution of PBS containing 2% low fat dried milk (Marvel) and 0.01% Triton X-100 (Sigma-Aldrich) for 30 minutes at 37 °C. Cells were then washed three times (leaving 10 minutes for each wash) with PBS (0.01% Triton X-100). Sheep anti-human TGN46 (Serotech) was the primary antibody used, diluted to 1:50 with PBS Triton. 100 µl primary antibody was added to each well of the 24-well plate and incubated at 37 °C for one hour. Coverslips were then washed three times with PBS Triton as above. The secondary antibody used was rabbit anti-sheep IgG conjugated to Texas Red (Abcam) and was diluted 1:100. 100 µl secondary antibody was added to each well and left for one hour at 37 °C. The coverslips were again washed three times and mounted in mowiol (0.1% citifluor).

## Confocal microscopy

Laser scanning confocal microscopy was carried out with a Leica TCS SP2 confocal microscope. Samples were viewed under oil with a 40x objective (numerical aperture 1.25) and pinhole diameter of Airy 1. Leica LCS software was used for image acquisition and to produce overlays of images from different channels. EGFP was excited at a wavelength of 488 nm and emission measured between 500-600 nm, Texas red was excited at 594 nm and emission measured between 605-700 nm, Alexa Fluor 594 was excited at 594 nm with emission measured between 605-670 nm and DAPI was excited at 350 nm and emission measured between 450-470 nm. All emission bandwidths were freely adjustable with the acousto optical beam splitter (AOBS). Ar/Kr, HeNe 543/594, HeNe 633 and Mai Tai multiphoton lasers were used to excite samples.

## 2.3 Protein techniques

### Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins by molecular weight. Gels consist of a stacking gel into which the samples are loaded, and a longer resolving gel through which they run. Different percentage gels can be made according to the size of the protein to be resolved; a higher molecular weight protein should be run on a lower percentage gel. Compositions of resolving and stacking gels are shown in tables 2.3 and 2.4 respectively. Acrylamide was added in the form of ProtoGel (National Diagnostics), composed of 37.5:1 acrylamide to bisacrylamide solution. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added immediately before pouring the gel. Resolving gels were first poured and left to set before the stacking gel was poured and combs to form the wells inserted. Before running, combs were gently removed and gels placed in tanks containing running buffer (5x running buffer: 15 g tris, 72 g glycine and 5 g SDS made up to 1 l with water). The wells were rinsed out using running buffer before loading. 1.0 mm gels were made using Biorad glass plates and were run in Biorad tanks. In some cases, pre-cast 14% Novex tris glycine gels were used and run in the XCell SureLock gel tank with Novex tris glycine SDS running buffer (all Invitrogen).

| | **Percentage Resolving Gel** | | |
|---|---|---|---|
| **For two gels:** | **10%** | **12%** | **15%** |
| ProtoGel | 3.3 ml | 4 ml | 4.9 ml |
| Tris HCl (1.5 M pH 8.8) | 1.7 ml | 1.7 ml | 1.7 ml |
| SDS (10%) | 100 µl | 100 µl | 100 µl |
| APS (25%) | 120 µl | 120 µl | 120 µl |
| TEMED | 5 µl | 5 µl | 5 µl |
| Water | 4.78 ml | 4.08 ml | 3.18 ml |

**Table 2.3 Composition of resolving gels for SDS-PAGE**

Amounts of each component of the resolving gels used in SDS-PAGE are shown. TEMED and APS were added last, immediately before gels were poured.

| For two gels: | Stacking Gel |
|---|---|
| ProtoGel | 0.6 ml |
| Tris HCl (3.6 M pH 9.3) | 1.6 ml |
| SDS (10%) | 40 µl |
| APS (25%) | 20 µl |
| TEMED | 4 µl |
| Water | 1.7 ml |

**Table 2.4 Composition of stacking gel for SDS-PAGE**

Quantities of components of the stacking gel are shown here. The same stacking gel was used regardless of the percentage of the resolving gel.

Sample buffer was composed of the following:

| | |
|---|---|
| Tris HCl (0.625 M, pH 6.8) | 1 ml |
| 10% SDS | 2 ml |
| Glycerol | 1 ml |
| β-mercaptoethanol | 0.5 ml |
| Water | 5.3 ml |

Bromophenol blue can be added to the buffer in order to visualise the samples as they are loaded into the gel.

## Preparation and running of samples on SDS polyacrylamide gels

HeLa and COS-7 cells used in western blots were transfected as described above, or left untransfected and plated onto Nunclon coated plates and grown for 48 hours. Media was removed from the plates on which cells were grown. Cells were harvested as detailed in results chapters. Where required, total protein concentrations of material were calculated using the Pierce BCA kit (as manufacturer's instructions for microplate procedure) and 96 well plates were read using Revelation software (Dynex Technologies). Following preparation and heating, samples for SDS-PAGE were briefly centrifuged in a microcentrifuge and loaded into

the gel with a SeeBlue Plus2 Pre-Stained Standard marker (Invitrogen) in one well. Samples were separated at 65 mA and 125 V until the buffer front reached the bottom of the gel.

### Transfer to nitrocellulose

In order to carry out a western blot, the samples must be transferred from the SDS polyacrylamide gel to a nitrocellulose membrane. The gels were placed onto Hybond-C nitrocellulose membrane (Amersham Biosciences) and placed between two sheets of filter paper, supported in the transfer apparatus by two nylon scouring pads. The tank was filled with transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol) and transfers were carried out at 500 mA and 100 V for 2 hours. Membranes were blocked in PBS tween (0.05%) supplemented with 5% low fat dried milk (Marvel) overnight before being analysed in a western blot.

### Western blot procedure

Following blocking, nitrocellulose membranes were washed three times in PBS tween (0.05%) allowing 10 minutes for each wash. Primary antibody (diluted in PBS tween to the appropriate concentration) was added and incubated with the membrane for 1 hour at room temperature. The membrane was then washed three times with PBS tween to remove any unbound primary antibody. Horseradish peroxidase (HRP) conjugated secondary antibody was added (diluted in PBS tween to the appropriate concentration) and incubated with the membrane for 1 hour. The membrane was washed three times with PBS tween before analysis. Proteins were detected using SuperSignal West Dura Extended Duration Substrate (Pierce) and a VersaDoc Model 3000 imaging system (Biorad).

### Stripping western blots

Western blots were stripped of antibodies to allow reprobing with different antibodies, for example a loading control. The membrane was incubated with stripping buffer (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris in water, pH 6.7) at 50 °C for 30 minutes. The membrane was then washed three times (allowing 10 minutes for each wash) with large

volumes of PBS tween (0.05%) to remove the stripping buffer and then blocked for 30 minutes with PBS tween supplemented with 5% low fat dried milk. The blot was washed three times with PBS tween before antibodies were applied as described above.

# 3. Searching for the ER retrieval signal of SERCA using chimeric proteins

## 3.1 Introduction

Chimeric proteins can be a useful tool in the search for sorting signals in proteins, and have been used to locate ER retrieval signals in SERCA[94,146,147]. By replacing sections of SERCA with corresponding sequence from the plasma membrane pump, PMCA, regions of SERCA that cause ER localisation can be detected. Due to the conservation of sequence, function and overall domain architecture between SERCA and PMCA, it is possible to select corresponding regions of the proteins to exchange by aligning the two sequences. A series of EGFP (enhanced green fluorescent protein) tagged SERCA/PMCA chimeras have been built, with the aim of locating the sequence(s) in SERCA that mediate its retrieval to the ER. The chimeras have been expressed in COS-7 cells and their localisation analysed using confocal microscopy and immunofluorescence. Conclusions can then be drawn based on the assumption that chimeras localised to the ER contain sequence information that mediates ER retrieval, whereas those localised to the plasma membrane do not.

EGFP tags have been used to determine the localisation of the chimeras in this study. Proteins were C-terminally tagged in order to avoid interference with ER targeting of the calcium pumps which is mediated by SRP recognition of their N-termini[38]. EGFP and other fluorescent proteins are ubiquitous in cell biological and biochemical research as they provide a means of detecting a protein of interest using fluorescence microscopy. By virtue of their intrinsic fluorescence, fluorescent proteins can be used to detect proteins inside living cells, eliminating the need for membrane permeabilisation and use of fluorescent antibodies. In addition to the use of fluorescent proteins as a tool for localising proteins in cells, they can also be used as reporters of gene expression and to detect protein interactions using techniques such as fluorescence resonance energy transfer and biomolecular fluorescence complementation in which the fluorescent protein is divided into two parts and requires interaction of two tagged proteins for fluorescence to be emitted[149,150].

Previous attempts have been made to pinpoint the ER localisation signal in SERCA using chimeric proteins. These studies have identified the N-terminus of SERCA as being important for ER localisation[94,146,147]. Carafoli and colleagues suggest that an ER localisation signal lies within the N-terminal 28 amino acids of SERCA, as chimeras in which these amino acids were added to the PMCA sequence were partially localised to the ER. Crucially however, removing these amino acids from SERCA and replacing them with PMCA sequence also produced an ER localised chimera, indicating that other regions of SERCA may also be important in its ER localisation[147]. The studies of this type carried out so far have only differentiated between ER and plasma membrane localisation[94,146,147]. It may be possible that chimeras not containing an ER retrieval signal could escape the ER but become held in a downstream compartment before reaching the plasma membrane. Therefore it may be important to look for localisation of the chimeras in the late Golgi apparatus to identify proteins that have escaped the ER retrieval process.

One important consideration when using chimeric proteins to study maintenance in the ER is the function of the ER in protein quality control. By creating proteins not made by the cell under normal conditions, there is a possibility that they will be recognised as misfolded by the quality control machinery of the cell, and maintained in the ER by this route[151]. ER localised chimeras could be construed as 'false positives' as they appear to contain an ER retrieval signal but in reality may be undergoing retrieval as a result of their propensity to misfold. To attend to this, several measures were taken when building the SERCA/PMCA chimeras in this study to maximise proper folding and exit (where appropriate) from the ER and ERGIC. Constructing chimeras from SERCA and PMCA is a logical starting point, as the two proteins have approximately 30% sequence identity, a common overall architecture and conserved functional domains[35,94]. This should increase the probability that the chimeras will fold correctly, as compared to equally complex membrane-spanning chimeras built from unrelated proteins. The chimeras in this study have been built in mirror pairs, containing opposite sections of SERCA and PMCA. Assuming there is no redundancy in the signal or misfolding of the proteins, one of the pair should be present in the ER and one at the plasma membrane. If both of the mirror pair chimeras are localised to the ER, then no conclusions can be made; one or both could be misfolded. Plasma membrane chimeras are the most informative, as they have

escaped the quality control of the ER and so are presumably correctly folded. This enables elimination of any section of SERCA in a plasma membrane localised chimera from the search for retrieval signals. Where possible (and in most cases), SERCA and PMCA sequences have been joined at regions that are conserved between the two proteins. This measure is taken to reduce the probability of misfolding, especially in the area surrounding the join (figure 3.1).

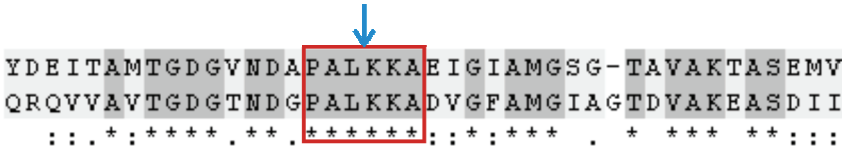



**Figure 3.1 Selecting a suitable position to join SERCA and PMCA sequences**
Sequences of SERCA (top line) and PMCA (bottom line) were aligned using ClustalW (at Uniprot). The conserved region selected is surrounded by a red box, and the blue arrow indicates the point at which the two sequences were joined.

To characterise the chimeras, trans-Golgi localisation was assessed. Although the distributions of SERCA and PMCA (shown in figure 3.5) appear very different, without a cell surface epitope on any of the chimeras able to be detected by an antibody, it is difficult to declare the absence of the reticular constructs at the plasma membrane. It is possible that some constructs appearing to be localised in the ER are present in small amounts in the plasma membrane. It is also possible that they have escaped the ER retrieval mechanism and continued to travel through the secretory pathway, becoming retrieved or retained somewhere upstream of the plasma membrane. Therefore, testing all of the reticular constructs for localisation in the trans-Golgi is a prudent step to take to ensure that those constructs appearing to be in the ER are indeed showing retrieval at an early stage in the secretory pathway. The trans-Golgi is arguably the last step in the pathway taken by plasma membrane proteins that is easy to detect using immunofluorescence. Although presence in the trans-Golgi is not an indication that the protein will definitely traffic to the plasma membrane, it does illustrate the loss of ER retrieval. Rer1p is a protein that is localised to the ERGIC and Golgi[122] but without locating compartments of the secretory pathway it is hard to ascertain where Rer1p ends its journey. By illuminating the trans-Golgi with antibodies, it becomes clear that Rer1p is present in the trans-Golgi, whereas SERCA is not (see figure 3.2). This example highlights the need for colocalisation experiments to determine how far chimeric constructs have travelled through

the secretory pathway; a technique which previous SERCA/PMCA chimera studies have not employed.

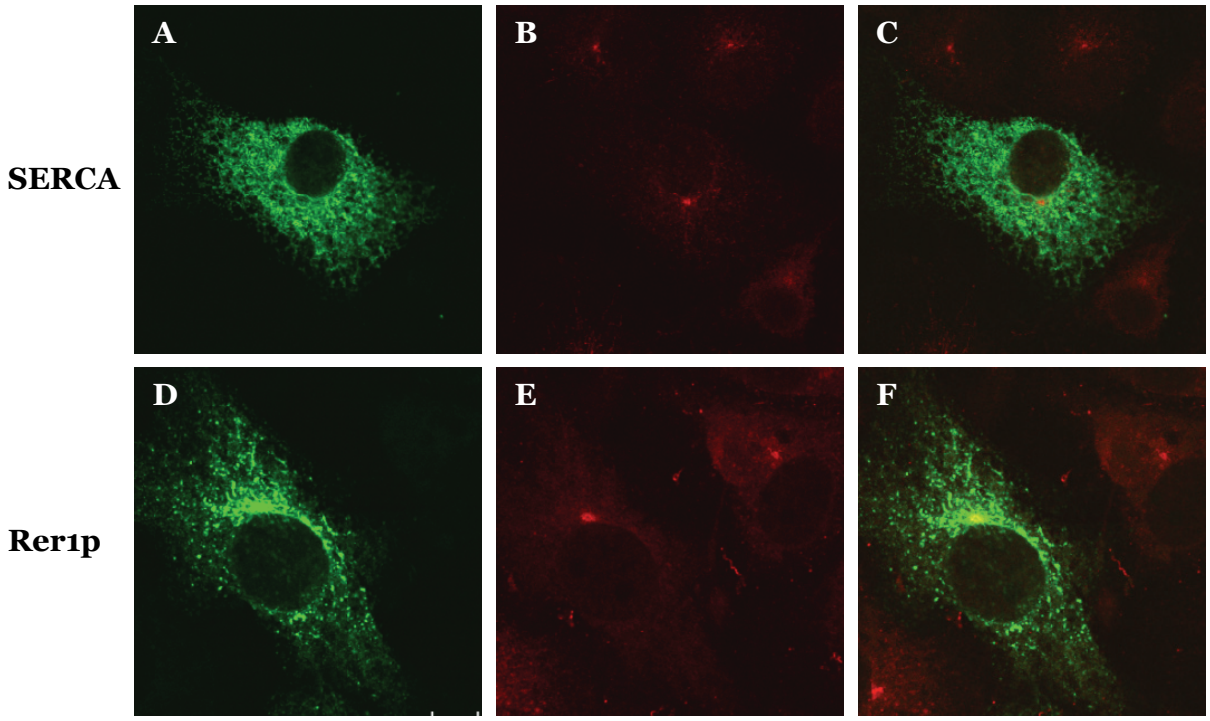



**Figure 3.2 Comparison of SERCA and Rer1p colocalisation with TGN46**
SERCA-EGFP (panels A-C) and Rer1p-EGFP (panels D-F) were expressed in COS-7 cells for 2 days (A and D) and then treated with BFA and antibodies against TGN46 which were visualised using a Texas Red conjugated secondary antibody (B and E). Overlay images of A and B, and D and E, are shown in panels C and F respectively. Images were acquired with a Leica TCS SP2 AOBS confocal microscope.

The trans-Golgi was detected in these studies with antibodies to the trans-Golgi marker protein TGN46 (trans-Golgi network protein of 46 kDa). Cells treated with these antibodies were pre-treated with brefeldin A (BFA) to improve visualisation of the compartment. BFA causes an increase in separation between the trans-Golgi, and the ER and rest of the Golgi stack, allowing discrimination between proteins that travel into the early but not late Golgi[94,148]. BFA targets the GEFs of Arf1. Arf1 is a small GTPase which plays a role in the formation of COPI vesicles that transport protein cargo from the Golgi to the ER. Despite this, a major result of BFA treatment is the accumulation of Golgi enzymes in the ER. This seems counterintuitive,

as inhibiting COPI coat formation should result in less retrograde transport from the Golgi to the ER. Reduction in COPI vesicle formation at the Golgi leads to accumulation of v-SNARES which would normally be incorporated into COPI vesicles. This increase of v-SNARE levels at the Golgi causes increased uncontrolled fusion between the Golgi and the t-SNARE containing ER membrane, leading to redistribution of the Golgi stack into the ER and separating it from the trans-Golgi[148,152]. Concanavalin A (conA) conjugated to Alexa Fluor 594 was also used to characterise the chimeras by highlighting the location of the plasma membrane. Protocols for anti-TGN46 antibody and conA treatment are described in chapter 2.

Creating constructs with an epitope on the extracellular side of the plasma membrane would allow determination of their presence at the cell surface upon addition of antibodies to non-permeabilised cells. Any construct present in the plasma membrane would be detected by a specific antibody and could be visualised using immunofluorescence, whereas constructs not present at the cell surface would be undetectable by treatment of non-permeabilised cells with antibodies. This was attempted by building constructs P/S2bM5-11 and P/S2bM11 from PMCA sequence and SERCA2b sequence. SERCA2b has an eleventh transmembrane domain and a lumenal C-terminus[22] which would be extracellular if the protein were able to travel to the plasma membrane. The C-terminal 7 (P/S2bM5-11) or 1 (P/S2bM11) transmembrane domains of SERCA2b were used to replace corresponding PMCA sequence with the aim of creating a plasma membrane localised chimera which had a extracellular EGFP tag. If one or both of these constructs were able to travel to the plasma membrane, the C-terminus of SERCA2b could then be fused to chimeric constructs and function as a tool to detect chimeric constructs at the plasma membrane. Treatment of non-permeabilised cells expressing these constructs with anti-GFP antibodies would then allow identification of those chimeras that are located at the plasma membrane. P/S2bM5-11 and P/S2bM11 constructs were characterised using selective permeabilisation experiments in which it is possible to permeabilise the plasma membrane whilst leaving the ER membrane intact[28]. Addition of anti-GFP antibodies to cells with non-permeabilised, partially permeabilised or completely permeabilised membranes allows determination of the localisation of the C-terminus of the protein in the ER lumen, cytoplasm or on the extracellular side of the plasma membrane. The selective permeabilisation protocol used in these experiments is detailed in the methods section of this chapter.

## 3.2 Methods

All general molecular biology, cell culture and transfection and microscopy protocols are described in chapter 2. cDNAs encoding rabbit SERCA1 and rat PMCA3 were provided by Dr. P. Adams and Prof. G. Shull as in Newton *et al.* (2003)[94]. The human SERCA2b construct was a gift from Prof. F. Wuytack[153]. In order to build the chimeric constructs detailed in this chapter, both single and multi-step polymerase chain reaction (PCR) methods were employed. Where possible, sections of DNA were amplified by PCR with unique restriction sites at each end of the fragment. For example, in the construction of chimera S/PNtermM1-2, (see figure 3.12) a section of PMCA DNA was amplified and cut with restriction enzymes (NheI and KpnI) to generate sticky ends. This fragment was ligated into SERCA EGFP in the pcDNA3.1 (+) expression vector (see chapter 2) which had been cut with the same restriction enzymes. This technique was used for all chimeras where it was possible to change sequence between restriction sites in the gene.

In the case of chimeras in which smaller fragments were exchanged between the two genes, it was not possible to simply insert these fragments using restriction digest and ligation. Restriction sites were not present in the genes at these points and finding an appropriate sequence to mutate into a unique restriction site (whilst keeping the protein sequence the same) was not possible in such a small window of DNA. This was overcome with the use of multi-step PCR[154]. An example of this is the construction of chimera S/PM1 (see figure 3.3) in which three small sections of DNA were 'stitched' together using PCR before the full length fragment was inserted into the vector. The primers at the very ends of the insert were composed of SERCA or PMCA sequence, a restriction site (NheI or KpnI in this example) and 6 or more random base pairs to allow efficient digestion of the resulting fragment by restriction enzymes. The primers within the insert (in this case flanking the sequence encoding M1) comprised SERCA or PMCA sequence (depending on the template used) with an overhang of sequence from the other gene to allow the resulting fragments to be annealed. The three initial PCRs were carried out separately, analysed by agarose gel electrophoresis, and cleaned using the Qiagen QIAquick PCR purification kit to remove all primers. The three products were combined in a fourth reaction, initially using five cycles of elongation and no

primers in order to produce a full length insert from the three fragments. The two primers flanking the restriction sites at each end of the insert were then added and a 30 cycle PCR was carried out to produce a full length insert. The primers used in the construction of chimera S/PM1 are shown in table 3.1. Primers for all chimeric SERCA/PMCA constructs are listed in appendix 1. The protein produced from these primers is shown in figure 3.4. The reagents and general protocol for PCR is detailed in chapter 2. For this example, the annealing temperatures and elongation times are shown in table 3.2.

| Primer | Sequence |
|---|---|
| A | 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′ |
| B | 5′-GTCACGTCCTGCAGGGCTTCTATCACCAGCTCCCACAGG-3′ |
| C | 5′-CCTGTGGGAGCTGGTGATAGAAGCCCTGCAGGACGTGAC-3′ |
| D | 5′-AAGGCAGTGATGGTCTCTCCCTCGTCTTCTGCCCCACCAG-3′ |
| E | 5′-GGGGCAGAAGACGAGGGAGAGACCATCACTGCCTTCGTTG-3′ |
| F | 5′-CCGCGATGTTGGTACCCGAG-3′ |

**Table 3.1 PCR primers used in the construction of chimera S/PM1**

Restriction sites are underlined in green, SERCA and PMCA sequence are shown in red and blue respectively and extra base pairs to ensure efficient restriction digest are in black. Primer F is composed entirely of SERCA sequence as the restriction site and bases either side are contained within the gene.

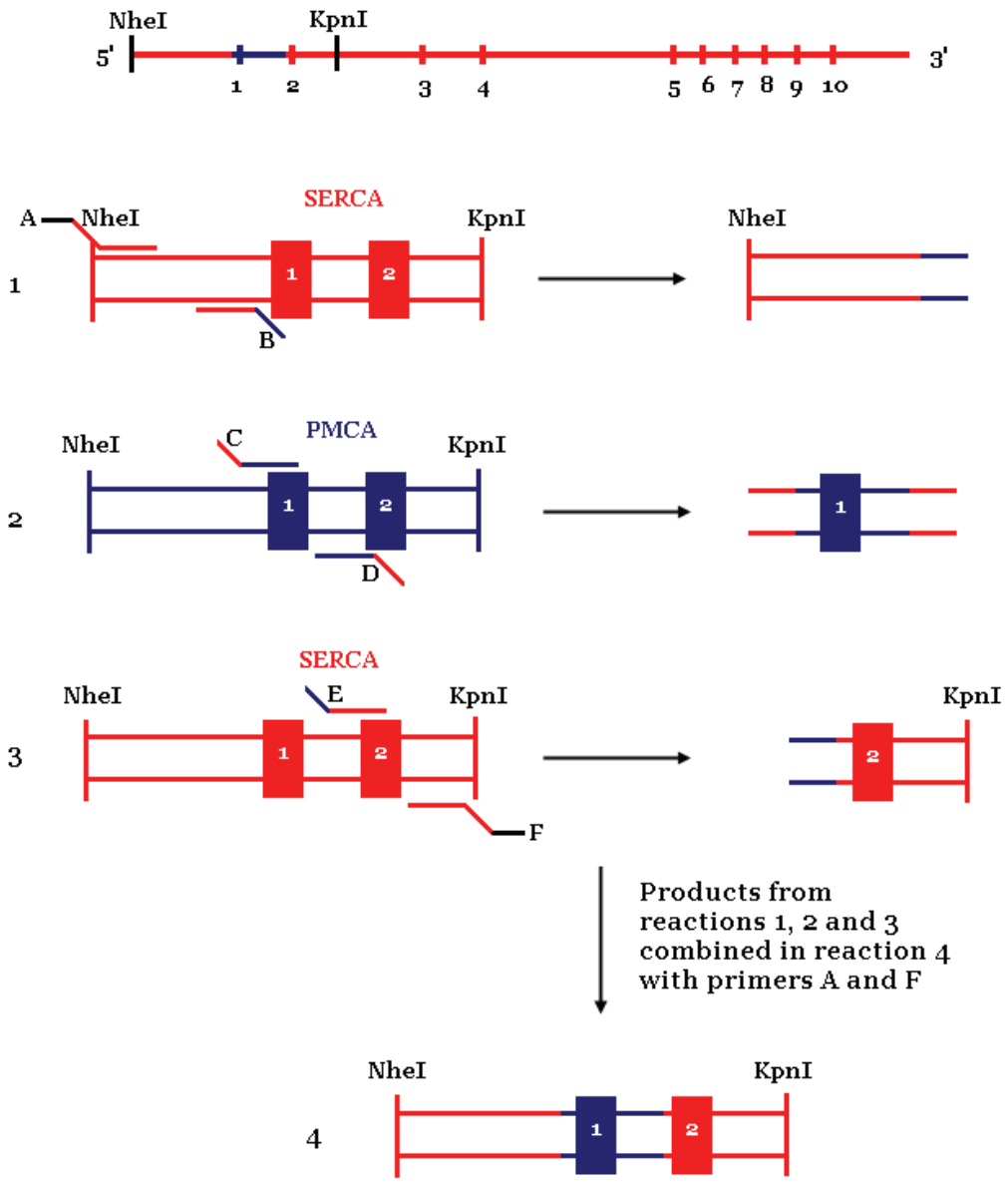



**Figure 3.3 PCRs carried out in the construction of S/PM1**

The architecture of chimera S/PM1 is shown at the top with the sequence encoding M1-M10 represented by vertical lines labelled 1-10. Red and blue show SERCA and PMCA sequence respectively. NheI and KpnI restriction sites and the 5' and 3' ends of the DNA are indicated. The first three reactions and corresponding primers (A-F) (see table 3.1) are shown on the left, and their products on the right. The black regions of the primers show 6 (or more) base pairs 5' of the restriction sites. 1 and 2 inside the filled rectangles denote sequence encoding M1 and M2 of the resulting protein.

| Reaction | Primers | Annealing (°C) | Elongation (min) | Cycles | Template |
|---|---|---|---|---|---|
| 1 | A, B | 55 | 1 | 30 | SERCA |
| 2 | C, D | 45 | 1 | 30 | PMCA |
| 3 | E, F | 59 | 1 | 30 | SERCA |
| 4a | None | 60 | 2 | 5 | Products 1, 2 and 3 |
| 4b | A, F | 60 | 2 | 30 | Products 1, 2 and 3 |

**Table 3.2 Details of the PCRs carried out to produce S/PM1**

Reaction 4 was carried out in two steps. The overlapping sections of the three previous products acted as primers in 4a, allowing amplification of the full length insert. Primers A and F were then added to the reaction and the fragment amplified in reaction4b.

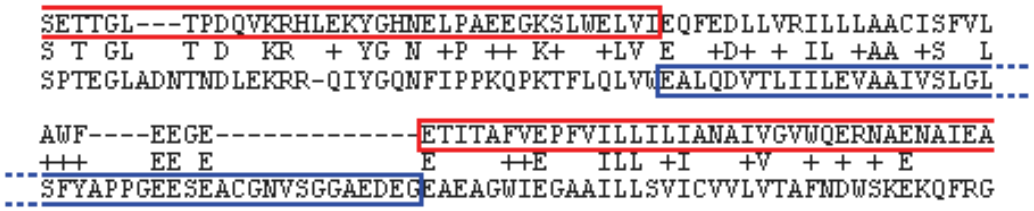



**Figure 3.4 Alignment of SERCA and PMCA to show the sequence of the S/PM1 protein**

The join between SERCA and PMCA in S/PM1 is shown. The alignment between SERCA (top lines) and PMCA (bottom lines) was carried out using BLAST (at NCBI). SERCA and PMCA sequences in the chimera are surrounded by red and blue boxes respectively. Dashed blue lines indicate continuation of the sequence on the second line.

Ligation of inserts into vectors is covered in chapter 2. Briefly, the insert (purified to remove primers) and the pcDNA3.1 (+) vector (in this example containing the SERCA EGFP construct) were both cut using restriction enzymes (NheI and KpnI in this example) to generate sticky ends. Ligation reactions were performed and the products of the reactions were transformed into DH5α *E. coli* cells. Ampicillin was used to select for positive transformants, and the presence and sizes of inserts was confirmed by restriction digest or colony PCR, followed by sequencing. DNA from colonies was purified using the Wizard™ Miniprep kit (Promega).

Correct constructs were used to transfect COS-7 cells using FuGENE-6 as detailed in chapter 2. In addition to observation of the distribution of the EGFP tag on the constructs themselves, further characterisation was carried out using antibodies to TGN46 (and Texas Red conjugated secondary antibodies) and concanavalin A conjugated to Alexa Fluor 594. Cells were viewed with a Leica TCS SP2 confocal microscope, described in detail in chapter 2.

For constructs containing the C-terminus of SERCA2b, characterisation was also carried out by a selective permeabilisation protocol modified from Butler *et al.* (2007)[28]. Two days after transfection in 24 well plates, COS-7 cells expressing the appropriate constructs were washed in PBS and fixed with 4% formaldehyde (in PBS) for 15 minutes. Cells were washed with PBS alone (no membrane permeabilisation), or supplemented with 0.01 mg/ml saponin (for plasma membrane permeabilisation) or 0.1% Triton X-100 (for entire membrane permeabilisation). Blocking was carried out with PBS supplemented with no detergent, saponin or Triton X-100 and 2% low fat dried milk (buffer P) for 30 minutes. Mouse anti-GFP antibodies (Roche) were added at a 1:100 dilution in the appropriate buffer P for 1 hour at 37 °C. Antibody was removed, and cells washed three times in buffer P (allowing 5 minutes for each wash). Anti-mouse Texas Red conjugated secondary antibody (GE healthcare) was added at 1:50 in Triton X-100 buffer P and incubated for 1 hour at 37 °C. Adding Trition at this step does not interfere with the primary antibody binding, and permeabilisation of all membranes allows faster movement of the antibody within the cells and more efficient removal of unbound antibody. Secondary antibody was removed and cells were washed three times in Triton X-100 buffer P and once in PBS before being mounted as described in chapter 2.

## 3.3 Results

All chimeras constructed were expressed in COS-7 cells and their subcellular distributions were assessed using confocal microscopy and immunofluorescence. A full list of all the chimeras constructed, and where they are located is shown in figure 3.12.

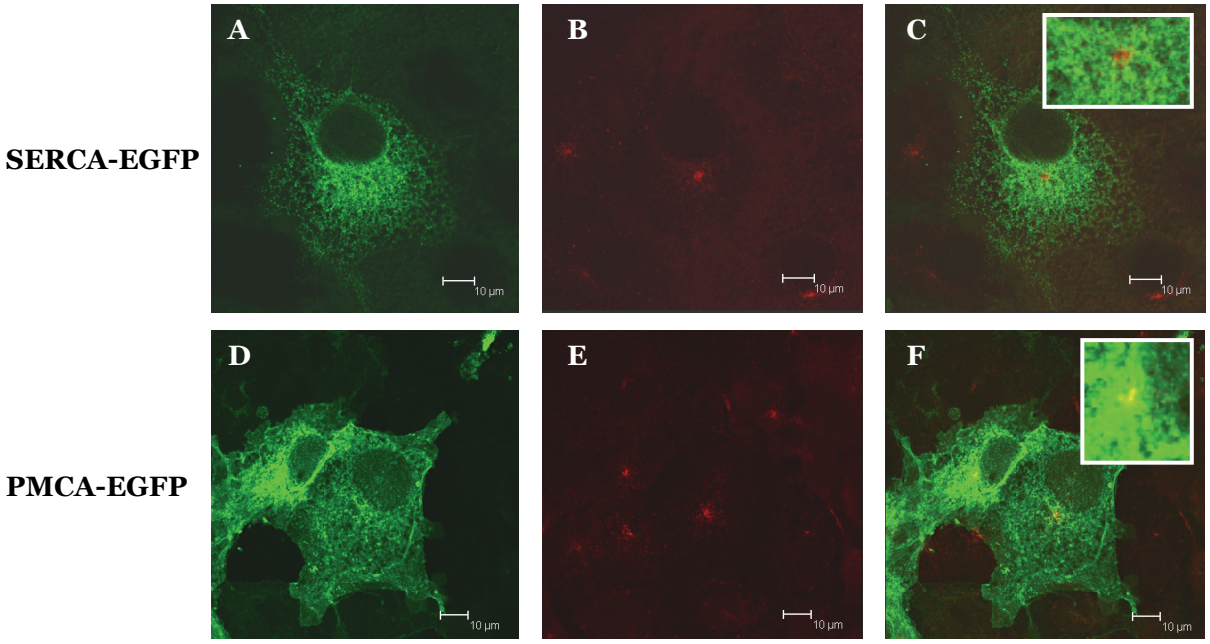



**Figure 3.5 Expression of SERCA-EGFP and PMCA-EGFP in COS-7 cells**

COS-7 cells were transfected with DNA encoding SERCA-EGFP (A-C) and PMCA-EGFP (D-F) in pcDNA3.1 (+). After 2 days, cells were treated with BFA and anti-TGN antibodies (B and E) and analysed by laser scanning confocal microscopy. Overlay images are shown in panels C and F, with inlaid images showing enlarged images of the trans-Golgi.

SERCA-EGFP (figure 3.5) shows a reticular pattern, typical of ER/ERGIC proteins. The lack of colocalisation with TGN46 shows that SERCA does not reach the trans-Golgi. Cells expressing PMCA-EGFP (figure 3.5) show a clear outline of the plasma membrane and colocalisation of the protein with the trans-Golgi marker TGN46.

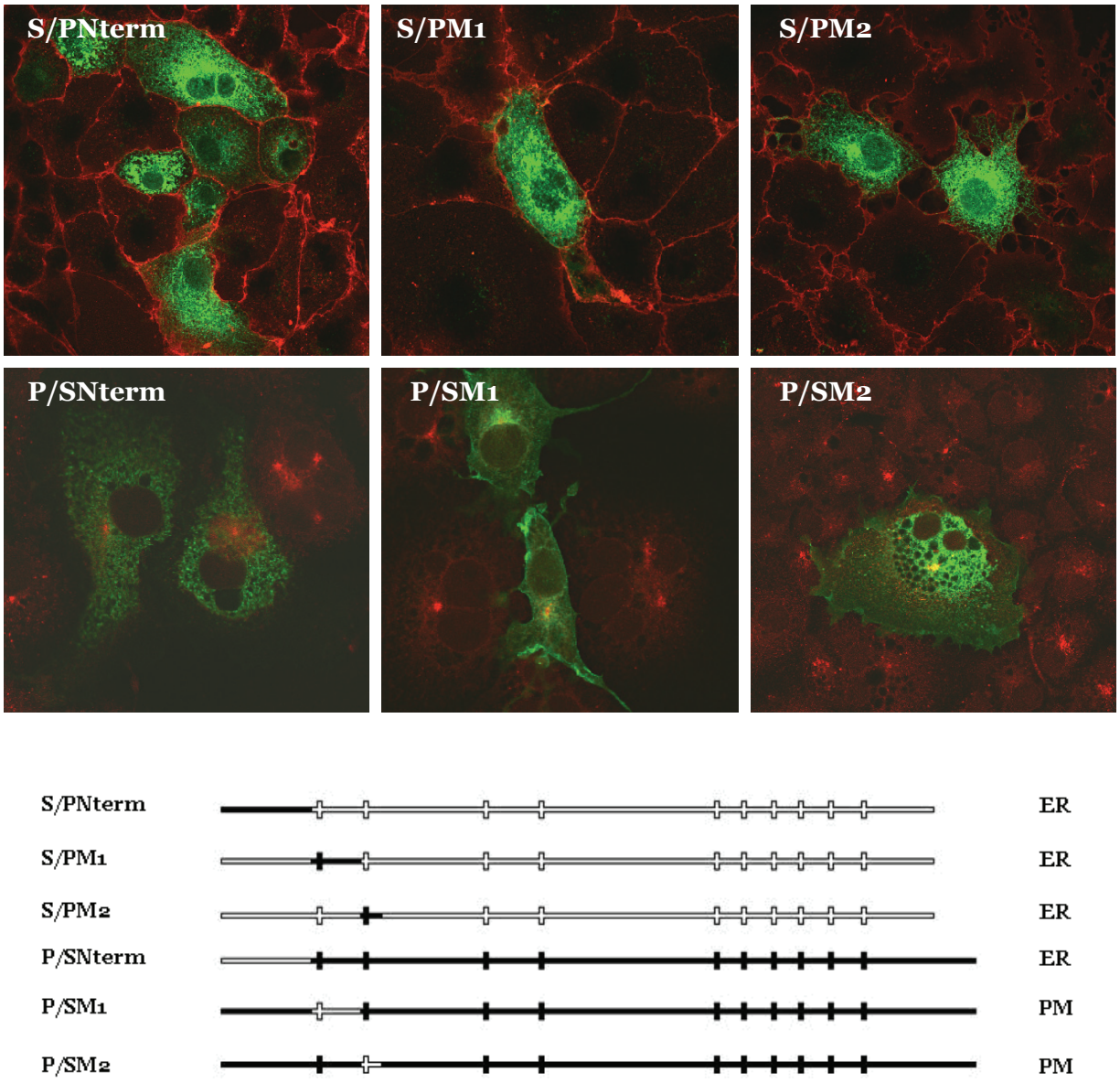



**Figure 3.6 Constructs built to detect a retrieval signal at the N-terminus of SERCA**

COS-7 cells were transfected with EGFP tagged chimeras as indicated. After 2 days, cells were treated with conA conjugated to Alexa Fluor 594 (top panels) or BFA and antibodies against TGN46 visualised using a Texas Red conjugated secondary antibody (bottom panels). Images were obtained using a Leica TCS SP2 confocal microscope. The architecture of the constructs and their locations are shown below, with SERCA and PMCA in white and black respectively, and transmembrane domains shown as vertical lines.

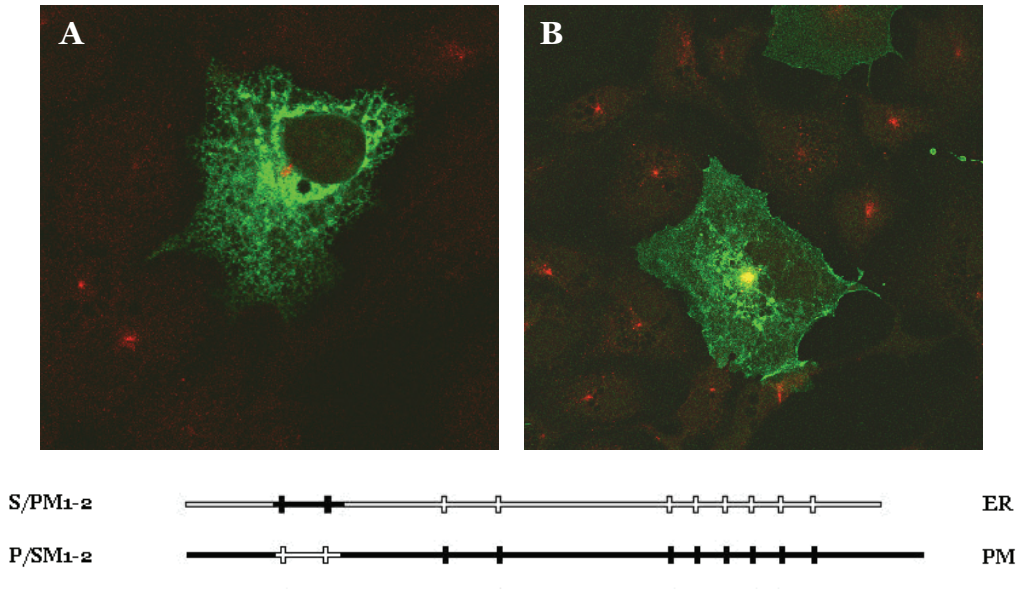



**Figure 3.7 Chimeras to determine the importance of M1 and M2 in retrieval**

COS-7 cells expressing S/PM1-2 (A) or P/SM1-2 (B) are shown 2 days after transfection. Cells were treated with BFA and anti-TGN46, followed by Texas Red conjugated secondary antibody. Analysis was carried out with confocal microscopy. SERCA sequence is shown in white and PMCA in black. Vertical lines represent transmembrane helices.

Previous studies of this type have highlighted the N-terminus of SERCA as containing sequence important for maintenance in the ER[94,146,147]. For this reason, the N-terminal portion of SERCA was divided into three sections and replaced with corresponding PMCA sequence (see figure 3.6). S/PNterm and P/SNterm are located in the ER. Replacing M1 or M2 of SERCA sequence with that of PMCA, results in the protein localising to the plasma membrane (P/SM1 and P/SM2). The opposite constructs (S/PM1 and S/PM2) show ER localisation. ConA surface labelling and TGN46 colocalisation were used to characterise these constructs. The effect of both M1 and M2 in combination was tested by building S/PM1-2 and P/SM1-2 which showed localisation in the ER and plasma membrane respectively (figure 3.7). These constructs do not indicate the presence of an ER localisation signal within the first two transmembrane domains of SERCA. In order to analyse the ability of the rest of the SERCA sequence to cause ER localisation, six constructs were then made in which the whole SERCA pump was divided into three sections and each one replaced with corresponding PMCA sequence.

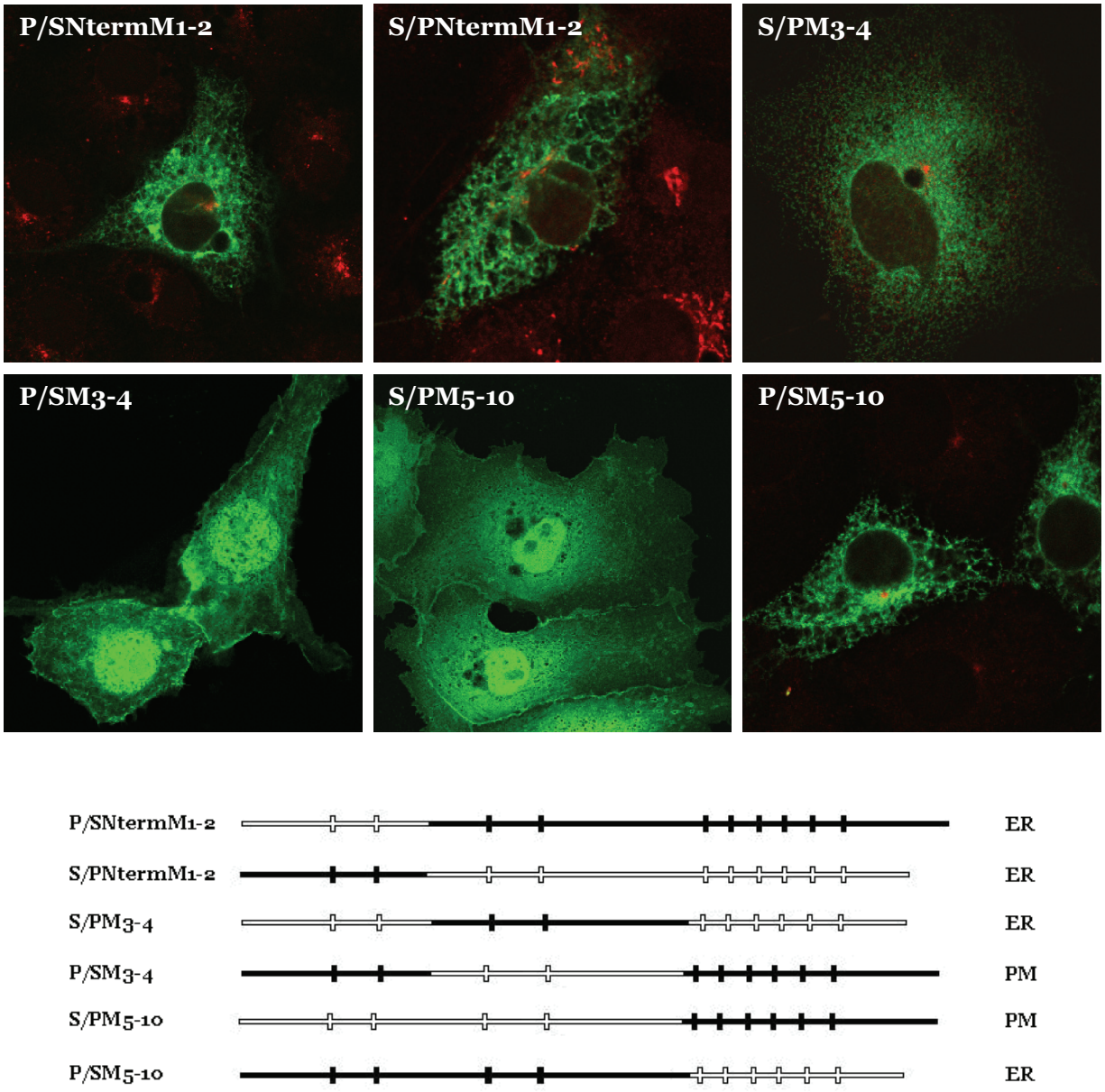



**Figure 3.8 Chimeras to search for a retrieval signal in the entire SERCA sequence**

COS-7 cells were transfected with the constructs shown above. After two days, the reticular constructs were treated with BFA and TGN46 antibodies which were visualised with a Texas Red conjugated secondary antibody. All images were obtained by confocal laser scanning microscopy. The compositions of the chimeras are shown below with SERCA in white, PMCA in black and transmembrane helices shown by vertical lines. The localisation of each is shown on the right.

The localisations of the six constructs in figure 3.8 suggest that the C-terminus of SERCA contains information dictating its retrieval to the ER. Replacement of the C-terminus of SERCA with that of PMCA (S/PM5-10) causes mislocalisation of the protein to the plasma membrane, whereas PMCA containing the C-terminus of SERCA (P/SM5-10) shows ER localisation and is not present in the trans-Golgi.

Further chimeras were constructed to dissect the C-terminus of SERCA in an attempt to locate the ER retrieval signal. These are shown in figure 3.9. Involvement of the last four and last two transmembrane helices of SERCA in retrieval was analysed. However, S/PM7-10, PM7-10, S/PM9-10 and P/SM9-10 were all localised in the ER and did not travel as far as the trans-Golgi. This may indicate misfolding of some or all of these constructs. This will be described in detail in the discussion section of this chapter. Chimeras SM1-2M9-10 and SM1-2/PM9-10 were constructed with the aim of determining whether M9 and M10 of SERCA play a role in retrieval. In both constructs, the first two helices of SERCA were included. M1 is required to cause initial ER targeting[38], and M2 was included to allow correct orientation of M9 and M10 in the membrane. A flexible ten amino acid linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ was used to connect M1-2 and M9-10. Both of these chimeras showed a reticular distribution and were not present in the trans-Golgi (see figure 3.9).

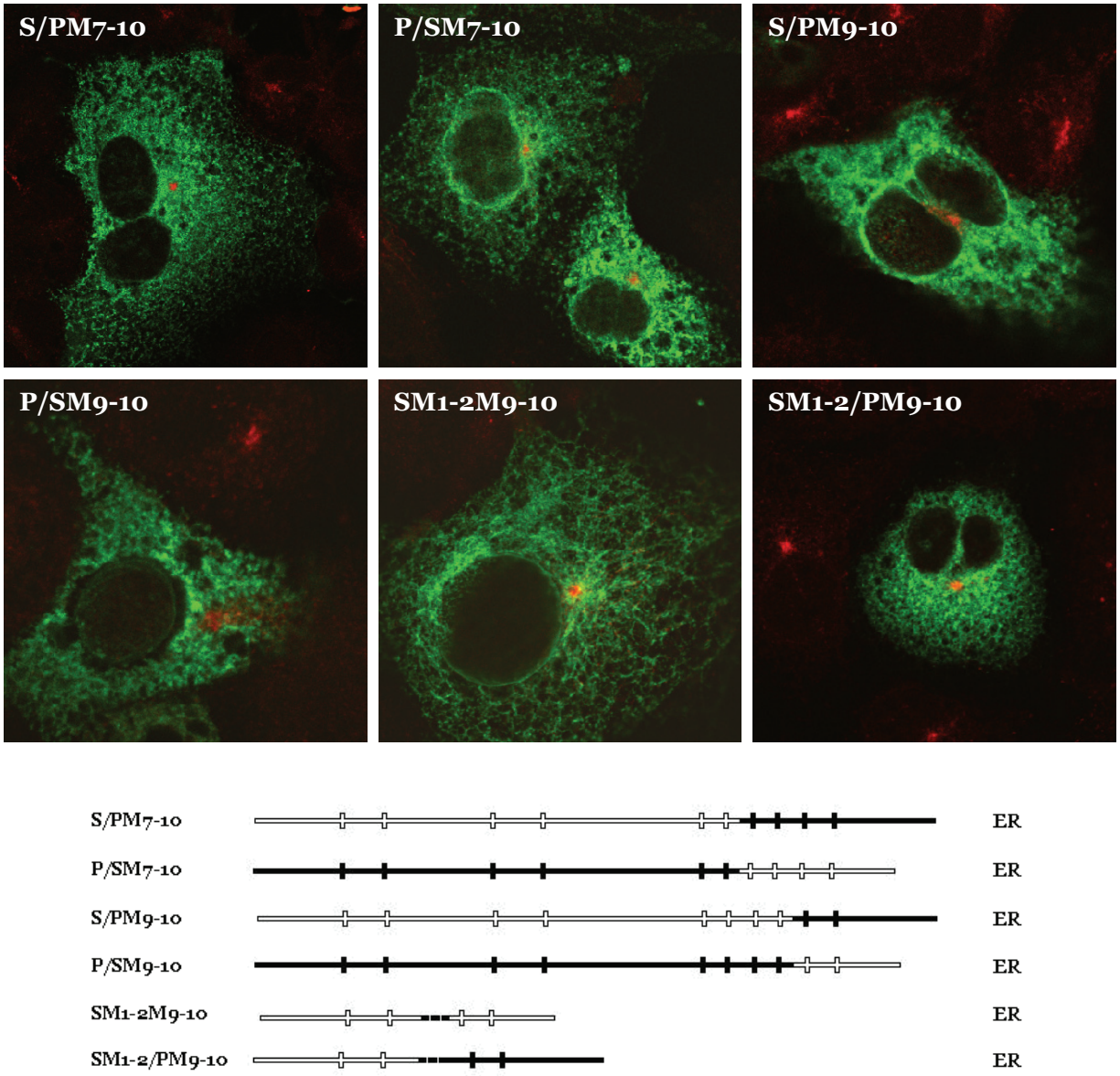



**Figure 3.9 Chimeras to detect a retrieval signal at the C-terminus of SERCA**

COS-7 cells were transfected with DNA encoding constructs as indicated. After 2 days, cells were treated with BFA and anti-TGN46 antibodies, visualised using a Texas Red conjugated secondary antibody. Images were acquired by confocal microscopy. The constructs and their localisations are shown below the panels (SERCA in white and PMCA in black).

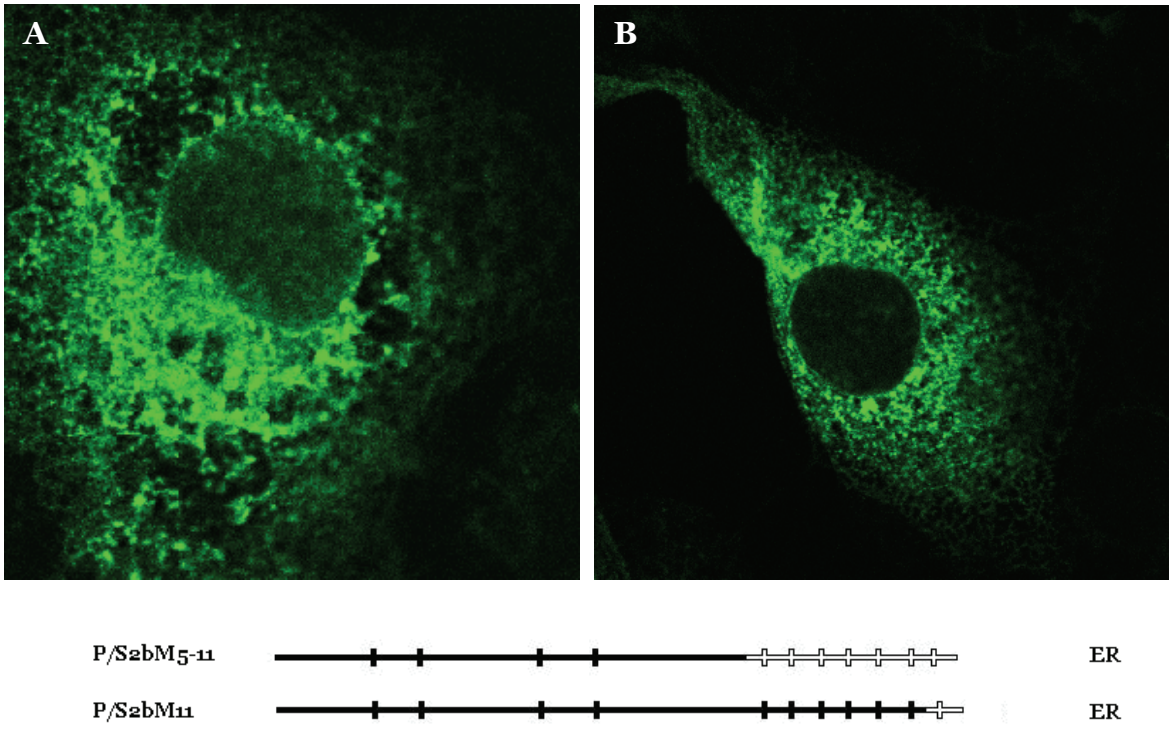
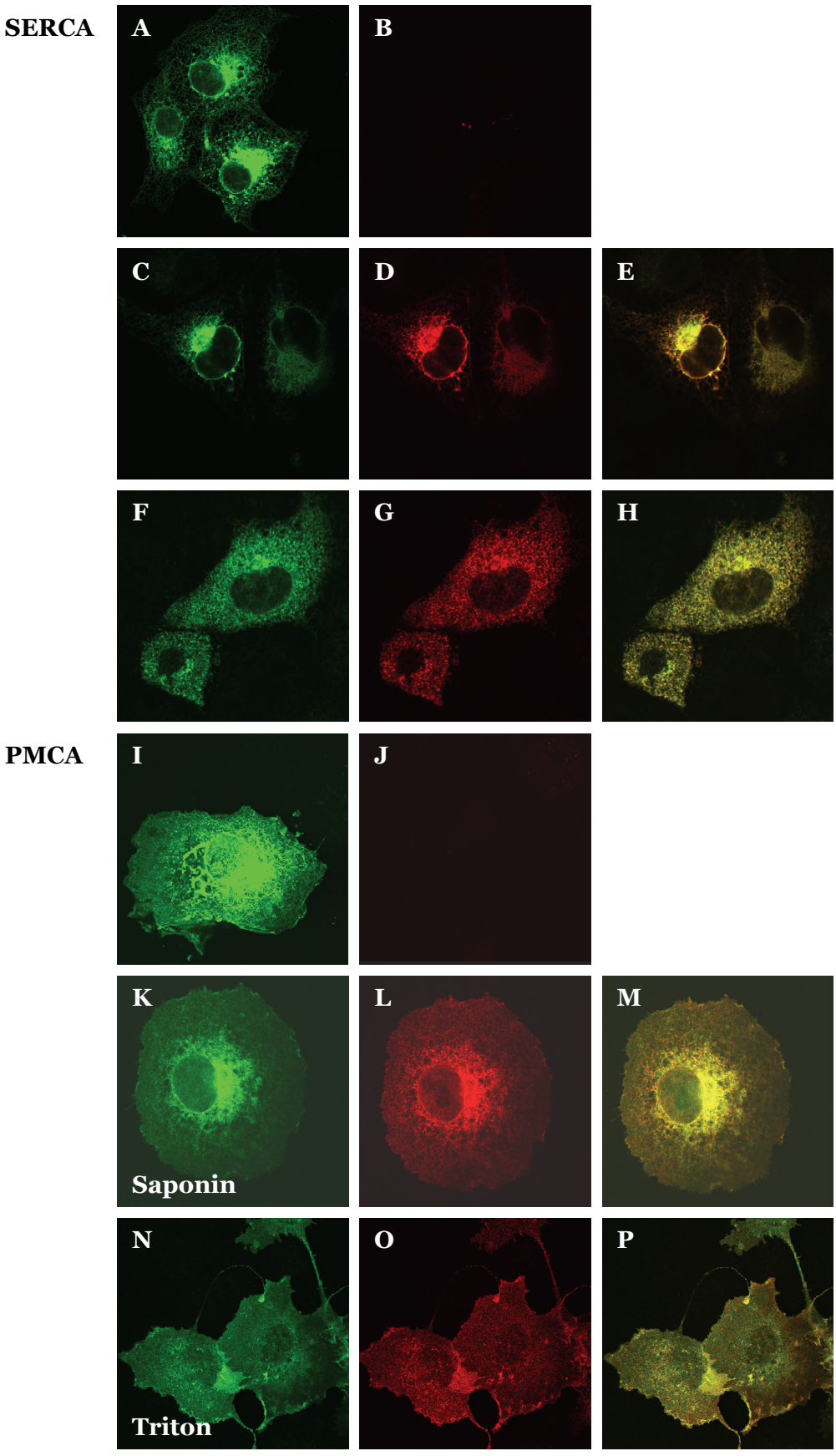



**Figure 3.10 SERCA2b based constructs**

COS-7 cells were transfected with DNA encoding either P/S2bM5-11 (A) or P/S2bM11 (B). They were analysed by confocal microscopy 2 days following transfection. The chimeras and localisations are shown with SERCA2b sequence in white and PMCA in black. Vertical lines represent the transmembrane helices.

SERCA2b constructs, P/S2bM5-11 and P/S2bM11, were built for reasons detailed above. They both appeared to be ER localised as shown in figure 3.10. To characterise the topology of these constructs, selective permeabilisation was carried out, as described above. Step-wise permeabilisation of COS-7 cells expressing SERCA-EGFP, PMCA-EGFP and P/S2bM5-11 is shown in figure 3.11. These results confirm that the C-terminus of SERCA2b in the P/S2bM5-11 construct is located inside the ER lumen in contrast with SERCA and PMCA which have cytoplasmic C-termini. This experiment also demonstrated that the construct is not present at the cell surface, as no signal was seen when non-permeabilised cells were treated with anti-GFP antibodies.


SERCA
A
B
C
D
E
F
G
H
PMCA
I
J
K
L
M
Saponin
N
O
P
Triton

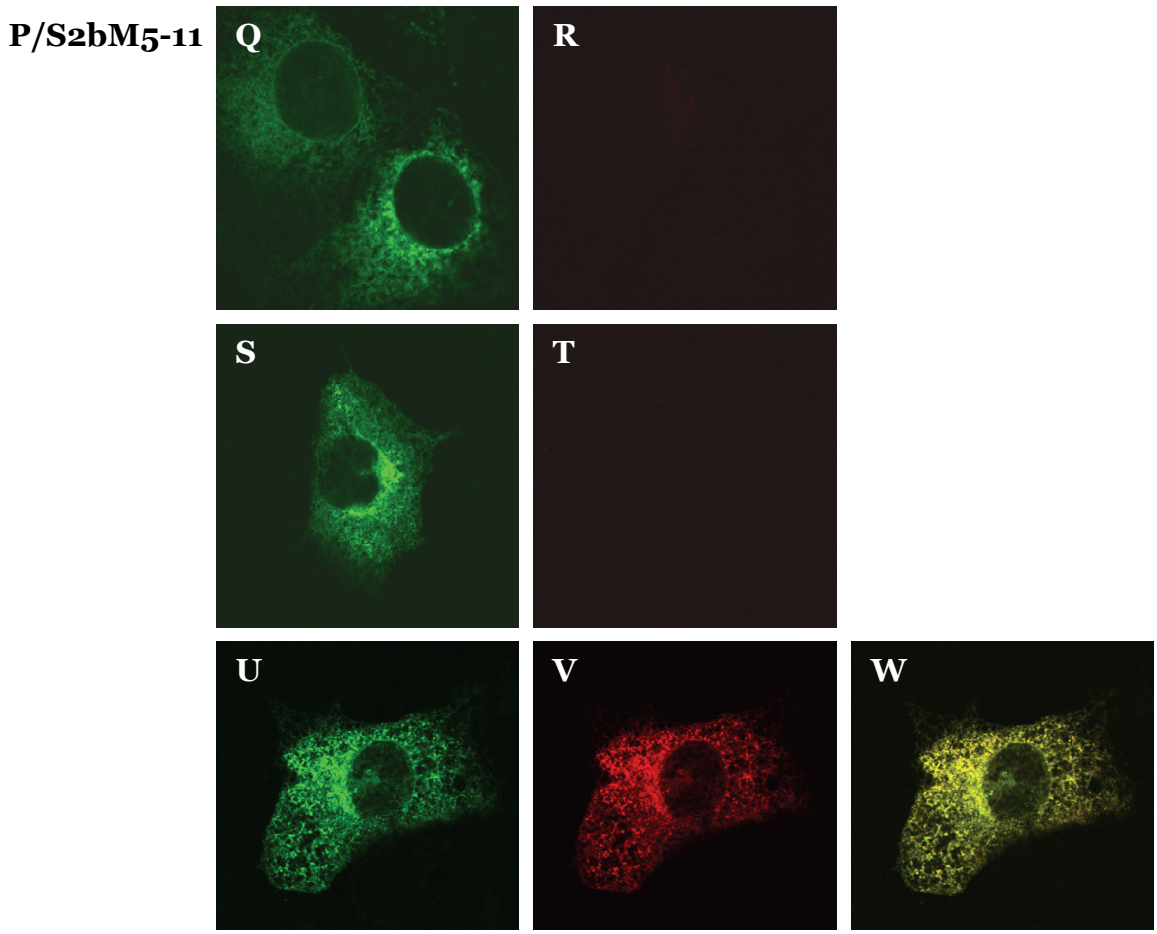



**Figure 3.11 Selective permeabilisation to determine protein topology**

COS-7 cells were transfected with SERCA-EGFP (A-H), PMCA-EGFP (I-P) or P/S2bM5-11 (Q-W). After 2 days, cells were treated with saponin to permeabilise the plasma membrane (C-E, K-M, S and T), Triton X-100 to permeabilise all membranes including the ER membrane (F-H, N-P and U-W) or PBS alone to keep all membranes intact (A, B, I, J, Q and R). Anti-GFP antibodies were added to detect the C-terminal EGFP tag on the proteins and visualised using a Texas Red conjugated secondary antibody (B, D, G, J, L, O, R, T and V). Overlay images where signal was seen with the antibody treatment are shown (E, H, M, P and W).

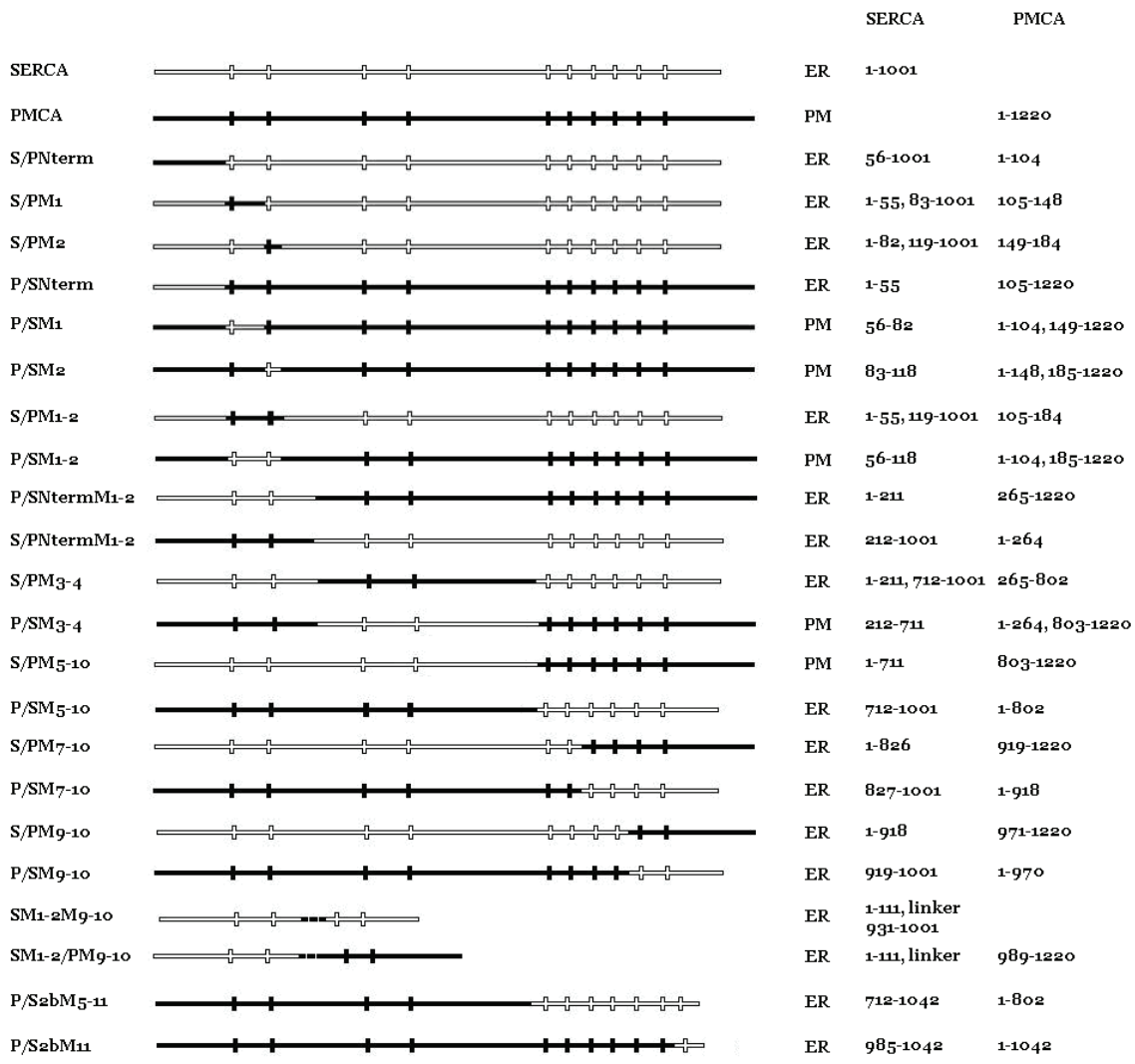



**Figure 3.12 Summary of all chimeras**

All chimeras constructed are shown, with names on the left hand side. They are all C-terminally tagged with EGFP. Their localisations in the ER or plasma membrane (PM) are shown, and their amino acid composition (numbers relating to SERCA and PMCA protein sequences) is detailed in the two columns on the far right. White sections correspond to SERCA (or SERCA2b) sequence and black to PMCA. Vertical lines indicate transmembrane helices.

## 3.4 Discussion

In this investigation, sections of SERCA and PMCA sequence have been joined in order to create a series of chimeric calcium pumps which have been used to detect regions of SERCA required for ER localisation. Previous studies on the trafficking of SERCA have made use of chimeras of this type. These studies have shown the N-terminus of SERCA is key to its maintenance in the ER[94,146,147]. The aim of the experiments reported in this chapter was to build on existing chimera studies in order to locate the sequence(s) in SERCA required for ER retrieval. Newton *et al.* reported the requirement for the first 211 residues of SERCA for its maintenance in the ER[94]. Two studies by a different group have also demonstrated a requirement for the N-terminus of SERCA. Foletti *et al.* used SERCA/PMCA chimeras to show that the first 85 amino acids of SERCA were sufficient to cause ER localisation[146]. This conclusion was drawn from an ER localised chimera consisting of PMCA sequence with a substitution of SERCA sequence at the N-terminus. Importantly, the opposite construct to this (which presumably would be localised to the plasma membrane) was not built in the study, leaving the possibility of ER maintenance by the quality control machinery open. More work from the same group in a paper by Guerini *et al.* suggests the first 28 amino acids of SERCA have a role to play in ER retrieval of the protein. However, the authors point out that there is likely to be sequence elsewhere in the pump involved in retrieval, as in cells expressing chimeras consisting of PMCA and the N-terminal 28 residues from SERCA, less than half showed ER localisation of the chimera[147].

Due to the interest raised by these studies in the N-terminus of SERCA, this investigation began with a detailed analysis of the role played by the N-terminal residues of SERCA in ER retrieval. Six chimeras were built in which amino acids at the N-terminus of the pump were mutated. S/PNterm, S/PM1 and S/PM2 are based upon SERCA sequence with PMCA N-terminus, M1 or M2 respectively substituted for corresponding SERCA sequence. These chimeras were all ER localised (see figure 3.6), suggesting that either none of these regions in SERCA are required for ER retrieval, or that the chimeras did not undergo proper folding. In order to clarify this situation, the mirror opposites of these constructs were built (P/SNterm, P/SM1 and P/SM2) and localisation in COS-7 cells was determined (also shown in figure 3.6).

Both P/SM1 and P/SM2 were localised at the plasma membrane, indicating that neither M1 nor M2 of SERCA are sufficient to cause ER retrieval. Like its mirror opposite, P/SNterm was also located in the ER, allowing no conclusion to be drawn at this stage with respect to the very N-terminal section of SERCA preceding M1. The ability of the combination of both M1 and M2 of SERCA to cause retrieval to the ER was also tested by building S/PM1-2 and P/SM1-2. The results (shown in figure 3.7) confirmed that M1 and M2 together are unable to cause ER localisation. Replacing M1 and M2 of PMCA with corresponding SERCA sequence produced a protein localised to the plasma membrane, whereas SERCA with corresponding PMCA sequence replacing M1 and M2 was still able to be retrieved to the ER.

As neither M1 nor M2 (both separately or in combination) of SERCA appear able to cause retrieval from the ER, the SERCA pump was then divided into three sections and six constructs were built to systematically test each third of the protein for ER retrieval capabilities. The localisations of these chimeras in COS-7 cells are shown in figure 3.8. Both P/SNtermM1-2 and S/PNtermM1-2 were located in the ER and did not travel as far as the trans-Golgi. As these mirror opposites are both located in the ER, no conclusions can be drawn here, and misfolding of one or both chimeras cannot be ruled out. The middle section of SERCA (amino acids 212-711) does not appear to be required for ER localisation, as replacement of this portion with corresponding PMCA sequence (S/PM3-4) results in an ER localised chimera and its introduction into PMCA (P/SM3-4) does not cause ER retrieval. The C-terminal section of SERCA was shown to be needed for ER localisation. Chimera S/PM5-10 in which the last third of SERCA was replaced with corresponding PMCA sequence showed plasma membrane localisation, indicating that this section of the protein is needed for correct localisation. P/SM5-10 however, showed ER localisation, as presumably the SERCA sequence within this construct is causing the protein to be retrieved to the ER. By analysing the localisations of these six chimeras, it seems likely that the sequence in SERCA which mediates its retrieval to the ER is located within residues 712-1001, at the C-terminus of the protein.

Further chimeras were then built to dissect the C-terminus in an attempt to narrow down the location of the ER retrieval signal, as shown in figure 3.9. Chimeras S/PM7-10 and P/SM7-10

were built to determine whether the ER retrieval signal lies within M7-10 of SERCA. Both of these constructs showed an ER distribution and did not travel to the trans-Golgi. S/PM9-10 and P/SM9-10 were constructed in an attempt to determine the role (if any) of the last two transmembrane helices of SERCA in ER localisation. Again, both of these constructs were located in the ER. As misfolding and subsequent ER retention by the quality control machinery of the cell may be accountable for the localisation of these chimeras, two constructs were then built consisting of only four transmembrane domains. The aim of this experiment was to determine whether the ER retrieval signal of SERCA is located within M9-10 of SERCA, and it was hoped that by using smaller, simpler constructs, the propensity of the proteins to misfold may be reduced. M1 and M2 of SERCA were connected by a flexible ten amino acid linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ to M9-10 of SERCA (SM1-2M9-10) or to M9-10 of PMCA (SM1-2/PM9-10). Both of these constructs were ER localised and were not present in the trans-Golgi. The results from these six constructs (all shown in figure 3.9) do not provide answers as to where in the C-terminal section of SERCA the retrieval signal is located. It is possible that some or all of these proteins are misfolded and that their ER localisation is due to the action of quality control mechanisms within the cell. Protein misfolding and possible ways to detect and circumvent it in studies such as this will be discussed in detail in chapters 4 and 5.

SERCA2b/PMCA chimeras P/S2bM5-11 and P/S2bM11 were built for reasons detailed above. They were expressed in COS-7 cells and were located in the ER, as shown in figure 3.10. The use of M11 from SERCA2b as a tool to detect cell surface localisation of chimeras was not pursued, as adding sequence for this extra transmembrane domain to PMCA (in P/S2bM11) resulted in the mislocalisation of PMCA to the ER. This may be a result of protein misfolding, but there is a possibility that information in this final transmembrane helix of SERCA2b may cause ER retrieval. Either way, adding this sequence to chimeric constructs would not have been a suitable assay to test for plasma membrane localisation. The selective permeabilisation experiment used to detect the C-terminal EGFP tag on the P/S2bM5-11 construct is shown in figure 3.11. This confirms that the protein is orientated correctly, as the EGFP tag appears to be in the ER lumen (as opposed to cytoplasmic in the case of SERCA and PMCA which both have 10 rather than 11 transmembrane helices). Anti-GFP antibodies

were only able to bind to the C-terminus of P/S2bM5-11 when all membranes had been permeabilised with Triton X-100. No signal was seen from antibodies added to cells in which only the plasma membrane had been permeabilised by treatment with saponin.

The chimeric proteins constructed in this investigation illustrate that the sequence(s) required for ER retrieval is located between residues 712-1001 of SERCA. Further dissection of the C-terminus yielded only ER localised chimeras from which no conclusions could be drawn. Although chimeric proteins can provide important information about targeting signals in proteins, the fact that the ER is a destination for misfolded proteins is problematic when searching for ER localisation signals. For this reason, plasma membrane localised constructs are required for drawing conclusions in this type of study. Where possible, SERCA and PMCA sequences were joined in conserved regions in an effort to avoid misfolding. However, with such complex polytopic membrane proteins it is impossible to predict how changing the sequence may affect the overall tertiary structure of the resulting protein, and whether misfolding will occur. Although SERCA and PMCA show significant homology, without the crystal structure of PMCA no accurate estimations can be made as to the structures of the resulting proteins. Figure 3.13 shows an example of a chimera used in this study and highlights the complexity of such a protein.

In addition to potential misfolding of the chimeras, other explanations may exist for the presence of both chimeras from a mirror pair in the ER. In some cases, redundancy in the retrieval signal may explain the localisation of opposite constructs to the ER. For example, in the case of chimeras S/PM9-10 and P/SM9-10 (see figure 3.12), it is possible that there is sequence either side of the join between SERCA and PMCA that is sufficient to retrieve the protein, and as a result both are retrieved to the ER.

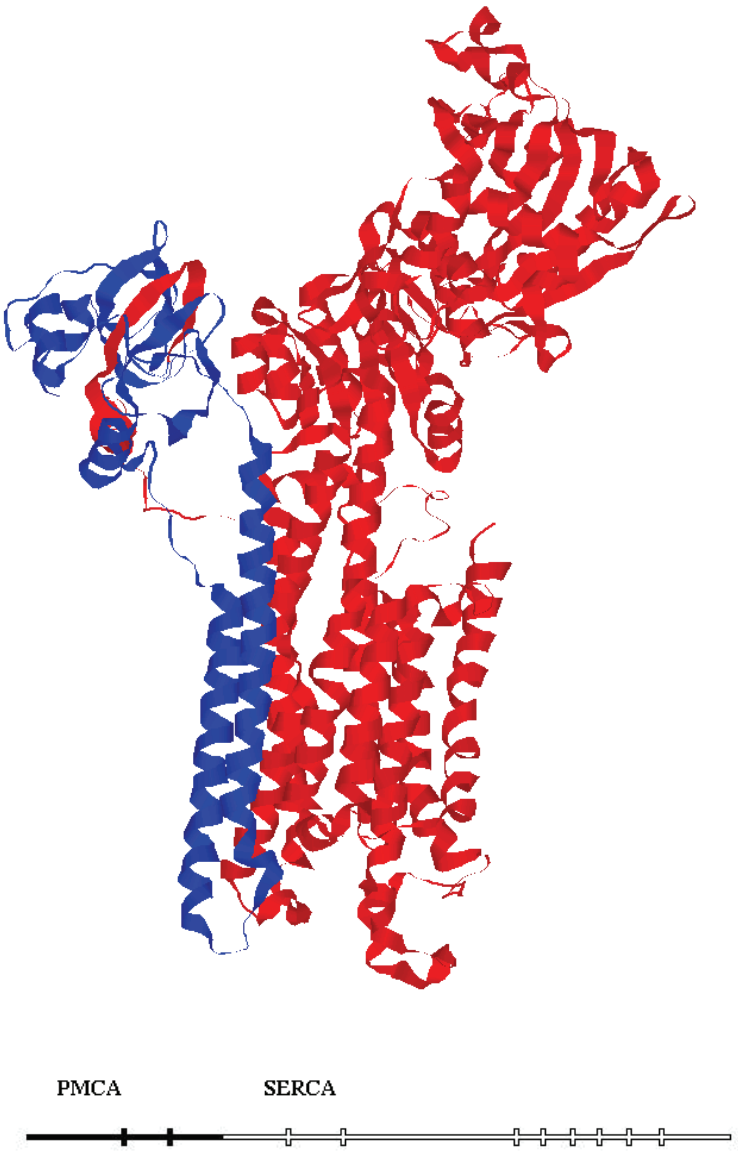



**Figure 3.13 Chimera S/PNtermM1-2**

In chimera S/PNtermM1-2, the first 211 residues of SERCA (shown in blue) were replaced with corresponding PMCA sequence. The rest of the pump consists of SERCA sequence (red). The primary structure of the chimera is shown below with PMCA and SERCA in black and white respectively and vertical lines indicating transmembrane helices. The SERCA structure (PDB code 1SU4) was rendered in Rasmol.

As mentioned above, the SERCA and PMCA pumps are large and complex proteins which are folded and held together by many intramolecular interactions. It is conceivable that PMCA contains the same or similar ER retrieval signal as SERCA, but in the correctly folded plasma membrane pump this sequence information may be masked by sequence elsewhere in the protein. Changing the sequence of PMCA by creating protein chimeras may expose this sequence, causing ER localisation of the pump mediated by PMCA not SERCA sequence. However, if the plasma membrane is indeed a 'default' localisation for proteins travelling through the secretory pathway[90], it seems illogical that more, rather than less, information is required to arrive there. The lines between misfolding and masking and unmasking of signals become blurred here, introducing potential for ambiguity in the interpretation of results. The importance of plasma membrane chimeras should be highlighted again here. By trafficking to the plasma membrane, these chimeras are able to escape the quality control machinery and are therefore not misfolded. Without additional studies to determine the folding of ER chimeras, plasma membrane chimeras are required for conclusions to be drawn from this type of study.

Over 20 crystal structures of SERCA in different conformations have now been published[18]. The availability of these structures means that the conformational changes of the pump during its catalytic cycle can be pieced together, rather like a flick-book of static images which come together to form an animation. If SERCA is indeed recognised by a receptor in the early Golgi in order to be retrieved to the ER, it would be easier to envisage this happening by recognition of a stationary rather than very dynamic portion of the pump. Looking at the collection of snapshots we now have of SERCA during calcium transport, it is clear that the protein undergoes very large conformational changes. The C-terminal transmembrane helices (M9 and M10) of SERCA appear to be the least dynamic part of the protein[18]. If transmembrane domains form part of the retrieval signal of SERCA then M9 and M10 seem likely candidates based on the fact that they do not move a great deal during calcium transport by SERCA. This fits well with the data from the chimeras presented here which show that the C-terminus of SERCA is necessary and sufficient for ER retrieval.

From observing the subcellular localisations of the chimeras built in this chapter, it can be concluded that the C-terminal section (residues 712-1001) of SERCA is required for its

retrieval to the ER. As so many of the chimeras constructed in this investigation (17 chimeras out of a total of 22) are localised in the ER, there is a significant possibility that at least some of these are misfolded and are maintained in the ER by quality control rather than specific sequence mediated retrieval. In the next chapter, possible ways of detecting misfolding in chimeric proteins will be discussed.

# 4. Detecting protein misfolding in chimeric calcium pumps

## 4.1 Introduction

Protein quality control is a vital part of cellular homeostasis. It is crucial that the cell can detect aberrant folding of proteins and take measures to allow re-folding or to activate degradation pathways in the case of terminally misfolded proteins[155]. This study makes use of mammalian systems to overexpress complex, heterologous proteins. An understanding of the quality control systems of the cell and methods to detect protein misfolding is therefore important, particularly when we may consider localisation of a chimera in the ER as a read-out of signal mediated protein retrieval.

The results presented in the previous chapter show that many of the SERCA/PMCA chimeras are located in the ER. In some cases, this ER localisation is likely to be due to protein misfolding rather than specific sequence mediated ER retrieval. For example, both P/SNtermM1-2 and S/PNtermM1-2 are located in the ER despite being constructed from opposite sections of SERCA and PMCA (see figure 3.12). Evidence from other chimeras in this study (S/PM5-10 and S/PM1-2) show that the N-terminal section of SERCA is not required for ER localisation, suggesting that P/SNtermM1-2 and possibly also S/PNtermM1-2 are maintained in the ER by quality control rather than signal mediated retrieval. It is also interesting to note that most of the chimeras in which the C-terminus of the protein has been mutated show ER localisation. The lower sequence homology between SERCA and PMCA C-termini in comparison with the rest of the protein sequences could conceivably increase the propensity of these chimeras to misfold. If a suitable assay could be developed to test for misfolding in these chimeras, it would be possible to gain more of an insight as to where in SERCA the ER retrieval signal is located.

Two mechanisms are used by the ER to detect and respond appropriately to misfolded proteins; the unfolded protein response (UPR) and ER-associated degradation (ERAD)[69]. The unfolded protein response (UPR) describes the changes the ER undergoes in order to tackle elevated levels of unfolded protein in the organelle. Under normal circumstances, the ER

employs a range of chaperones to facilitate protein folding in the lumen. These chaperones are often calcium dependent and bind to proteins to allow folding, as well as catalysis of post-translational modifications including N-linked glycosylation and disulphide bond formation[69]. BiP is a chaperone which binds to unfolded proteins in order to hold them in a folding-competent state. It uses cycles of ATP hydrolysis to bind and unbind the unfolded protein, allowing an opportunity for correct folding with each cycle. BiP exists as both a monomer (which binds unfolded proteins) and an oligomer (which acts as a pool of unbound BiP in the ER). A marked increase in unfolded protein leads to depletion of the oligomeric pool of BiP, a change which may begin a signalling pathway ultimately resulting in adaptation of the ER and initiation of the UPR[155]. Other sensors of the UPR include IRE1 and PERK; transmembrane kinases able to communicate the presence of unfolded proteins in the ER lumen to downstream activators in the cytoplasm. The UPR is characterised by activation of signalling pathways which increase the protein folding capacity of the ER. As a result of the UPR, the role of the ER as a site of protein synthesis is compromised, with a decrease in transcription of genes involved in translation. This allows upregulation of transcription of genes encoding ER resident chaperones. An increase in the size of the ER is also observed during the UPR[69,155].

The ERAD pathway is distinct from the UPR and is responsible for the degradation of terminally misfolded proteins[69]. This degradation is carried out by the ubiquitin proteasome system located in the cytoplasm. Any protein in the ER lumen or membrane must first be transported across the membrane and into the cytoplasm where it is then conjugated to ubiquitin; a small protein which functions as a tag for destruction by the proteasome. Terminally misfolded proteins are recognised by chaperones in the lumen, including BiP, and escorted to a channel which is able to retrotranslocate the misfolded protein back across the ER membrane into the cytoplasm. It is not clear whether this channel is the same Sec61 translocon that allows synthesising peptides into the ER lumen, or if another protein pore is required. After crossing the ER membrane, misfolded proteins are targeted for destruction by the addition of polyubiquitin chains, catalysed by enzymes located on the cytoplasmic side of the ER membrane. The ubiquitinated protein is then degraded in the catalytic core of the 26S proteasome[69,156,157].

The UPR is characterised by specific upregulation of certain proteins[69]. Misfolding of chimeras in this study could feasibly be detected by measuring expression levels of UPR proteins including the chaperone BiP. In order to measure this in cells expressing SERCA/PMCA chimeras, suitable controls have been used. The cystic fibrosis transmembrane conductance regulator protein (CFTR) is a chloride ion transporter usually localised to the plasma membrane. The ΔF508 mutation of the CFTR gene causes the protein to be recognised as misfolded and maintained in the ER. This mutation, causing deletion of a phenylalanine in the CFTR channel, manifests as cystic fibrosis in individuals homozygous for the mutant allele[157,158]. ΔF508 CFTR is an appropriate positive control to use here for detecting misfolding in the chimeras. Both CFTR and PMCA are multi-spanning membrane proteins which, when correctly folded, arrive at the plasma membrane. ΔF508 CFTR has been shown previously to elicit the UPR and to cause BiP upregulation, as measured by increased BiP mRNA levels[158]. Another control that has been used in this study is the treatment of cells with tunicamycin; a pharmacological agent which causes aberrant folding of proteins in the ER by inhibiting N-linked glycosylation, resulting in the induction of the UPR[159]. The experiments described here attempt to detect misfolding in calcium pump chimeras by observing expression levels of the UPR marker protein BiP in cells expressing various constructs.

## 4.2 Methods

All general techniques (molecular biology, cell culture, transfection, microscopy and western blotting) are described in chapter 2. The GFP-tagged ΔF508 CFTR construct was a gift from Prof. B. Stanton[160].

Pharmacological induction of the UPR was carried out by incubating cells with tunicamycin (Sigma-Aldrich) at a final concentration of 5 μg/ml for 24 hours, as described in Bartoszewski *et al.* (2008)[158]. COS-7 and HeLa cells for western blotting were grown and transfected on 10 cm culture dishes (as detailed in chapter 2). Cells were harvested by washing twice with ice cold PBS before adding 400 μl sample buffer (as in chapter 2, but without bromophenol blue) at 60 °C to each plate, and scraping cells from the plates. Sample buffer was supplemented with 40 μl mammalian protease inhibitor cocktail (Sigma Aldrich). Samples were sonicated for 1 minute and aliquots frozen in liquid nitrogen. Protein concentrations were estimated using the Pierce BCA kit as described in chapter 2. Densitometry of western blots to quantify protein levels was carried out using the Versadoc Model 3000 imaging system (Biorad) and Quantity One software. Actin was used as a loading control for western blotting.

Antibodies and dilutions used in western blots were as follows: mouse anti-GFP, 1:500 (Roche); rabbit anti-BiP, 1:1000 (Abcam); mouse anti-actin, 1:10000 (Sigma-Aldrich); sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP), 1:2000 (GE Healthcare) and goat anti-rabbit IgG conjugated to HRP, 1:3000 (Abcam). GE Healthcare HRP conjugated secondary antibodies were affinity adsorbed (against rat, human and mouse).

Antibodies and dilutions used in immunofluorescence were rabbit anti-BiP, 1:50 (Abcam) and donkey anti-rabbit IgG conjugated to Texas Red, 1:100 (GE Healthcare). Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 1 μg/ml for 10 minutes to visualise nuclei.

Two mirror pairs of chimeras were selected for these experiments. P/SM1-2 and S/PM1-2 are localised to the plasma membrane and ER respectively, so presumably at least P/SM1-2 is

correctly folded. P/SNtermM1-2 and S/PNtermM1-2 are both located in the ER, and the SERCA sequence in P/SNtermM1-2 has been shown by other constructs not to be required for ER localisation, suggesting that one or both of these constructs may be misfolded.

## 4.3 Results

HeLa cells were transfected with various constructs, empty expression vector, treated with tunicamycin or left untransfected (all were left 48 hours before harvesting). Cell lysates were prepared and samples were analysed by western blotting to measure the levels of BiP and determine whether any constructs elicit the UPR in cells.

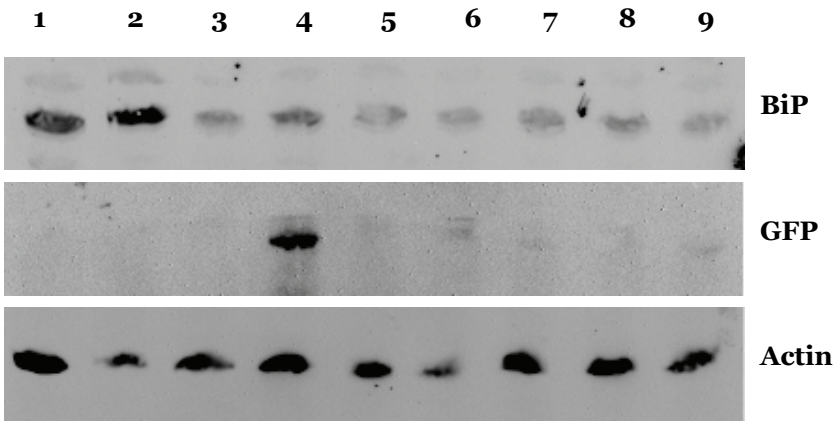



**Figure 4.1 Measurement of BiP levels in HeLa cells expressing chimeric constructs**
HeLa cells were transfected with constructs for 48 hours, or left untransfected or treated with tunicamycin. Lanes are as follows: (1) HeLa cells only, (2) tunicamycin treated, (3) empty pcDNA3.1 vector, (4) SERCA-EGFP, (5) PMCA-EGFP, (6) P/SM1-2, (7) S/PM1-2, (8) P/SNtermM1-2, (9) S/PNtermM1-2. Samples were analysed by western blotting to measure levels of BiP expression under different conditions. GFP antibodies were used to detect calcium pump expression and actin was used as a loading control.

Figure 4.1 shows the levels of BiP expression in HeLa cells expressing different constructs, treated with tunicamycin or left untreated. The expression of EGFP tagged constructs was also confirmed with fluorescence microscopy (not shown). Densitometry was then carried out on these blots to determine the expression of BiP relative to actin.

| Lane | Construct | Actin | BiP | BiP/Actin | Localisation |
|---|---|---|---|---|---|
| 1 | None | 67 | 37 | 0.6 | - |
| 2 | None + tunicamycin | 17 | 52 | 3.1 | - |
| 3 | pcDNA3.1 (empty vector) | 32 | 8 | 0.25 | - |
| 4 | SERCA-EGFP | 62 | 16 | 0.26 | ER |
| 5 | PMCA-EGFP | 36 | 8 | 0.22 | PM |
| 6 | P/SM1-2 | 14 | 4 | 0.29 | PM |
| 7 | S/PM1-2 | 47 | 7 | 0.15 | ER |
| 8 | P/SNtermM1-2 | 51 | 6 | 0.12 | ER |
| 9 | S/PNtermM1-2 | 29 | 4 | 0.14 | ER |

**Table 4.1 Densitometry to quantify BiP expression in HeLa cells expressing chimeras**
The expression of BiP in HeLa cells under each condition was calculated by densitometry relative to actin levels. Numbers indicate volumes of each band, compared to a zero value taken from a section of the blot containing no bands. The lane numbers correspond to the blot shown in figure 4.1. Localisations of constructs in the ER or plasma membrane (PM) are also shown.

Table 4.1 shows quantification of the blots in figure 4.1. Only cells treated with tunicamycin show an increase in BiP levels over that seen in untreated, untransfected cells. The GFP antibody detected only very low levels of chimeric calcium pump expression. Due to these low expression levels of the chimeric constructs in HeLa cells, the experiment was repeated in COS-7 cells which express higher amounts of protein from this expression vector due to the expression of the SV40 large T-antigen in this cell line[161] (see Invitrogen pcDNA3.1 manual). Protein concentration assays were carried out on the samples and an equal amount (12 μg) of protein was loaded from each condition (see figure 4.2). Again, as with HeLa cells, the only detectable BiP upregulation was seen in cells treated with tunicamycin, with no increase in BiP levels seen in cells transfected with any of the calcium pump constructs or empty vector.

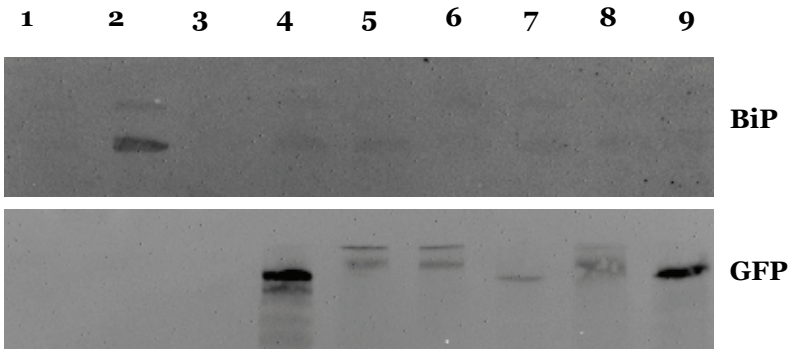
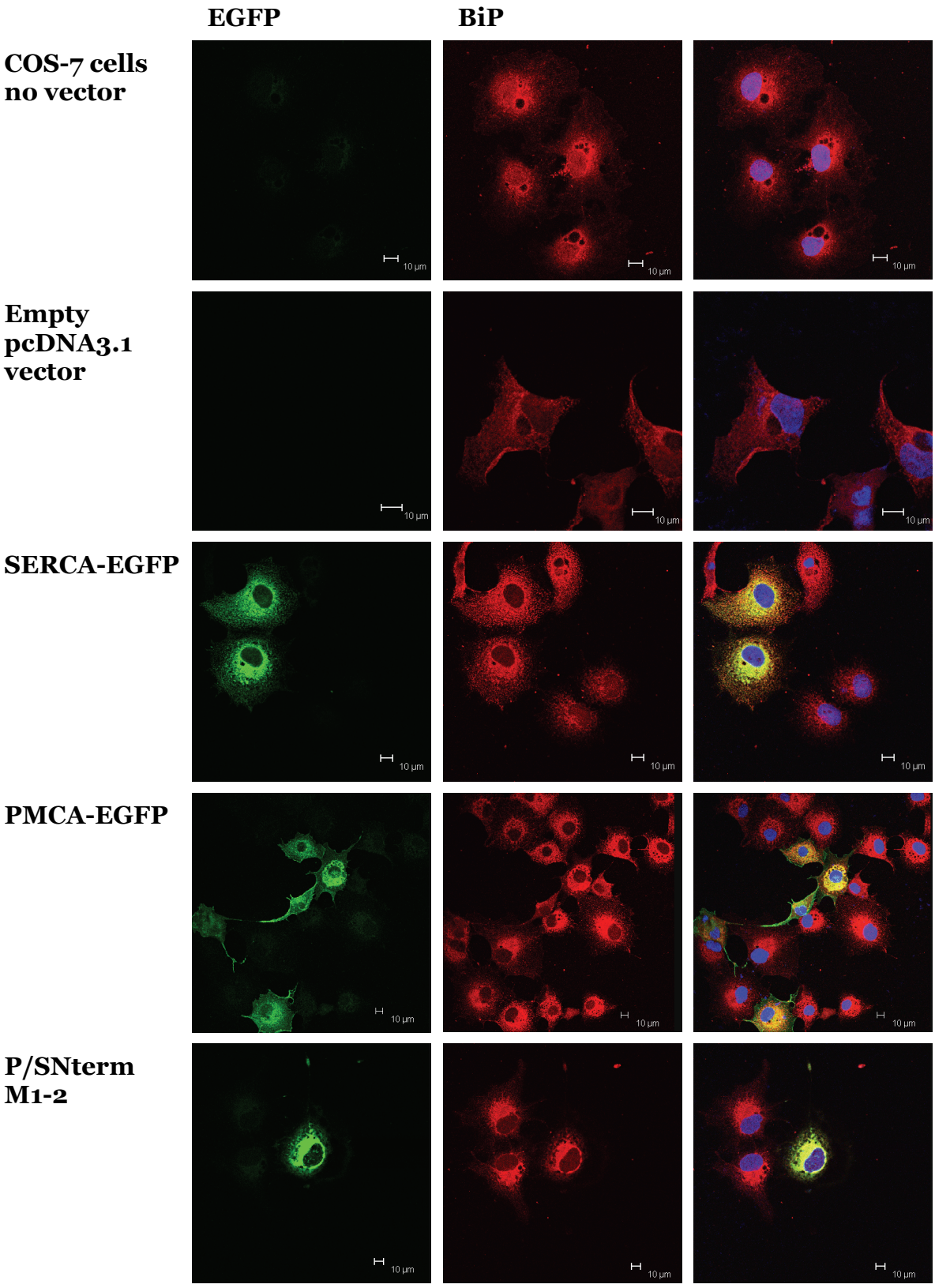



**Figure 4.2 Expression of BiP in COS-7 cells expressing chimeras**

COS-7 cells were transfected with constructs for 48 hours, or left untransfected or treated with tunicamycin. Lanes are as follows: (1) COS-7 cells only, (2) tunicamycin treated, (3) empty pcDNA3.1 vector, (4) SERCA-EGFP, (5) PMCA-EGFP, (6) P/SM1-2, (7) S/PM1-2, (8) P/SNtermM1-2, (9) S/PNtermM1-2. Samples were analysed by western blotting to measure levels of BiP expression under different conditions. GFP antibodies were used to detect calcium pump expression.

As no increase in BiP levels was seen with any of these constructs by western blotting, immunofluorescence was employed in an attempt to observe BiP expression. It is possible that the transfection efficiency (approximately 10%) of these constructs may dilute any BiP overexpression to a point at which it becomes undetectable by western blot. By using immunofluorescence, it may be possible to observe differences in BiP expression between transfected and untransfected cells containing chimeric calcium pumps. COS-7 cells were transfected with the same constructs as above and treated with BiP antibodies, visualised using a Texas Red secondary antibody. This is shown in figure 4.3. No notable difference can be observed between BiP levels in transfected and untransfected cells, even in cells transfected with CFTR ΔF508 which has been shown to upregulate BiP expression[158].


EGFP
BiP
COS-7 cells no vector
Empty pcDNA3.1 vector
SERCA-EGFP
PMCA-EGFP
P/SNterm M1-2
10 µm

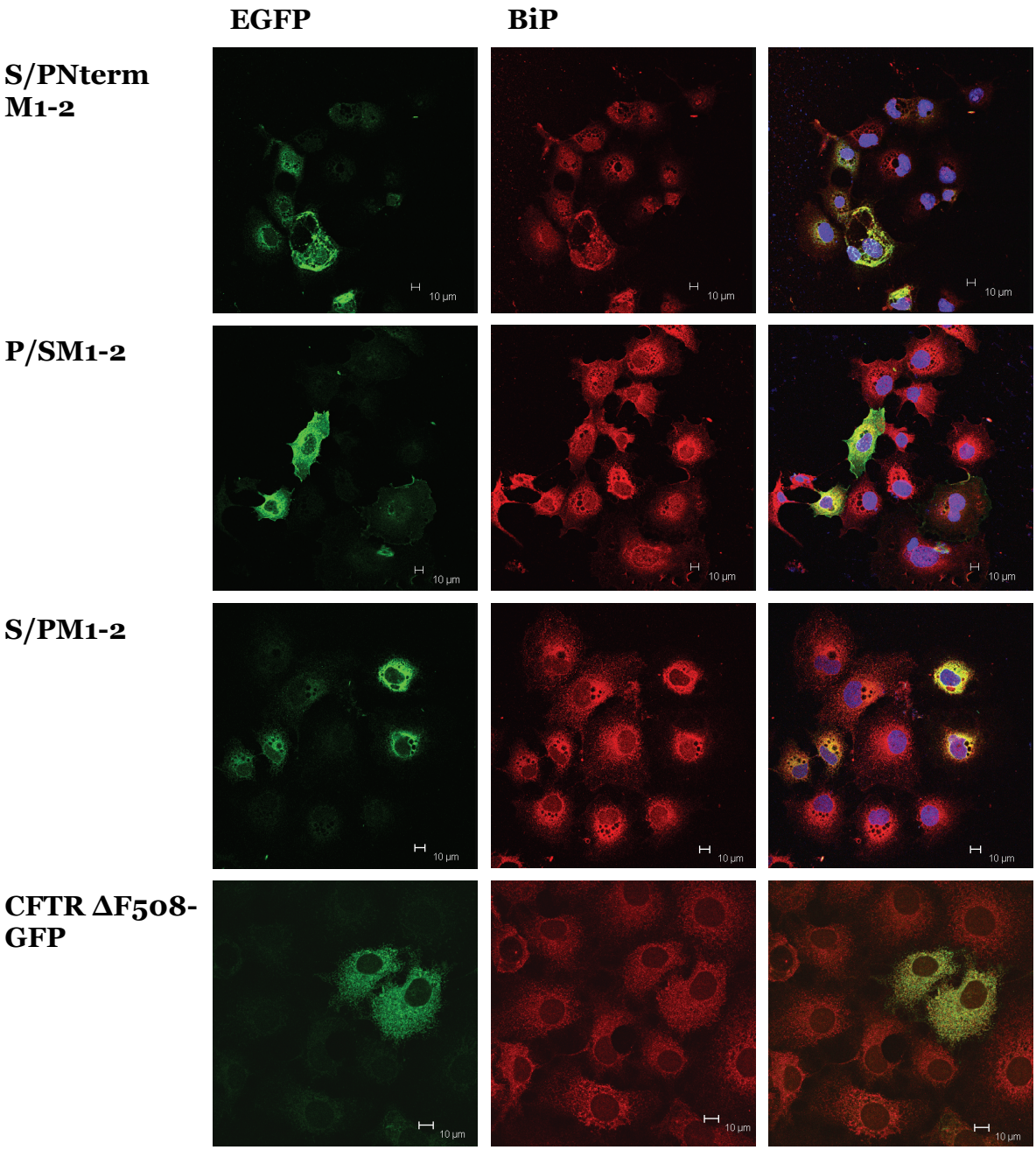



**Figure 4.3 BiP detection in COS-7 cells expressing constructs by immunofluorescence**
COS-7 cells were transfected with constructs as indicated for 48 hours, or left untransfected. EGFP fluorescence is shown in the left hand columns. BiP was visualised by immunofluorescence using a Texas Red conjugated secondary antibody (middle columns). DAPI was used to stain the nuclei. Overlay images are shown in the third columns. Images were acquired by confocal microscopy. Scale bars are 10 µm.

## 4.4 Discussion

The experiments described here were carried out in an attempt to detect activation of the UPR by expression of misfolded proteins in mammalian cells. As explained in section 4.1, it is possible that at least some of the ER localised chimeric calcium pumps constructed in this study are maintained in the ER as a result of protein misfolding rather than specific signal mediated retrieval. More information could be gained from the localisations of the set of chimeras if it were known which proteins were located in the ER as a result of misfolding. Plasma membrane localised proteins (PMCA and P/SM1-2) were used in these experiments as controls for correctly folded proteins, and tunicamycin and the misfolded CFTR ΔF508 were employed as known inducers of the UPR[158,159]. In addition, some cells were transfected with empty pcDNA3.1 (+) vector only, to ensure that the transfection procedure did not elicit the UPR.

Western blotting was used to detect increases in BiP expression in HeLa cells transfected with chimeric constructs. Figure 4.1 shows very low expression of all EGFP tagged constructs in HeLa cells (detected with a GFP antibody) except SERCA-EGFP. BiP and actin levels were measured by densitometry and the relative amount of BiP (adjusted according to actin) was calculated (as shown in table 4.1). The results show that only cells treated with tunicamycin showed a marked increase in the amount of BiP expressed over untreated and untransfected cells. This positive control demonstrates that BiP levels are indeed increased in the UPR when elicited by tunicamycin, but similar effects were not seen with any of the transfected constructs.

The same experiment was then carried out COS-7 cells in order to increase the expression levels of recombinant proteins. The pcDNA3.1 expression vector used throughout this study (see chapter 2) contains the SV40 origin of replication, allowing episomal replication of the vector in cell lines containing the large T-antigen. The COS-7 cell line contains the large T-antigen and so is able to replicate the vector, allowing for higher gene expression in comparison to other cell lines such as HeLa which lack the large T-antigen[161] (see Invitrogen pcDNA3.1 manual). Equal amounts of total protein from COS-7 cells transfected with EGFP

tagged constructs, tunicamycin treated, or untransfected were analysed by western blotting with a BiP antibody. These blots are shown in figure 4.2 and demonstrate that, as in HeLa cells, the only condition which causes a detectable increase in BiP expression is treatment with tunicamycin.

The western blotting data presented in this chapter indicate that none of the constructs expressed in HeLa or COS-7 cells cause an increase in BiP levels. This could simply reflect the proper folding of all constructs, or it could be that some of the constructs cause an upregulation of BiP, but due to the proportion of cells which are transfected (around 10%) this increase is not detectable by western blot. In the case of tunicamycin treated cells, a clear response is seen. All cells in the sample will come into contact with tunicamycin and so all cells should show an increase in BiP levels. In the case of the transfected cell samples, it is feasible that any increase in BiP expression is diluted by the untransfected majority. This problem highlights the requirement for near 100% transfection in experiments where protein expression is quantified in this way. This can be achieved using stable transfectants selected by antibiotic treatment (see Invitrogen pcDNA3.1 manual).

Immunofluorescence was used as an alternative method to observe BiP expression. Untransfected cells in transfected conditions served as internal controls to which cells expressing EGFP tagged constructs could be compared. Cells were treated with BiP antibodies, visualised with a Texas Red conjugated secondary antibody and analysed by confocal microscopy. Figure 4.3 shows no observable difference in BiP abundance in cells expressing any of the constructs, as compared to untransfected cells. EGFP tagged CFTR ΔF508 was used as a control, as it has previously been shown to cause the UPR and upregulate BiP expression[158]. However, no difference was seen in BiP between cells expressing the mutant chloride channel and untransfected neighbouring cells. This result suggests that immunofluorescence is not a suitable technique to measure increases in protein expression in this system, as it is not possible to detect upregulation of BiP in cells transfected with CFTR ΔF508.

The inconclusive nature of the data presented in this chapter suggests that these methods may not be subtle enough or appropriate for detecting misfolded proteins in this system. ER localisation of at least some of the chimeric calcium pumps may be a result of misfolding, but it has not been possible to detect misfolding with the experiments described here. The mechanisms of how non-glycosylated proteins (such as the calcium pumps described here) are maintained in the ER due to misfolding is not well understood. Interactions with BiP and calnexin are thought to play a role, with the possibility that the KDEL motif of BiP is partially responsible for ER retention of the misfolded protein[162]. It may be possible to determine which chimeric calcium pumps are misfolded by measuring interactions with BiP or calnexin, but without a clearer understanding of these mechanisms of ER retention, finding a definitive test for misfolding in these proteins is not straightforward. The next chapter will describe the building of constructs likely to have reduced propensities to misfold, in order to circumvent this problem of ER retention as a result of protein misfolding.

# 5. Searching for an ER retrieval signal in the tenth transmembrane domain of SERCA

## 5.1 Introduction

The localisations of the chimeric constructs described in chapter 3 demonstrate that the sequence in SERCA required for ER localisation is contained within residues 712-1001. Constructs built to dissect this section of the pump yielded only ER localised proteins, and experiments to test for misfolding in the chimeras (see chapter 4) were inconclusive. This chapter describes the attempts made to overcome the problem of misfolding by building simpler constructs and constructs in which only small changes have been made to the SERCA sequence. In addition, bioinformatics has been used to compare SERCA sequences with those of PMCA and the Golgi calcium pump SPCA1 and to locate the positions of specific residues within the tenth transmembrane domain (M10) of SERCA.

Structural studies of SERCA in different conformations have yielded much information on the movement of the pump during its catalytic cycle[18]. When searching for localisation signals in SERCA, it is important to consider the pump as a dynamic, three-dimensional protein within the lipid bilayer, rather than simply a static primary protein sequence. It is unknown what protein machinery is responsible for maintaining SERCA in the ER. However, if the mechanism is analogous to KDEL or di-lysine mediated retrieval, then it is likely to involve a membrane spanning receptor[91,99]. Assuming this is the case for SERCA, a signal located close to the membrane spanning domains of the protein would seem most likely, and a signal located in the dynamic cytoplasmic A, P or N domains[18] would be unexpected. As shown in chapter 1 (figure 1.3), structural information suggests that M9 and M10 of SERCA move very little during the transport of calcium, in comparison with the rest of the protein[18]. It is easier to envisage the recognition of SERCA by a membrane spanning receptor if the retrieval signal is located in a static, rather than mobile, portion of the protein. Any large conformational changes in the vicinity of the retrieval signal would alter the location of the signal relative to the membrane and the membrane spanning receptor, suggesting that static sections of the protein are most likely to contain retrieval information.

It is possible that the retrieval signal in SERCA is located within the membrane spanning regions of the pump. If SERCA is recognised by Rer1p or a similar receptor, then polar or charged residues in one of the transmembrane helices could be responsible for ER localisation[130]. Unfortunately, it is not possible to simply detect a retrieval signal by searching for polar or charged residues within membrane spanning helices, as polytopic, ion transporting membrane proteins like SERCA have a high prevalence of charged residues in their membrane spanning regions. By looking at the structures and conformational changes of SERCA, it is possible to predict which, if any, charged or polar residues would be accessible from the bilayer. This will be discussed later in the chapter by looking at the structure of the pump, but it seems likely that a lysine at position 972 points out into the membrane, making M10 of SERCA a candidate for recognition by Rer1p or a similar receptor. Interestingly, this lysine is conserved amongst SERCAs but not in PMCAs. PMCA3 contains a phenylalanine at the corresponding position (see figure 5.1). There are charged and polar residues in M1 and M2, but as shown in chapter 3, these transmembrane domains are not required for ER localisation of the protein. Charged residues are also present in the other transmembrane domains, but many of these are involved in calcium binding and are not accessible from the bilayer[12,18].

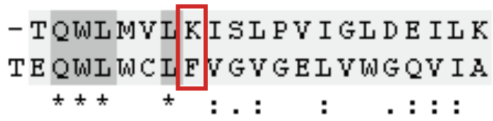



**Figure 5.1 Alignment of M10 sequences from SERCA and PMCA**

Human SERCA1 (residues 965-985; top) and PMCA3 (residues 1035-1056; bottom) sequences were aligned using ClustalW (at Uniprot). Lysine 972 in SERCA and the corresponding phenylalanine in PMCA are marked with a red box. The sequences of rabbit SERCA1 and rat PMCA3 M10s (used in this study) are identical to these human sequences.

Mutagenesis studies will be used here to determine the importance of K972 for SERCA localisation. The length of M10 will also be increased by adding leucine residues in an attempt to disrupt the position of any signal within this helix in the membrane. This technique has been used previously to cause mis-targeting of cytochrome $b_5$[74] and the SERCA modulators

phospholamban and sarcolipin[28] to the plasma membrane. Plasma membrane localisation caused by elongating transmembrane segments suggests that the signal causing the protein to remain in the ER is within the membrane. Using this approach, it may be possible to determine whether the ER retrieval signal of SERCA is within M10. Another technique employed here to compare SERCA and PMCA M10 sequences is the use of a CD8 reporter construct[163]. This plasma membrane localised construct has a single transmembrane domain and has been fused to an EGFP tag in this study. By replacing the CD8 transmembrane domain with SERCA or PMCA M10 sequence and observing the localisation of the protein, it may be possible to determine the ability of these sequences to cause ER retrieval.

M10 of SERCA has been selected for further investigation in the search for the retrieval signal of the pump. It is less mobile than the rest of the protein during the catalytic cycle, increasing the chance of recognition by a membrane spanning receptor[18]. It contains a conserved charged residue (lysine 972) which could be recognised by Rer1p or a similar receptor[130]. Due to its location at the end of the protein sequence, it is accessible from the bilayer, is not totally surrounded by other helices, and it is not involved in binding of calcium as M4, M5, M6 and M8 are[18]. In addition, the data presented from the chimeras constructed in this study (see chapter 3) show that the C-terminal portion of SERCA is required for ER localisation.

## 5.2 Methods

General methods (molecular biology, cell culture and microscopy) are detailed in chapter 2.

To produce the SERCA K972F and SERCA M10 3Leu constructs, site-directed mutagenesis was carried out according to the QuikChange protocol (Stratagene).

The following reagents were used:

| | |
|---|---|
| Sterile distilled water | 38.0 µl |
| *Pfu* DNA polymerase buffer | 5.0 µl |
| dNTPs (25 mM each dNTP) | 1.0 µl |
| DNA template (100 ng/µl) | 1.0 µl |
| Primer 1 (100 ng/µl) | 2.5 µl |
| Primer 2 (100 ng/µl) | 2.5 µl |
| *Pfu* DNA polymerase (2.5 U/µl) | 1.0 µl |

The reactions were carried out in a thermocycler as shown in table 5.1, with step 2 repeated 20 times. The template used in all mutagenesis in this chapter was the SERCA-EGFP construct.

| **Step** | **Process** | **Temperature (°C)** | **Duration** |
|---|---|---|---|
| 1 | Denaturation | 95 | 30 seconds |
| 2 | Denaturation | 95 | 30 seconds |
| | Annealing | Primer $T_m$ -5 | 1 minute |
| | Elongation | 72 | 19 minutes |

**Table 5.1 QuikChange mutagenesis cycle**

The cycle used for the QuikChange mutagenesis is shown here. Annealing temperatures were adjusted according to the primers used.

The products from the QuikChange reactions were analysed by agarose gel electrophoresis. Parental DNA was removed from successful products by adding 1 µl DpnI (10 U/µl; Promega) to entire reaction volumes and incubating for one hour at 37 °C. This step allows removal of any non-mutated DNA, as DpnI selectively cleaves only methylated DNA. The newly made

mutated DNA will be unmethylated and so remain uncleaved. DpnI digested products were then used to transform DH5α *E. coli*. by electroporation (as described in chapter 2), and positive transformants were selected and DNA extracted for sequencing.

The primers used in the construction of K972F (in which lysine 972 in SERCA is replaced with phenylalanine) and SERCA M10 3Leu (in which 3 leucine residues are added into the middle of SERCA M10) are shown in table 5.2. Primers were designed using the Oligo software (Molecular Biology Insights) to incorporate mutations into the SERCA sequence. The three extra leucines in SERCA M10 3Leu were positioned between L975 and P976 in M10.

| **Primer** | **Sequence** |
|---|---|
| K972F_fwd | 5′-TGGCTGATGGTTCTGTTTATCTCTCTGCCAGTTATCGGTC-3′ |
| K972F_rev | 5′-AACTGGCAGAGAGATAAACAGAACCATCAGCCATTGAGTC-3′ |
| M10 3Leu_fwd | 5′-CTCTCTGCTGCTGCTGCCTGTTATCGGTCTGGACGAAATC-3′ |
| M10 3Leu_rev | 5′-TAACAGGCAGCAGCAGCAGAGAGATCTTCAGAACCATCAG-3′ |

**Table 5.2 Primers used in the production of K972F and M10 3Leu SERCA mutants**
The forward (fwd) and reverse (rev) primers used to make SERCA K972F and SERCA M10 3Leu are shown, with 5' and 3' ends marked. The mutated sequences are shown in red. In the case of K972F, the mutation was a substitution, and for M10 3Leu, an insertion.

The CD8 reporter constructs were used to test the ability of the M10 sequences of SERCA and PMCA to cause ER retrieval. The original CD8 construct was a gift from Dr. M.N.J. Seaman[163]. The CD8 gene was amplified from this construct using PCR and inserted into pcDNA3.1 (+) between NheI and HindIII. This allowed insertion of the CD8 gene upstream of a myc epitope linker sequence (EQKLISEEDLPVAT) and the EGFP tag, resulting in the C-terminally tagged CD8-EGFP protein as shown in figure 5.2. This was constructed to ensure that the EGFP tag would not disrupt the trafficking of CD8 to the plasma membrane.

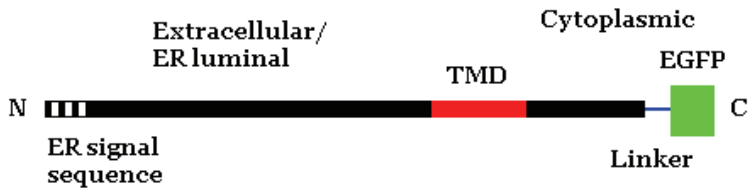



**Figure 5.2 Structure of CD8 reporter constructs**

The general structure of the CD8 constructs is shown here. All constructs contain a cleaved N-terminal ER signal sequence (black and white striped), transmembrane domain (TMD; in red) which was replaced with SERCA and PMCA M10 sequence to produce CD8 SERCA M10 and CD8 PMCA M10 respectively, and a C-terminal EGFP tag (green). The N-terminal section is extracellular (or ER luminal in ER localised constructs), and the C-terminal tail is cytoplasmic.

In addition to CD8-EGFP, CD8 SERCA M10 and CD8 PMCA M10 were also built, in which the transmembrane domain of CD8 was replaced by SERCA or PMCA M10 sequence. These constructs were built using multi-step PCR as described for the SERCA/PMCA chimeras in chapter 3. Briefly, DNA encoding the extracellular and cytoplasmic domains of CD8 was amplified by PCR, using primers with sequence overlapping the beginning and end of the M10 of SERCA or PMCA. The desired M10 (SERCA or PMCA) was amplified with flanking sequence from the CD8 extracellular and cytoplasmic domains at the 5' and 3' ends respectively. These reactions produced three fragments which were 'stitched' together in a fourth reaction to produce the full length insert. This was ligated into the vector between NheI and HindIII, upstream of the EGFP gene, to produce constructs as shown in figure 5.2, with SERCA or PMCA transmembrane domains in the place of the CD8 transmembrane domain. The primers used in the production of these constructs are listed in appendix 2.

## 5.3 Results

Bioinformatics approaches were used to compare SERCA M10 to the M10 sequences of PMCA and the Golgi pump SPCA, and to analyse the structure of M10.


**Figure 5.3 M10 sequences of ER, Golgi and plasma membrane calcium pumps**
The sequences of human SERCA1 (AT2A1), SPCA1 (AT2C1) and PMCA3 (AT2B3) were aligned using ClustalW (Uniprot). The lysine at position 972 in SERCA, and the corresponding residues at the same position in SPCA and PMCA are shown with a red box. The aspartate at position 981 in SERCA is also shown with a green box.

Figure 5.3 shows an alignment of M10 sequences from SERCA, SPCA and PMCA. Neither the Golgi or plasma membrane pumps contain a lysine or any positively charged amino acid at the same position. There is an aspartate at position 981 in SERCA which is not present in SPCA or PMCA. Uncharged amino acids are located in the corresponding positions in these pumps.

The structure of the pump was then studied to determine if either of these charged residues would be accessible from the bilayer. Figure 5.4 shows where on the tenth transmembrane domain these charged residues lie, and that only K972 appears to be exposed to the lipid bilayer. Helical wheel projections (shown in figure 5.5) were used to show the location of K972 and D981. Like the crystal structure, the projection shows that these residues are on opposite sides of the M10 helix. SERCA M10 was compared to the transmembrane domain of nicastrin which has been shown to interact with Rer1p. The transmembrane domain of nicastrin has a polar face which has been proposed to be involved in its interaction with Rer1p[128].

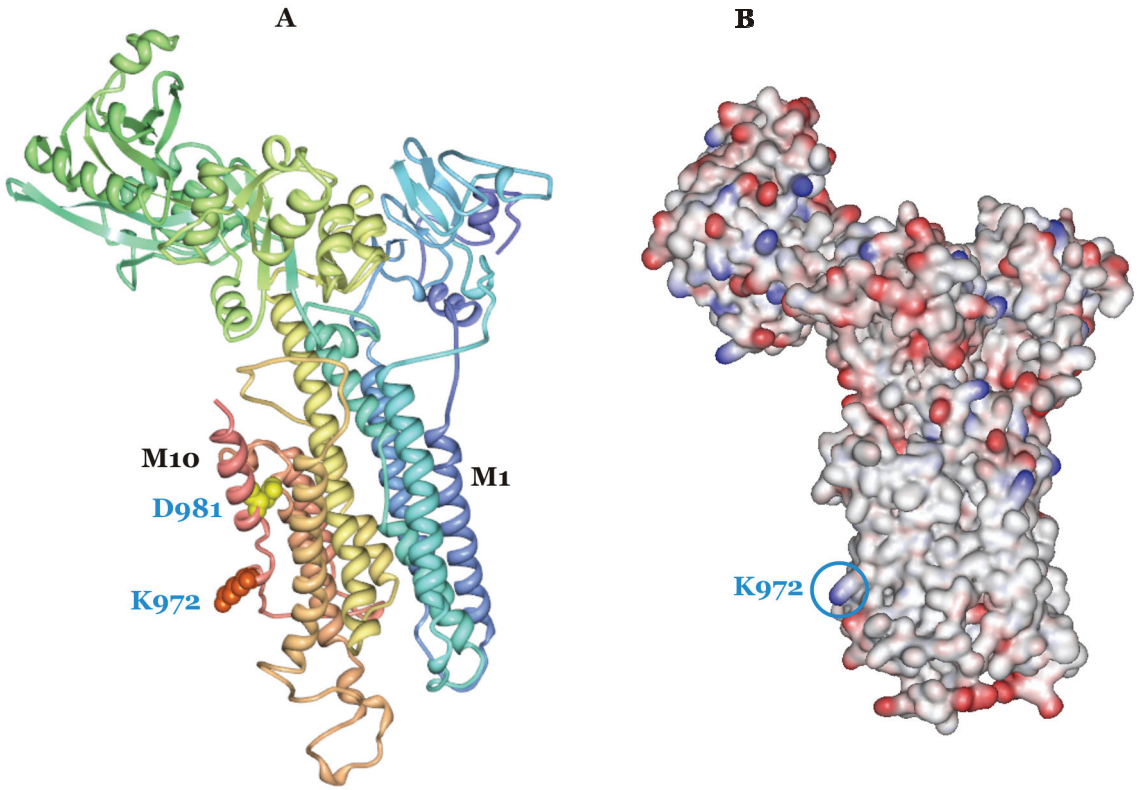



**Figure 5.4 Location of charged residues in M10 of SERCA**

Structures of SERCA in the E1 calcium bound conformation (PDB code 1SU4) to show charged residues in M10. (A) shows the structure of SERCA from blue at the N-terminus to red at the C-terminus. K972 (orange) and D981 (yellow) are shown in space fill, and the first (M1) and last (M10) transmembrane helices are labelled. (B) shows the surface of SERCA in the same orientation as (A) with red and blue indicating negatively and positively charged residues respectively. K972 is circled in blue. (A) and (B) were created with PDB Protein Workshop and WebLab ViewerPro respectively.

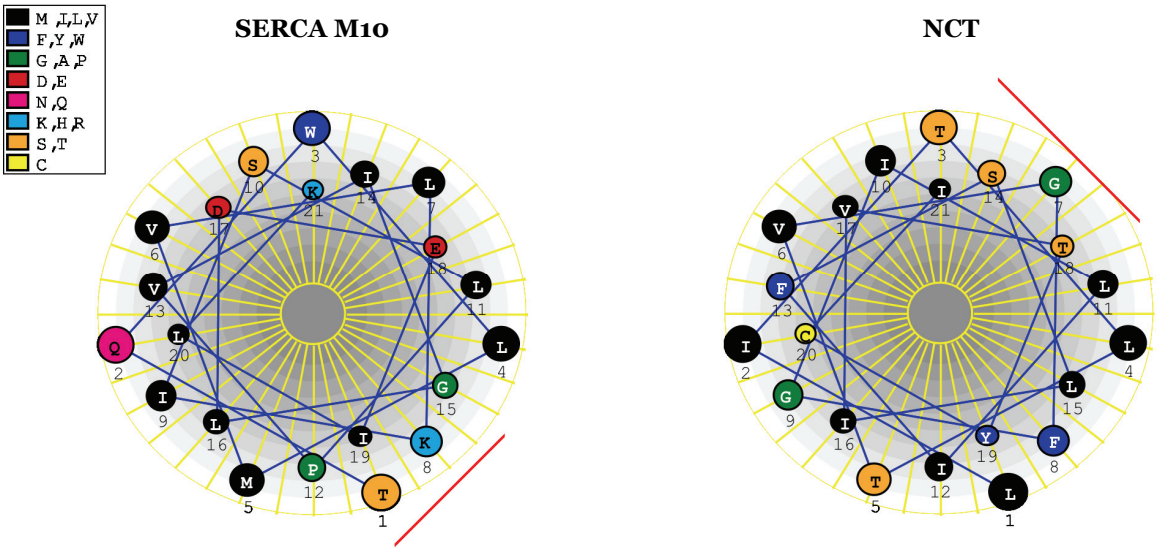



**Figure 5.5 Helical wheel projections of SERCA M10 and NCT transmembrane domain**
Helical wheel projections of SERCA M10 and the transmembrane domain of nicastrin (NCT) showing the positions of polar and charged residues within the α-helices. Red bars indicate possible interaction sites of Rer1p with these transmembrane domains. In SERCA M10, this corresponds to the region close to K972 (residue 8 in the helix), as all other polar or charged residues point into the structure rather than out into the bilayer. D981 (residue 17) is located on the opposite face of the helix. Helical wheel projections were created with Protean (DNASTAR).

To determine the role of K972 in the tenth transmembrane domain of SERCA in ER retrieval, the residue was mutated to phenylalanine (as in PMCA) to create SERCA K972F. In addition, the SERCA M10 3Leu construct was created in which three extra leucine residues were added to SERCA M10. This elongation of the transmembrane domain by adding three hydrophobic residues will most likely cause a change in the position of M10 in the bilayer and may disrupt interaction with a retrieval receptor such as Rer1p. These constructs (both carrying C-terminal EGFP tags) were expressed in COS-7 cells and fluorescence microscopy was used to determine their localisations. Both of these constructs were located in the ER (as shown in figure 5.6), showing a reticular pattern indistinguishable to that produced by SERCA-EGFP.

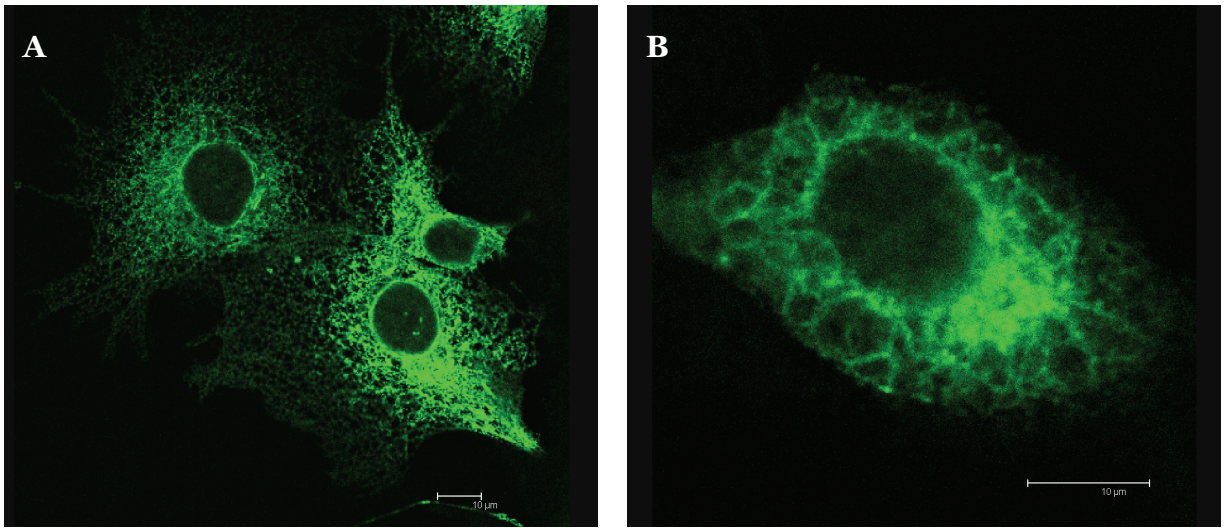



**Figure 5.6 COS-7 cells expressing SERCA K972F or SERCA M10 3Leu**
COS-7 cells were transfected with DNA encoding either SERCA K972F (A) or SERCA M10 3Leu (B). Cells were analysed by confocal microscopy 2 days following transfection. Scale bars are 10 µm.

To determine the ability of M10 sequences from SERCA and PMCA to cause ER retrieval, the CD8 constructs were built as discussed above. Figure 5.7 shows the distribution of EGFP tagged CD8 in COS-7 cells. It colocalises with the trans-Golgi marker and appears to be present at the plasma membrane. Cells expressing CD8 constructs in which the transmembrane domain has been replaced by M10 of SERCA or PMCA are shown in figure 5.8. Both CD8 SERCA M10 and CD8 PMCA M10 show reticular localisation and are not present in the trans-Golgi.

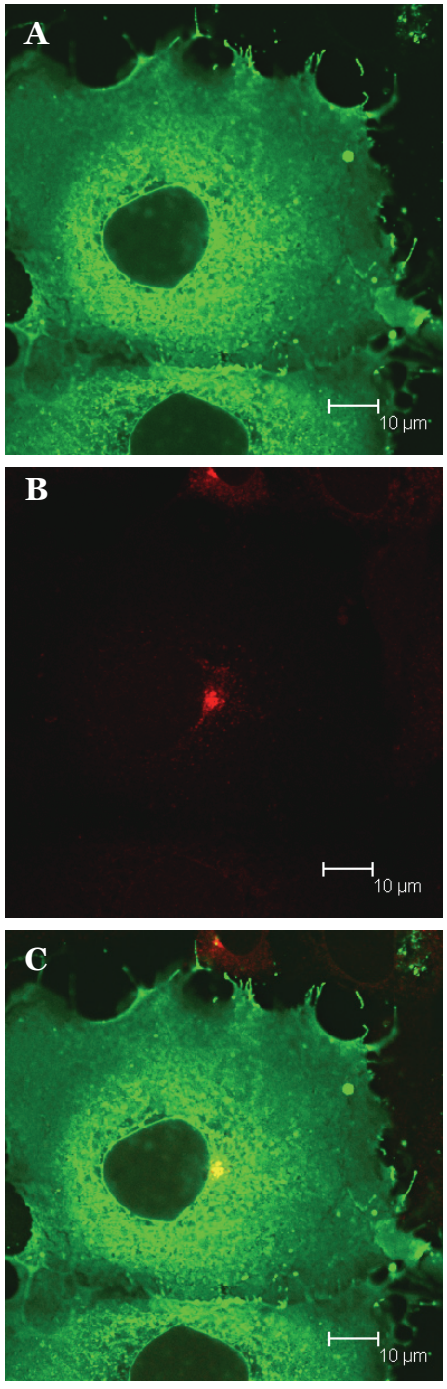



**Figure 5.7 COS-7 cells expressing CD8-EGFP**

COS-7 cells were transfected with the CD8-EGFP construct (A). After 2 days, cells were treated with BFA and anti-TGN46, visualised with a Texas Red conjugated secondary antibody (B). An overlay is shown (C). Images were obtained by confocal microscopy. Scale bars are 10 µm.

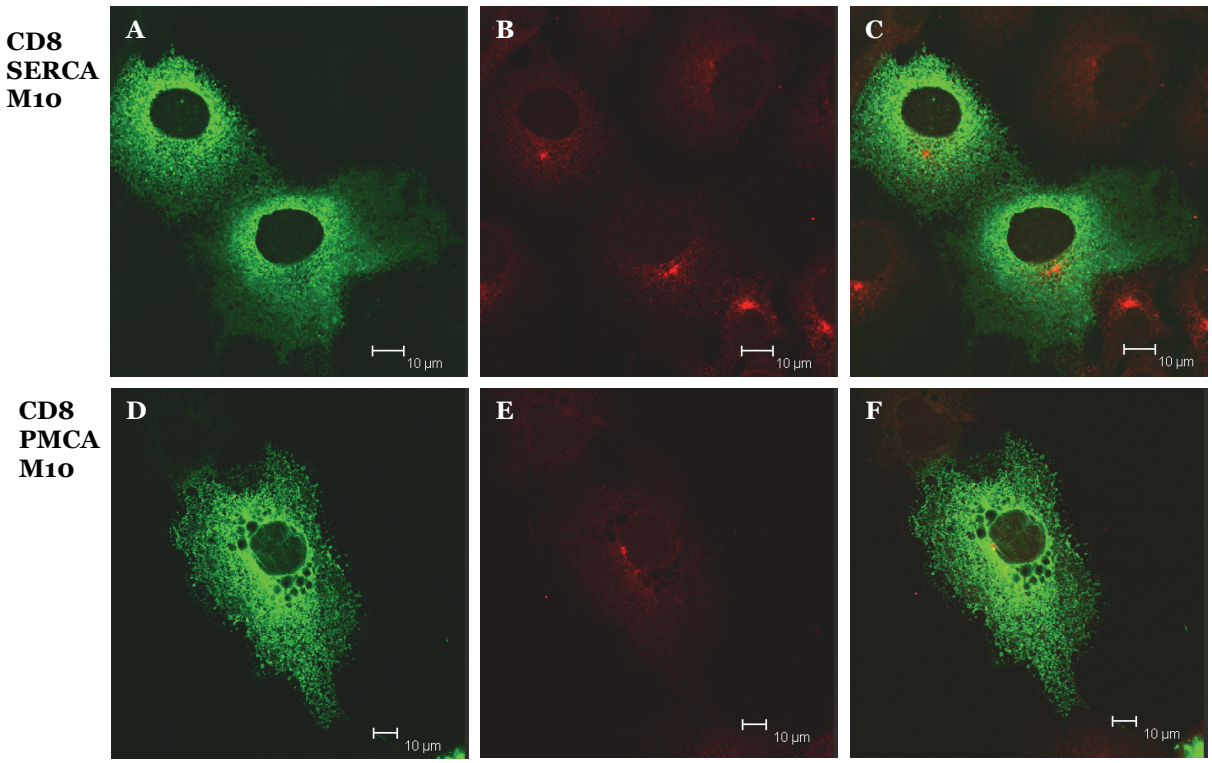



**Figure 5.8 COS-7 cells expressing CD8 SERCA M10 or CD8 PMCA M10**

COS-7 cells are shown 2 days following transfection with CD8 SERCA M10 (A-C) or CD8 PMCA M10 (D-F). Cells were treated with BFA and TGN46 antibodies to locate the trans-Golgi, revealed using a Texas Red conjugated secondary antibody (B and E). Overlay images are shown (C and F). Images were acquired using confocal microscopy. Scale bars are 10 µm.

## 5.4 Discussion

The constructs described in this chapter were built in an attempt to circumvent the problem of ER localisation as a result of misfolding. The chimeric constructs shown in chapters 3 and 4 were built from two proteins which, although similar, are both complex and large. The interactions between different domains of SERCA and PMCA that were joined together are undoubtedly numerous and are also largely unpredictable. For this reason, smaller scale mutations were used in the experiments described here, and single transmembrane domains were isolated for insertion into the CD8 reporter construct.

Using the crystal structures of SERCA and sequence analysis tools, it is possible to make predictions about where individual residues are positioned within the protein structure. This is of use when considering possible interaction sites for retrieval receptors such as Rer1p. Rer1p has been shown to interact with polar residues in transmembrane domains and is a possible candidate for the retrieval of SERCA to the ER[128,130]. Figure 5.3 shows two charged residues (K972 and D981) in SERCA M10 that are not present in the M10 sequences of the plasma membrane (PMCA) or Golgi (SPCA) calcium pumps. The absence of these residues in the other pumps may indicate that they are involved in ER localisation. To investigate this further, the positioning of these residues was determined in the 3-dimensional structure of SERCA. Figure 5.4 shows the position of these charged residues in M10. K972 appears to be accessible from the bilayer, whereas D981 faces into the protein and cannot be seen on the outside surface. This suggests that while K972 may be able to be recognised by a membrane spanning receptor such as Rer1p, D981 most probably could not as it is hidden from the bilayer. Helical wheel projections have also been used to determine how the primary protein sequence is distributed in the membrane spanning α-helix of M10. The helical wheel projection of SERCA M10 (figure 5.5) shows that K972 is on the opposite face of the helix to D981. This is consistent with the positioning of these residues in the crystal structure.

M10 of SERCA has been analysed here for its ability to cause ER retrieval. Reasons for selecting this region of SERCA for further investigation are explained in the introduction to this chapter (section 5.1). Structural analysis of SERCA shows that K972 faces into the bilayer and could feasibly be recognised by a membrane spanning retrieval receptor, such as Rer1p.

For this reason, site-directed mutagenesis was used to substitute this lysine for a phenylalanine residue. Phenylalanine was selected as a replacement on account of its presence at the corresponding position in the M10 of PMCA, which is not capable of ER retrieval. Figure 5.6 shows that the SERCA K972F mutant is ER localised and does not escape to the plasma membrane. This could be due to the fact that K972 is not involved in retrieval and so mutation does not lead to mis-targeting. Another possibility is that K972 is involved, but that redundancy is built into the system so loss of this residue alone does not cause mis-targeting. The ER localisation of the K972F mutant could also reflect misfolding of the protein as a result of this amino acid substitution. Without presence of this protein at the plasma membrane, it is not possible to unequivocally determine the involvement of K972 in the ER localisation of SERCA.

Another approach used to assess the contribution made by M10 in the retrieval of SERCA to the ER was extension of the transmembrane helix. By elongating the helix, its position within the bilayer is likely to change, and a retrieval motif within the transmembrane domain may be distorted, resulting in mis-targeting of the protein. As shown in figure 5.6, this is not the case. Adding three extra hydrophobic residues to M10 of SERCA does not result in a loss of ER localisation. Again, there is more than one explanation for this result. M10 may not contain a retrieval signal, so disruption of this helix has no effect on the localisation of the pump. Adding extra residues to the transmembrane domain may also disrupt intramolecular interactions with other sections of the pump, and the protein may be retained in the ER by quality control mechanisms. It is also possible that three leucine residues are not sufficient to distort M10 enough to cause mis-targeting. This could be tested by introduction of more hydrophobic residues to the helix to elongate it further. Other studies using this technique have inserted 4 (phospholamban), 7 (sarcolipin) or 5 (cytochrome $b_5$) hydrophobic residues to create transmembrane domains of lengths 27, 26, and 22 amino acids respectively[28,74]. The addition of three leucine residues to SERCA M10 results in a transmembrane domain of 24 residues, which may not be a significant enough increase in length to cause mis-targeting to the plasma membrane. In addition, phospholamban, sarcolipin and cytochrome $b_5$ are all single-pass membrane proteins[28,74]. The presence of elongated mutants of these proteins at the plasma membrane shows that they are not recognised as misfolded. However, disruption of

one of the 10 transmembrane domains in SERCA is presumably more likely to result in protein misfolding due to the much greater complexity of the membrane spanning region of the calcium pump, compared to single-spanning membrane proteins.

The CD8 reporter construct was used as a tool to determine the role played by M10 of SERCA in ER localisation. The first experiment conducted was to ensure that the EGFP tag fused to the C-terminus of CD8 did not disrupt its trafficking to the plasma membrane. The tag was added to the C-terminus of CD8, as the N-terminus of the protein contains a cleaved ER signal sequence[163]. It is possible that the effectiveness of this signal sequence could be compromised if the EGFP tag was present at the N-terminus of the protein. Figure 5.7 shows that EGFP tagged CD8 is present in the trans-Golgi and plasma membrane. This suggests that the EGFP tag does not have an effect on the trafficking of the protein through the secretory pathway.

Sequence encoding SERCA M10 was then used to replace the transmembrane domain of CD8. The resulting construct, CD8 SERCA M10 was expressed in COS-7 cells and its localisation analysed by confocal microscopy. Unlike the CD8-EGFP construct, CD8 SERCA M10 showed an ER localisation and was not present in the trans-Golgi (figure 5.8) suggesting that it cannot travel through the late secretory pathway. The reason for this ER localisation could be that M10 is causing specific retrieval of the reporter construct, or it could be that the protein is misfolding or aggregating. M10 of SERCA would not be found alone in the bilayer under normal circumstances, and it is possible that the charged residues that would normally be hidden are revealed, thus causing recognition by quality control machinery. To investigate this, M10 of PMCA (which is unable to cause ER retrieval of the intact plasma membrane calcium pump) was inserted into the CD8 reporter construct in the same position as SERCA M10. This construct was also located in the ER (figure 5.8) and did not show colocalisation with the trans-Golgi marker. It can be assumed that the localisation of CD8 PMCA M10 in the ER must be due to misfolding or aggregation, as this helix cannot cause retrieval of PMCA to the ER. Importantly, localisation of CD8 PMCA M10 in the ER does not necessarily suggest that CD8 SERCA M10 is located in the ER as a result of misfolding, as the two transmembrane domains have different sequences. It is possible that the M10 of SERCA

causes specific sequence mediated retrieval of the CD8 reporter, but without testing other transmembrane domains of SERCA it is not clear if this is the case.

Despite attempting to circumvent the problem of ER localisation in chimeric proteins as a result of possible misfolding, the smaller mutations and simpler constructs described in this chapter were unable to provide an answer as to whether M10 of SERCA is involved in ER retrieval. The experiments shown here demonstrate that mutating and isolating regions of a complex polytopic membrane protein such as SERCA with the aim of finding sequences mediating ER localisation is not straightforward. Therefore, to pursue the question of how SERCA is retrieved to the ER, further experiments were subsequently carried out to focus on the machinery, rather than the sequences, responsible for this process.

# 6. Characterisation of antibodies raised against human Rer1p

## 6.1 Introduction

Rer1p is a possible candidate for the retrieval of SERCA to the ER from the ERGIC or early Golgi. It has been shown to interact with several membrane proteins in both yeast and mammalian systems, and interaction with transmembrane regions of these proteins seems key to the retrieval process[119,123-126,128,129]. Details of what is known about the mechanisms and targets of Rer1p is detailed in chapter 1, section 1.6. Aside from Rer1p and possibly BAP31 (also discussed in section 1.6), no other known proteins seem likely candidates for the retrieval of SERCA to the ER. For this reason, polyclonal antibodies have been raised and affinity purified (by Eurogentec, Belgium) against two epitopes in human Rer1p with a view to investigating possible interactions between Rer1p and SERCA.

An antibody against human Rer1p would allow several different techniques to be used in an attempt to determine whether the protein is involved in the maintenance of SERCA in the ER. Techniques such as RNA interference, cross-linking and immunoprecipitation all require an antibody which can detect the protein of interest in western blots. In addition, function of the antibody in immunofluorescence experiments is of use to ascertain subcellular localisation of Rer1p and detect any colocalisation with SERCA. Before the newly raised antibody could be used in experiments to detect protein interactions, it first had to be characterised. This was done by testing the ability of the antibody to detect endogenous and tagged Rer1p in western blots, and using immunofluorescence to determine the subcellular localisation of the protein recognised by the antibody.

Rer1p is predicted to have a W-shaped topology with four transmembrane domains (M1-4), cytosolic N- and C-termini, very small luminal loops and a large cytosolic loop between M2 and M3[130] as shown in figure 6.1. Two peptides were selected from the sequence of human Rer1p to be synthesised and used as antigens in the production of the anti-Rer1p antibodies. The residues chosen for the first peptide were within the large cytosolic loop, and the second peptide was made from amino acids at the very C-terminus of the protein. The amino acids

and positions are detailed in section 6.2 below. The positions of these peptides within the whole protein are shown in figure 6.1. These portions of the protein were selected for their hydrophilicity and predicted flexibility[164]. Short peptides (~15 residues) were used to avoid the formation of any secondary structures not formed in the full-length protein. These peptides were synthesised and fused to the carrier protein keyhole limpet hemocyanin (KLH), which helps the peptides elicit a strong immune response. Details of how peptides are selected for antibody production by Eurogentec can be found at http://www.eurogentec.com/eu-home.html. Database searches were also carried out to ensure that the selected sequences were not present in any other proteins.

The following chapter describes the characterisation of antibodies raised against two peptides from human Rer1p. At the beginning of this study, no commercial antibodies were available to Rer1p, so raising one was necessary in order to pursue this line of investigation. During the project, a commercial antibody became available (from Everest Biotech), raised to the same C-terminal epitope.

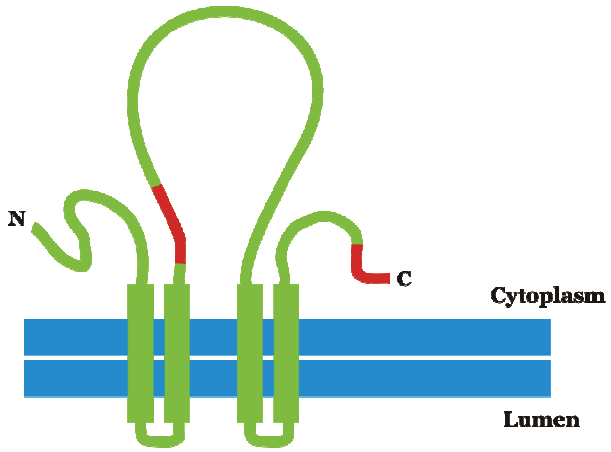



**Figure 6.1 Rer1p topology and epitopes selected for antibody production**
The predicted topology of Rer1p (green) is shown with the ER or Golgi membrane shown in blue. The N- and C-termini of the protein are labelled. The peptides selected as antigens in the production of anti-Rer1p antibodies are shown in red. Based on Sato, K., *et al.* (2003)[130].

## 6.2 Methods

All general methods are detailed in chapter 2 (materials and methods). The Rer1p-EGFP and His-Rer1p constructs were obtained from Genecopoeia (MD, USA). The Rer1p-YFP2 construct was built as part of a separate project. In this construct, Rer1p was tagged at the C-terminus with the C-terminal section (amino acids 159-239) of yellow fluorescent protein (YFP), connected by the flexible ten amino acid linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$.

The antibodies to human Rer1p were raised in two rabbits against the following peptides: PKVDPSLMEDSDDGP (loop; residues 85-99) and RRYRGKEDAGKAFAS (C-terminus; residues 182-196). KLH was used as a carrier protein and fused to the N-termini of the peptides onto a cysteine residue added to the beginning of each peptide. Serum was affinity purified against either the loop or C-terminal peptides. Preimmune serum was also supplied and was used in western blots to ensure that the animals used showed no existing immunity to Rer1p. Eurogentec (Belgium) carried out the peptide synthesis and antibody production.

Lysates of COS-7, HeLa and Caco-2 cells were used in western blots to detect endogenous Rer1p. In addition, various Rer1p constructs were expressed in COS-7 cells and cells harvested for western blotting. All cells were cultured and transfected as described in chapter 2. Cells grown on 10 cm culture dishes were washed twice with ice cold PBS followed by addition of 400 µl sample buffer (as in chapter 2, but without bromophenol blue) at 60 °C, supplemented with 40 µl mammalian protease inhibitor cocktail (Sigma Aldrich). Cells were scraped from the plate and sonicated for 1 minute. Aliquots were frozen in liquid nitrogen. Further sample buffer (see chapter 2) was added to lysates and samples were heated to 70 °C for 10 minutes before analysis by SDS-PAGE and western blotting.

Antibodies and dilutions used in western blots were as follows: rabbit anti-Rer1p (loop or C-terminal epitopes), 1:500 (Eurogentec); mouse anti-GFP, 1:500 (Roche); goat anti-Rer1p, 1:3000 (Everest Biotech); goat anti-rabbit IgG conjugated to HRP, 1:3000 (Abcam); sheep anti-mouse IgG conjugated to HRP, 1:2000 (GE Healthcare); donkey anti-sheep IgG

conjugated to HRP, 1:3000 (Abcam). GE Healthcare HRP conjugated secondary antibodies were affinity adsorbed (against rat, human and mouse).

In immunofluorescence experiments carried out to illuminate the ERGIC, a 'cold block' pre-treatment was used in which cells were incubated at 15 °C for one hour immediately before methanol fixation. This procedure enhances visualisation of the ERGIC, which usually shows widespread distribution throughout the cell, by concentrating the compartment close to the Golgi[165]. Cells treated with anti-TGN46 antibodies were pre-treated with BFA for one hour (as described in chapter 2).

Antibodies and dilutions used in immunofluorescence were as follows: rabbit anti-Rer1p (both loop and C-terminal epitopes), 1:50 (Eurogentec); sheep anti-TGN46, 1:50 (Serotec); mouse anti-ERGIC-53, 1:50 (Alexis); donkey anti-rabbit IgG conjugated to Texas Red, 1:100 (GE Healthcare); sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC), 1:100 (GE Healthcare); donkey anti-sheep IgG conjugated to Texas Red, 1:100 (Abcam); donkey anti-rabbit IgG conjugated to FITC, 1:100 (GE Healthcare).

In situations where double labelling was used, for example cells in which Rer1p and ERGIC-53 were labelled simultaneously, care was taken to avoid cross-reactivity of secondary antibodies with inappropriate primary antibodies. Figure 6.2 shows how this was carried out for the two different scenarios. In addition, control experiments were carried out to test all primary antibodies in combination with the secondary antibodies to ensure that no cross-reactivity was occurring. For example, anti-ERGIC-53 (raised in mouse) was tested against the secondary anti-rabbit IgG antibody (raised in donkey). No Texas Red fluorescence was seen, indicating that the anti-rabbit IgG secondary antibody does not react with the anti-ERGIC-53 antibody (raised in mouse). All antibodies that were used in double labelling were tested in this manner.

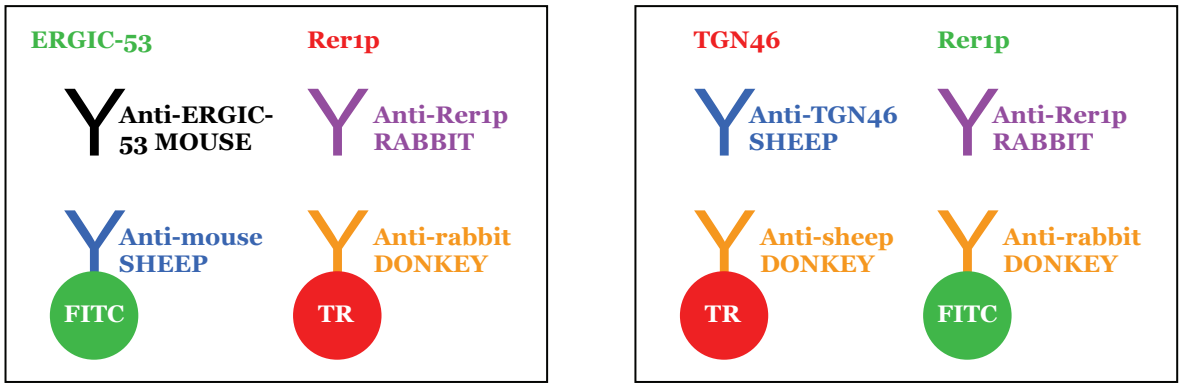



**Figure 6.2 Double labelling immunofluorescence**

The black rectangles indicate two different double labelling scenarios. Proteins which were labelled (ERGIC-53, Rer1p and TGN46) are at the top, coloured in green or red for FITC or Texas Red labelling respectively. Primary antibodies are on the top line and secondary antibodies below. All antibodies are shown by a 'Y' and are coloured according to the species in which they were raised (also shown in uppercase lettering beside each antibody). Conjugations of secondary antibodies are shown in green for FITC or red for Texas Red (TR).

## 6.3 Results

Characterisation of the antibodies raised against human Rer1p was initially carried out by western blotting. Lysates of different cell lines were tested with the antibody that had been affinity purified against the C-terminal epitope of Rer1p. As shown in figure 6.3, the antibody raised against the C-terminal epitope of Rer1p detected a protein at approximately 50 kDa in Caco-2, COS-7 and HeLa cell lysates. This is incongruous with the molecular weight of Rer1p which is predicted to be 23 kDa[122].

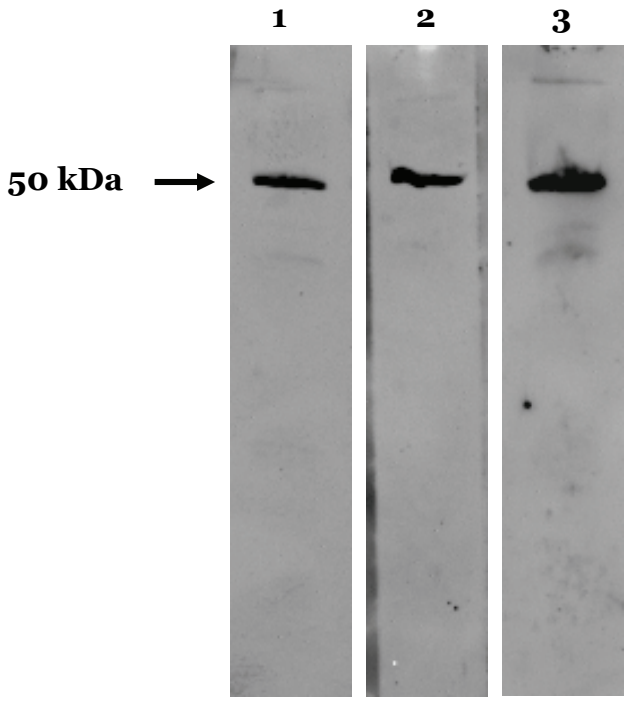



**Figure 6.3 Detection of a 50 kDa protein by anti-Rer1p antibody in cell homogenates**
Lysates of Caco-2 (1), COS-7 (2) and HeLa (3) cells were separated by SDS-PAGE and analysed by western blotting with affinity purified antibody raised against the C-terminus of human Rer1p. The three bands are from different gels and have been aligned according to the molecular weight markers run alongside the samples.

To further characterise the antibody, western blots were carried out to test the ability of the antibody to recognise tagged forms of Rer1p. COS-7 cells were transfected with His-Rer1p, Rer1p-EGFP or Rer1p-YFP2 and analysed by SDS-PAGE and western blotting (figure 6.4). The Rer1p antibody detected all three of these tagged proteins at the correct predicted molecular weights, approximately 24 kDa (His-Rer1p), 50 kDa (Rer1p-EGFP) and 33 kDa (Rer1p-YFP2). The same gel was then probed with anti-GFP antibodies. Both Rer1p-EGFP and Rer1p-YFP2 contain the epitopes for the GFP antibody used, whereas His-Rer1p does not. Anti-GFP antibodies detected Rer1p-EGFP and Rer1p-YFP2 at the same sizes as the Rer1p antibody.

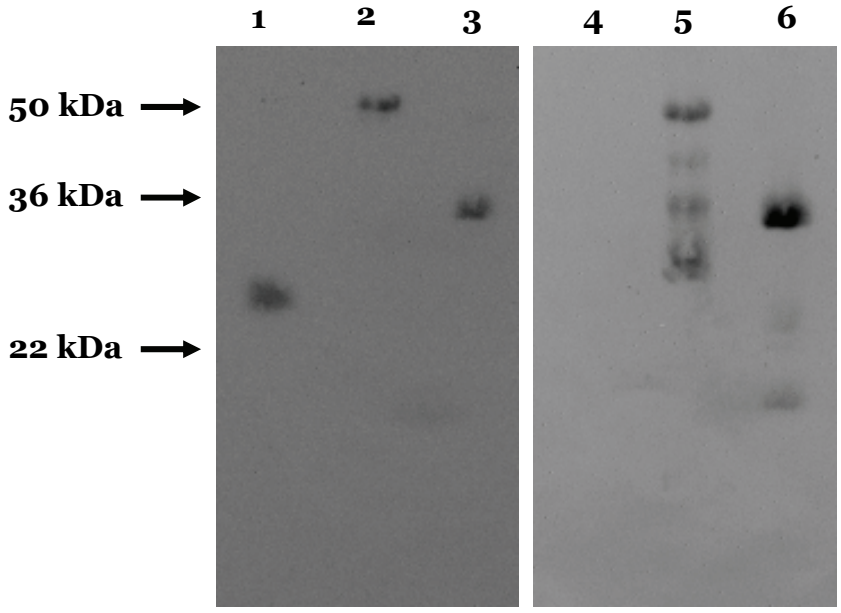



**Figure 6.4 Detection of His-Rer1p, Rer1p-EGFP and Rer1p-YFP2 by anti-Rer1p**
COS-7 cells were transfected with His-Rer1p (1 and 4), Rer1p-EGFP (2 and 5) or Rer1p-YFP2 (3 and 6) and cell lysates were analysed after 2 days. Lanes 1-3 show western blots with the antibody raised to the loop epitope of Rer1p. 4-6 shows the same gel as 1-3, reprobed with the anti-GFP antibody. Approximate molecular weights are indicated.

During this study, a commercial antibody to human Rer1p became available from Everest Biotech (Oxford, UK). This antibody was also raised against the C-terminus of the protein. It was tested in western blots to determine whether it would detect the same sized protein as that recognised by the antibodies raised by Eurogentec. The commercial antibody also detected a protein at 50 kDa in both Caco-2 and HeLa cell lysates, not the predicted weight of 23 kDa (figure 6.5). The results from the commercial antibody are indistinguishable from those seen with the antibody raised by Eurogentec.

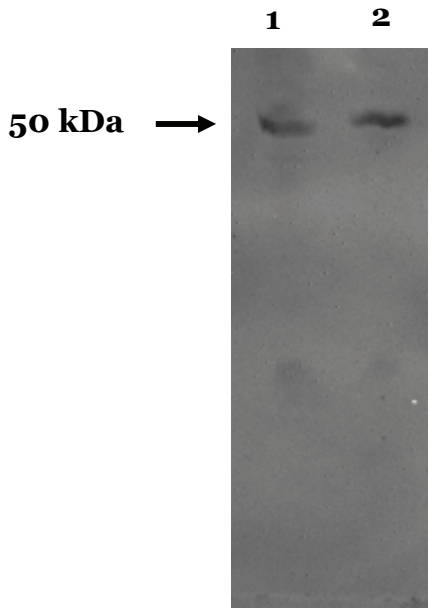



**Figure 6.5 Detection of a 50 kDa protein by a commercially available Rer1p antibody**
Caco-2 (lane 1) and HeLa (lane 2) cell homogenates were separated by SDS-PAGE and analysed by western blotting with anti-Rer1p antibodies (Everest Biotech). The position of the 50 kDa band from the molecular weight marker is indicated.

Immunofluorescence and microscopy were also used to characterise the Rer1p antibodies. HeLa cells were transfected with Rer1p-EGFP and treated with the antibody raised to the C-terminal epitope of Rer1p. Figure 6.6 shows colocalisation with Rer1p-EGFP and the Rer1p antibody, visualised using a Texas Red conjugated secondary antibody.

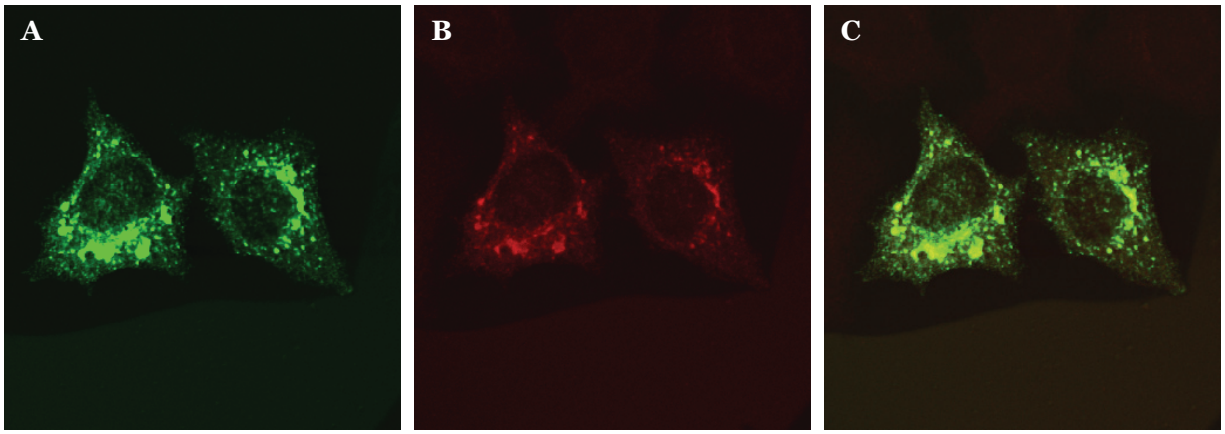



**Figure 6.6 Detection of Rer1p-EGFP by anti-Rer1p in immunofluorescence**
HeLa cells were transfected with DNA encoding Rer1p-EGFP (A). After 2 days, cells were treated with anti-Rer1p antibodies, visualised using a Texas Red conjugated secondary antibody (B). An overlay image is shown (C). Images were acquired by confocal microscopy.

Endogenous Rer1p could not be detected using the C-terminal or loop anti-Rer1p antibodies, so COS-7 cells were transfected with His-Rer1p and a strong signal was seen in transfected cells. This strategy was used to assess colocalisation of Rer1p with markers of the ERGIC and trans-Golgi as well as SERCA-EGFP. Figure 6.7 illustrates the colocalisation of His-Rer1p, detected with the anti-Rer1p loop antibody, with the ERGIC marker ERGIC-53 in cells that had been incubated at 15 °C for one hour before antibody treatment. Figure 6.8 demonstrates colocalisation of Rer1p with TGN46 in BFA treated cells, indicating the presence of Rer1p in the late Golgi.

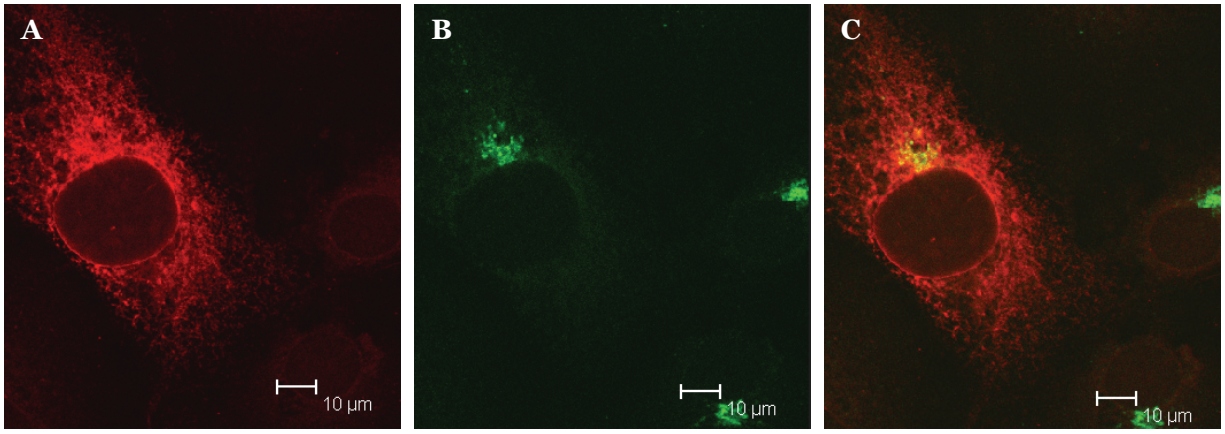



**Figure 6.7 Colocalisation of His-Rer1p with the ERGIC marker ERGIC-53**

COS-7 cells were transfected with His-Rer1p. After 2 days, cells were incubated at 15 °C for one hour and then double labelled with anti-Rer1p (loop epitope), visualised with a Texas Red conjugated secondary antibody (A) and anti-ERGIC-53, visualised with a FITC conjugated secondary antibody (B). An overlay is shown (C). Scale bars are 10 µm. Images were obtained with confocal microscopy.

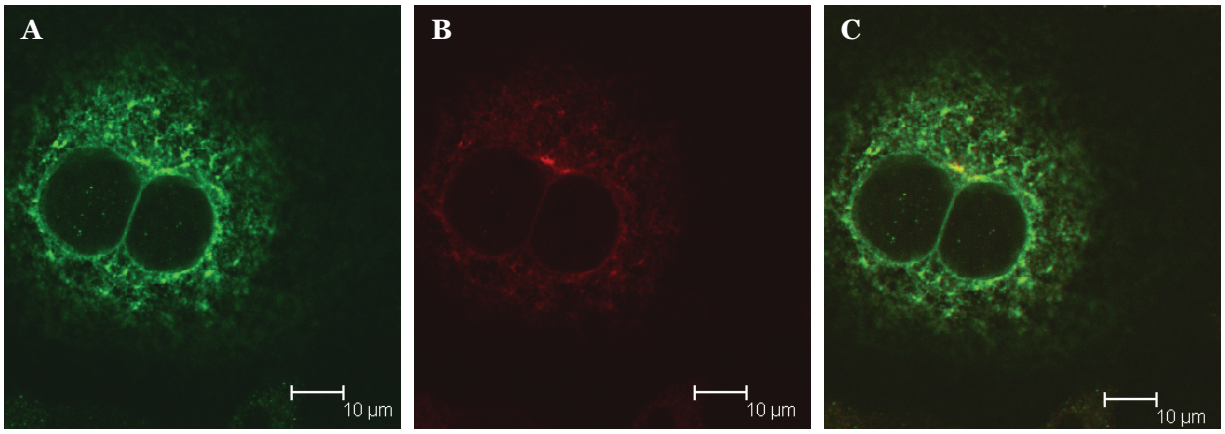



**Figure 6.8 Colocalisation of His-Rer1p with the trans-Golgi marker TGN46**

COS-7 cells were transfected with DNA encoding His-Rer1p for 2 days. Cells were treated with BFA and double labelled with anti-Rer1p (loop epitope), visualised with a FITC conjugated secondary antibody (A) and anti-TGN46, visualised with a Texas Red conjugated secondary antibody (B). An overlay is shown (C). Scale bars are 10 µm. Images were obtained with confocal microscopy.

Colocalisation of SERCA with Rer1p was assessed by transfecting COS-7 cells with both SERCA-EGFP and His-Rer1p, visualised by immunofluorescence. Figure 6.9 shows significant, but not total, colocalisation of SERCA and Rer1p. The majority of the reticular network illuminated contains both SERCA and Rer1p, but some distinct areas exist which contain only Rer1p. This is demonstrated by the inlay image in panel C in figure 6.9.

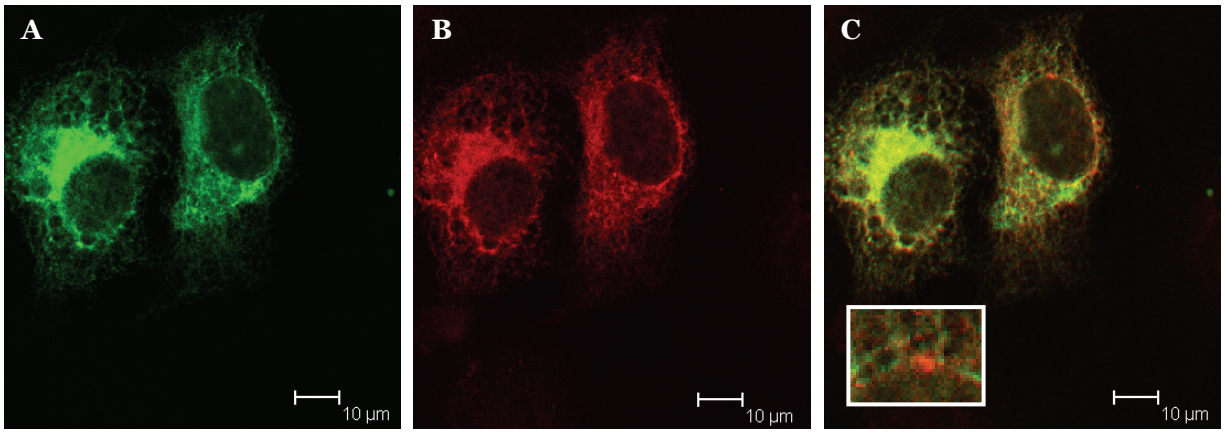



**Figure 6.9 Colocalisation of SERCA-EGFP with His-Rer1p**

COS-7 cells were transfected with SERCA-EGFP and His-Rer1p constructs. EGFP fluorescence is shown in panel A. After 2 days, cells were treated with anti-Rer1p antibodies (loop epitope), visualised with a Texas Red secondary antibody (B). An overlay is shown in panel C. The inlay image shows an expansion of a section of panel C in which colocalisation of SERCA and Rer1p was not seen. Scale bars are 10 µm. Images were obtained with confocal microscopy.

## 6.4 Discussion

Human Rer1p has a predicted molecular weight of 23 kDa[122]. The western blots shown in figure 6.3 demonstrate that in the three cell lines tested, the protein detected by the antibody raised against human Rer1p has an approximate molecular weight of 50 kDa. No bands are seen at 23 kDa or any other size. This apparently specific interaction of the antibody with a protein of 50 kDa is unexpected, and to investigate this further, western blots were carried out on homogenates from cells expressing tagged forms of Rer1p. Figure 6.4 demonstrates the ability of the anti-Rer1p antibody to detect His-Rer1p, Rer1p-EGFP and Rer1p-YFP2 at their predicted molecular weights of approximately 24 kDa, 50 kDa and 33 kDa respectively. In addition, the blot in figure 6.3 was also probed with anti-GFP antibodies which can recognise Rer1p-EGFP and Rer1p-YFP2. The anti-GFP antibodies detect bands at the same size as those detected by the anti-Rer1p antibody in samples from cells expressing either Rer1p-EGFP or Rer1p-YFP2. The results from this experiment strongly suggest that the Rer1p antibody is specifically recognising the Rer1p protein. The specific detection of three different tagged forms of Rer1p at three different sizes, two of which are also detected with GFP antibodies, indicates that the antibody is recognising these heterologously expressed proteins, all of which have Rer1p in common. If the antibody was unable to recognise Rer1p, it is almost impossible that these three differently sized bands would be detected, given that all that differs between the three samples is the tag attached to the transfected Rer1p construct.

During the course of this investigation, a commercial anti-Rer1p antibody became available, raised to the same C-terminal epitope on Rer1p. Figure 6.5 demonstrates that in a western blot, this antibody also specifically detects a band at approximately 50 kDa in both Caco-2 and HeLa cell lysates. Assuming that the 50 kDa protein detected by all three of these antibodies tested is endogenous Rer1p, what explanations exist for this unexpected size? 50 kDa is close to twice the size of the predicted 23 kDa, suggesting that Rer1p may exist as a dimer. That the tagged forms of Rer1p appear to be monomeric could be due to the tags interfering with the dimerisation of the protein. A range of temperatures were used in the preparation of the cell homogenates for western blotting, from 60 °C to 100 °C, and reducing agents were added to the samples in an attempt to disrupt any dimeric protein. Despite this, a band of 50 kDa was always seen, with no band at 23 kDa. Post-translational modifications can also cause proteins

to be detected at unexpected sizes in western blots. Post-translational processing of proteins can both increase and decrease the molecular weight of a protein predicted from its primary amino acid sequence. Addition of groups such as fatty acids, phosphates and carbohydrates can all increase the molecular weight of a protein, but such a large increase in size (from 23 to 50 kDa) in the case of Rer1p is unlikely to be entirely due to post-translational modifications[166]. A third possible explanation as to why Rer1p is detected at a much larger size than expected is that the detected protein may be a splice variant of Rer1p. Alternative RNA splicing describes the RNA rearrangements that occur in order to include or exclude certain exons from the final mRNA[166]. Inclusion of one or more extra exons into the sequence of the gene encoding Rer1p may result in a much longer mRNA product and result in the 50 kDa protein detected by the antibodies used here. Several Rer1p isoforms are reported in the databases, but the largest is 214 amino acids in size (accession number Q9P0H9-1) and is therefore not large enough to explain the 50 kDa protein seen here. It is possible that there are other, as yet undiscovered splice variants of the RER1 gene which result in a larger protein.

Immunofluorescence was also employed to characterise the anti-Rer1p antibodies. Figure 6.6 demonstrates that, as expected, the antibody to Rer1p colocalises with Rer1p-EGFP. Untransfected cells did not show a signal with anti-Rer1p antibodies, presumably due to the insufficient amount of Rer1p in these cells combined with the fact that the antibody was not raised against a conformational epitope. For this reason, cells were transfected with His-Rer1p and the protein was then illuminated using anti-Rer1p antibodies. That the transfected cells showed a signal with the antibody but untransfected cells did not (most clearly demonstrated in figure 6.7) suggests that the antibody can specifically recognise Rer1p. In figure 6.7, His-Rer1p was detected using anti-Rer1p antibodies and visualised using a Texas Red conjugated secondary antibody. In addition, antibodies directed against the ERGIC marker, ERGIC-53, were used to ascertain whether Rer1p was present in this compartment. Colocalisation of His-Rer1p and ERGIC-53 was indeed seen, indicating that Rer1p travels to the ERGIC, in agreement with previous reports of Rer1p localisation[122]. This is consistent with the role of the protein as a retrieval receptor in this part of the secretory pathway.

Colocalisation of His-Rer1p and the trans-Golgi marker TGN46 was also tested. Figure 6.8 shows His-Rer1p transfected cells which were double labelled to detect Rer1p (visualised using a FITC conjugated secondary antibody) and TGN46. This demonstrated that Rer1p is present in the trans-Golgi. Previous investigations have shown Rer1p to be distributed throughout the Golgi, comparable to the distribution of the KDEL receptor which fulfils a similar role[122,167]. Cells expressing SERCA-EGFP were treated with anti-Rer1p antibodies to determine the extent of colocalisation between the two proteins. Figure 6.9 shows a large amount of colocalisation between SERCA and Rer1p, presumably in the ER and ERGIC. The colocalisation is not complete however, as some areas show Rer1p but not SERCA localisation. This is most likely to indicate later sections of the secretory pathway, the medial- and trans-Golgi, in which Rer1p is present but SERCA is absent. This is consistent with the finding that SERCA is absent from the trans-Golgi (see figure 3.5, chapter 3) and, as shown here, Rer1p is present in the trans-Golgi (figure 6.8).

Despite the detection of a protein of 50 kDa in cell lysates by western blot, it seems likely from the other experiments described here that the Rer1p antibody is capable of specifically detecting Rer1p. All three tagged forms of Rer1p tested were detected at the correct sizes, and the two constructs containing the GFP antibody epitopes were also detected at the same size as with the GFP antibody (figure 6.4). These results suggest that although endogenous Rer1p is not detected at its predicted size of 23 kDa, the antibody can detect Rer1p in western blots. The antibody produced by Everest Biotech, raised against the C-terminus of human Rer1p, also detected a band at approximately 50 kDa in lysates of Caco-2 and HeLa cells. The immunofluorescence results show that the antibody can detect both Rer1p-EGFP (figure 6.6) and His-Rer1p (figures 6.7, 6.8 and 6.9) and that these proteins show subcellular localisation consistent with that of Rer1p. The large overlap in distribution between Rer1p and SERCA (figure 6.9) is agreeable with the potential recognition and retrieval of SERCA by Rer1p. The antibodies raised to human Rer1p have been characterised and are most probably able to recognise Rer1p specifically. This allows immunoprecipitation and cross-linking experiments to be carried out in an attempt to detect any interactions between SERCA and Rer1p. These experiments will be discussed in the next chapter.

# 7. Searching for interactions between SERCA and potential retrieval receptors

## 7.1 Introduction

It has so far not been possible to identify the precise sequence(s) in SERCA which cause it to be retrieved to the ER. For this reason, the study has focussed on identification of the protein machinery that is involved in the retrieval process, rather than the protein sequences that facilitate it. Many techniques exist for detecting protein-protein interactions. Coimmunoprecipitation and cross-linking have been selected for use in this investigation in order to test whether SERCA interacts with the retrieval receptor Rer1p[125] or the putative cargo receptor BAP31 which has been shown to interact with both anterograde and retrograde cargo between the ER and Golgi[136,142,144].

Coimmunoprecipitation and cross-linking are able to detect interactions between membrane proteins[168]. Coimmunoprecipitation makes use of specific antibodies to separate the protein of interest from a complex mixture of proteins such as a cell lysate. The antibodies are added to the lysate and specifically bind the protein of interest. By adding antibody binding protein such as protein A or G attached to sepharose or agarose beads, it is possible to immobilize the antibody-antigen complex and separate the proteins from the lysate by centrifugation. The antigen and any other proteins that have been coimmunoprecipitated are then separated from the antibodies and beads by heating. Proteins that interact with the antigen can be identified by SDS-PAGE and western blotting or mass spectrometry[168]. In these experiments, EGFP was used as the antigen with which to isolate the proteins of interest. This technique allowed immunoprecipitation of several different EGFP-tagged proteins with the use of one antibody. The other advantage of this method is that the EGFP tag will presumably not be involved in the interaction between the protein of interest (such as SERCA) and an interacting protein (such as Rer1p). This should allow the anti-GFP antibody to bind to the protein of interest regardless of whether the target protein is interacting with other proteins.

The second technique used here to search for protein-protein interactions was chemical cross-linking which allows low-affinity interactions to be strengthened and detected[168]. The cross-linker selected for this investigation was DTBP (dimethyl-3,3'-dithiobispropionimidate). DTBP is a water soluble, bifunctional, membrane permeable cross-linking agent containing two imidoester groups that react with amine groups on proteins. DTBP is cleavable upon reduction of the disulphide linkage in the centre of the molecule[128,169]. By adding DTBP to microsomal membranes, it is possible to capture protein-protein interactions between membrane proteins. Immunoprecipitation can then be carried out to enrich the sample for the protein of interest, and by cleaving the cross-linker, in this case by reduction, it is possible to identify proteins that interact with the protein of interest. This can be done by SDS-PAGE and western blotting if candidate proteins are known and antibodies to these proteins exist.

The only known candidate proteins for the process of retrieval of SERCA to the ER are Rer1p and BAP31. As discussed in detail in section 1.6 (chapter 1) Rer1p has been shown to retrieve membrane proteins from the Golgi to the ER, with interactions between the membrane spanning regions of Rer1p and target proteins being an important factor in this process[119,123-126,128,129]. BAP31 has been implicated in the anterograde and retrograde transport of proteins between the ER and Golgi[136,142,144]. This is discussed in detail in section 1.6 (chapter 1). Therefore, there is a possibility that BAP31 may interact with SERCA in the ERGIC or early Golgi and mediate its retrieval to the ER, presumably via the di-lysine motif at the C-terminus of BAP31[132]. Antibodies to BAP31 are already commercially available, and the antibodies raised to Rer1p (discussed in the previous chapter) have been characterised and appear to be specific for Rer1p. Therefore, through the use of coimmunoprecipitation and cross-linking techniques, it may be possible to detect any interaction between SERCA and Rer1p or BAP31. BAP31 is thought to be involved in the maturation and trafficking of both the wild-type and the ΔF508 mutant of the CFTR chloride ion channel[144]. GFP-tagged ΔF508 CFTR has been incorporated into these experiments as a protein which is likely to show an interaction with BAP31.

## 7.2 Methods

All general molecular biology, cell culture and transfection protocols are detailed in chapter 2. The GFP-tagged ΔF508 CFTR construct was a gift from Prof. B. Stanton[160]. The calnexin-EGFP construct was obtained from Genecopoeia (MD, USA).

COS-7 cells for coimmunoprecipitation experiments were grown on 15 cm culture dishes and transfected with DNA constructs encoding SERCA-EGFP, PMCA-EGFP or CFTR ΔF508-GFP. After 2 days, media was removed from plates and cells were washed twice in ice-cold PBS. RIPA buffer (Pierce) (1.9 ml) supplemented with 19 µl mammalian protease inhibitor cocktail (Sigma Aldrich) was added to each 15 cm dish and cells scraped off. Cells were sonicated for 30 seconds and centrifuged at 16 000 g for 15 minutes. The BCA protein concentration kit (Pierce) was used to estimate the protein concentration of the supernatant, according to manufacturer's instructions. The supernatant was then divided into aliquots and frozen in liquid nitrogen. 8 µg (20 µl) mouse anti-GFP antibody (Roche) was added to approximately 100 µg of transfected COS-7 sample. A control was also carried out in which antibody was added to 100 µl RIPA buffer. These mixtures were incubated overnight at 4 °C. Immobilized protein G (Pierce) was washed twice in RIPA buffer and 50 µl washed resin slurry was added to each sample of COS-7 and antibody. The protein G, antibody and sample mixtures were mixed end-over-end at room temperature for 2 hours. 0.5 ml RIPA buffer was added to each sample before centrifugation at 2500 g for 3 minutes. The supernatants were discarded and this step was repeated a further three times. The washed pellet was then resuspended in 25 µl sample buffer (as in chapter 2) and heated to 80 °C for 10 minutes. Samples were centrifuged at 2500 g for 3 minutes. Supernatants were collected and loaded onto a SDS PAGE gel for separation and analysis of immunoprecipitated proteins by western blotting.

The cross-linking protocol used was modified from that described by Spasic, D. *et al.* (2007)[128]. Microsomes were first made from COS-7 cells expressing constructs. COS-7 cells were transfected with SERCA-EGFP, PMCA-EGFP or calnexin-EGFP on 15 cm culture dishes. Media was removed from plates and cells were washed twice in ice-cold PBS. 500 µl PBS supplemented with 5 µl protease inhibitor cocktail was added to each plate, and cells

were scraped off the plates. Cells from four 15 cm culture dishes (all expressing the same construct) were combined and pelleted by centrifugation at 800 g for 10 minutes. Cells were resuspended in homogenisation buffer (250 mM sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.4) supplemented with protease inhibitor cocktail. Cells were then homogenised and centrifuged at 400 g for 10 minutes to pellet nuclei. The supernatant was ultracentrifuged at 100 000 g for 1 hour and microsomal membranes were resuspended in 100 µl microsome buffer (125 mM NaCl, 50 mM Hepes, pH 7.4) and homogenised. Protein concentrations of microsomes were determined using the BCA kit, supplementing samples with SDS (final concentration 2%) to solubilise membrane proteins.

Microsomes were cross-linked by adding 3 mM DTBP (Pierce) and incubating for 30 minutes at room temperature. Cross-linking reactions were quenched with 50 mM Tris at room temperature for 30 minutes. Triton X-100 was added (at a final concentration of 1%) and samples were incubated for 30 minutes at 4 °C to extract the proteins from the membranes. Centrifugation at 16 000 g for 15 minutes produced cleared cell extracts which could then be used in the coimmunoprecipitation procedure described above. Pellets of cross-linked immunoprecipitated material were resuspended in sample buffer (as in chapter 2 but without β-mercaptoethanol). Resuspended samples were split into two equal parts. One part was reduced (to cleave the DTBP cross-linker) by adding β-mercaptoethanol. The other half of the sample was left non-reduced. These samples were then analysed by SDS-PAGE and western blotting.

The antibodies and dilutions used in western blots were as follows: rabbit anti-Rer1p (loop or C-terminal epitopes), 1:500 (Eurogentec); mouse anti-GFP, 1:500 (Roche); rabbit anti-BAP31, 1:1000 (Abcam); donkey anti-rabbit IgG conjugated to HRP, 1:10 000 (GE Healthcare); sheep anti-mouse IgG conjugated to HRP, 1:2000 (GE Healthcare). GE Healthcare HRP conjugated secondary antibodies were affinity adsorbed (against rat, human and mouse).

## 7.3 Results

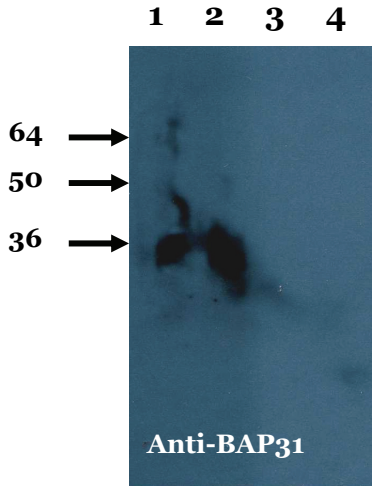



**Figure 7.1 Immunoprecipitated EGFP-tagged SERCA, ΔF508 CFTR and PMCA and western blot with anti-BAP31**

COS-7 cells were transfected with SERCA-EGFP (1), ΔF508 CFTR-GFP (2) or PMCA-EGFP (3). Immunoprecipitation was carried out with an anti-GFP antibody and immunoprecipitates analysed by SDS-PAGE and western blotting with anti-BAP31 antibodies. Lane 4 shows a RIPA buffer (cell-free) control. Approximate molecular weights (kDa) are indicated.

Coimmunoprecipitation experiments were carried out on lysates from COS-7 cells expressing SERCA-EGFP, ΔF508 CFTR-GFP or PMCA-EGFP. The immunoprecipitated material was analysed by SDS-PAGE and western blotting. Western blotting with anti-BAP31 (figure 7.1) detected a coimmunoprecipitated protein close to the size of BAP31 (31 kDa) with SERCA-EGFP and ΔF508 CFTR-GFP but not PMCA-EGFP or the cell-free control. The result shown in figure 7.1 was not reliably reproducible, so cross-linking with DTBP was used in an attempt to stabilise any interaction between SERCA and BAP31. In addition, microsomes were purified from transfected cells to enrich samples for proteins of interest. Cross-linked samples were analysed by SDS-PAGE and western blotting with anti-GFP and anti-BAP31 antibodies. The anti-GFP blot in Figure 7.2 shows the increase in size of EGFP tagged protein as a result of cross-linking to other proteins. The anti-BAP31 blot in Figure 7.2 also demonstrates the shift of cross-linked material to much larger molecular weights, and the presence of a band at approximately 60 kDa which is likely to represent the BAP29/BAP31 heterodimer[133,135].

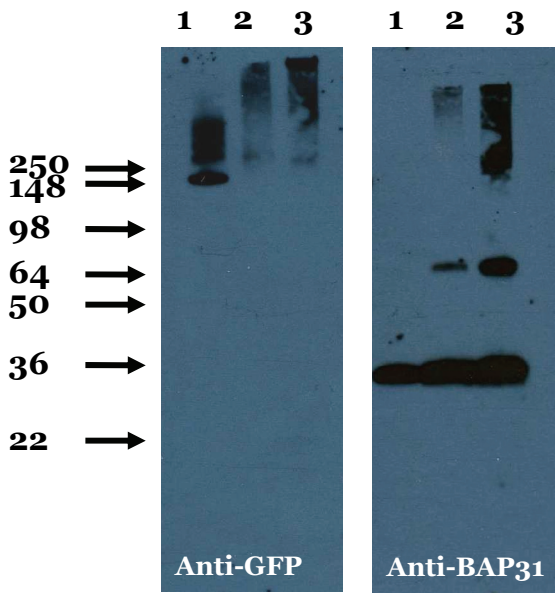



**Figure 7.2 Cross-linking of microsomes from COS-7 cells expressing SERCA-EGFP**
Microsomes from COS-7 cells expressing SERCA-EGFP were treated with the cross-linker DTBP. SDS-PAGE and western blotting with anti-GFP and anti-BAP31 was used to analyse the samples. Anti-GFP and anti-BAP31 blots were carried out on the same gel. Lanes are as follows: (1) non-cross-linked SERCA-EGFP microsomes, (2) cross-linked reduced SERCA-EGFP microsomes and (3) cross-linked non-reduced SERCA-EGFP microsomes. Approximate molecular weights (kDa) are indicated.

The results in figure 7.2 confirm that cross-linking with DTBP is effective in this system. Blots with both anti-GFP and anti-BAP31 reveal an increase in higher molecular weight protein complexes under cross-linking conditions. To investigate the possibility that SERCA interacts with BAP31, immunoprecipitation with anti-GFP antibodies was carried out on SERCA-EGFP microsomes that had been cross-linked with DTBP. Figure 7.3 shows a western blot of such a sample with anti-BAP31 antibodies. Under both reducing and non-reducing conditions, a band at the predicted size of BAP31 was detected. The non-reduced sample shows more material at higher molecular weights, consistent with the cross-linker being uncleaved. This again suggests that BAP31 is interacting with SERCA, but like the immunoprecipitation shown above, this result was not consistently reproducible.

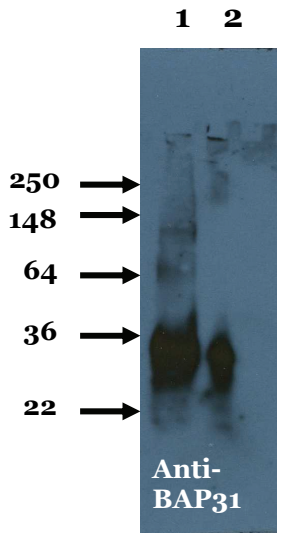



**Figure 7.3 Cross-linking and immunoprecipitation of SERCA-EGFP microsomes**
Microsomes purified from COS-7 cells expressing SERCA-EGFP were cross-linked with DTBP and protein was immunoprecipitated with anti-GFP antibodies. Reduced (1) and non-reduced (2) cross-linked and immunoprecipitated material was analysed by SDS-PAGE and western blotting with anti-BAP31 antibodies. Approximate molecular weights (kDa) are indicated.

In order to determine whether the potential interaction seen between SERCA and BAP31 in the cross-linking experiment described above is specific to an ER retrieval pathway, PMCA-EGFP and calnexin-EGFP were introduced as controls. Neither protein should interact with ER retrieval receptors as neither undergo significant ERGIC to ER transport. The combination of cross-linking and immunoprecipitation was again used here. In this experiment, no interaction was seen with any of the three EGFP-tagged proteins and either Rer1p or BAP31. Figure 7.4 shows a western blot with anti-GFP antibodies on these samples. The non-cross-linked, non-immunoprecipitated samples show the EGFP-tagged proteins only. The cross-linked, reduced samples show the heavy and light IgG chains as well as SERCA-EGFP and PMCA-EGFP, confirming they have been immunoprecipitated. Calnexin-EGFP is not detected here. Any EGFP-tagged proteins in cross-linked, non-reduced samples are obscured by a large amount of non-dissociated IgG at 150 kDa.

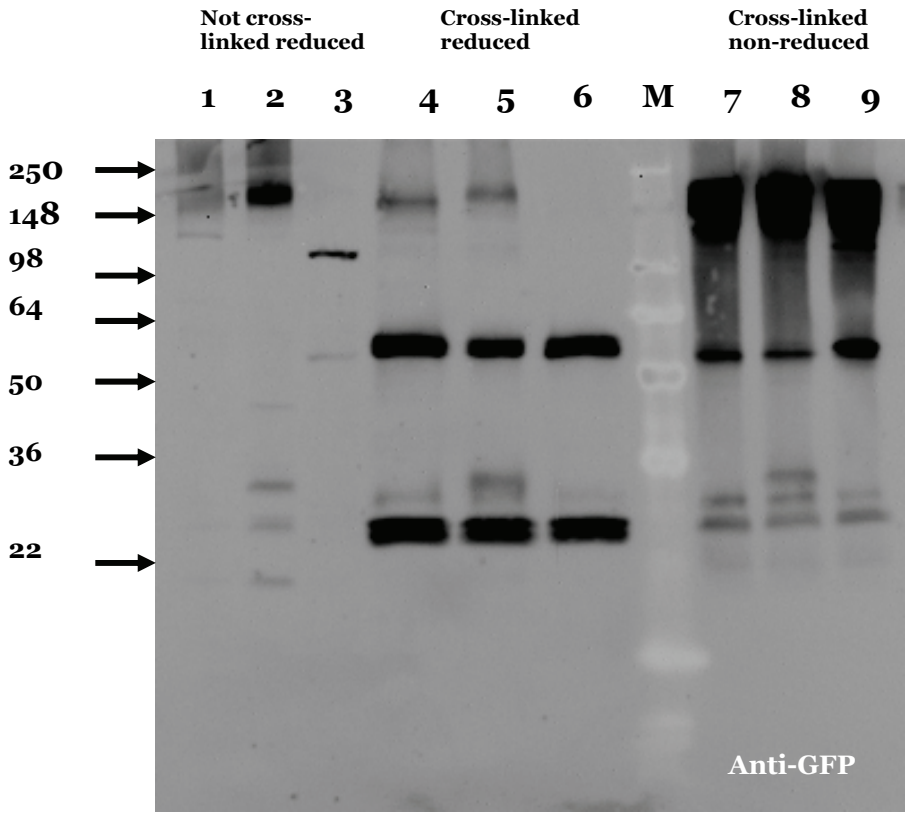



**Figure 7.4 Cross-linking and immunoprecipitation of SERCA, PMCA and calnexin**
Microsomes were purified from COS-7 cells expressing SERCA-EGFP (lanes 1, 4 and 7), PMCA-EGFP (lanes 2, 5 and 8) or calnexin-EGFP (lanes 3, 6 and 9). Samples were left untreated (1-3) or were cross-linked with DTBP (4-9). Following cross-linking, samples were immunoprecipitated with anti-GFP and either reduced (4-6) or left non-reduced (7-9). Samples were analysed by SDS-PAGE and western blotting with anti-GFP antibodies. A molecular weight marker (M) is also shown between reduced and non-reduced samples. Approximate molecular weights (kDa) are indicated.

## 7.4 Discussion

The experiments described in this chapter were carried out in an attempt to detect interactions between SERCA and BAP31 or Rer1p, either of which could potentially be involved in the retrieval of SERCA to the ER. Although no interaction between SERCA and Rer1p could be detected, there was a potential interaction between SERCA and BAP31 observed in some experiments.

Immunoprecipitation was first used as a means of detecting interactions between SERCA and possible retrieval receptors. Western blotting of immunoprecipitated material with BAP31 antibodies (figure 7.1) shows that BAP31 is coimmunoprecipitated with both SERCA and ΔF508 CFTR but not PMCA. This suggests that BAP31 interacts with SERCA and ΔF508 CFTR and could feasibly be involved in the trafficking of these proteins between the ER and Golgi. PMCA is the only protein out of the three tested that is localised to the plasma membrane and presumably undergoes no retrograde transport from the Golgi to the ER. That BAP31 is not coimmunoprecipitated with PMCA makes its role in the trafficking of these proteins more likely to be in retrograde, rather than anterograde, transport between the Golgi and ER. This result was not reliably replicable. For this reason, chemical cross-linking was then used as a means of stabilising any interaction between SERCA and BAP31 before immunoprecipitation. In addition, microsomes, rather than total cell lysates, were used in an attempt to enrich the samples for proteins located in the ER and Golgi membranes.

The ability of DTBP to cross-link proteins in this system was tested. Western blots of cross-linked microsomes with both anti-GFP and anti-BAP31 antibodies (figure 7.2) show an increase in molecular weight of detected proteins. Interestingly, BAP31 also shows a prominent band close to 60 kDa under cross-linked conditions. This band is likely to represent the heterodimer formed from BAP31 and the related 29 kDa protein, BAP29[133,135]. Consistent with this, the 60 kDa band was not seen under non-cross-linked conditions when samples were blotted with anti-BAP31 (figure 7.2, lane 1).

A combination of cross-linking and immunoprecipitation of microsomes from transfected cells was used to investigate the possibility that SERCA interacts with BAP31. As seen with the

immunoprecipitation experiment, western blotting with anti-BAP31 revealed a coimmunoprecipitated protein at the correct size for BAP31. Again, this result was not easily reproducible, so further work is required to unequivocally show interactions between SERCA (and ΔF508 CFTR as detected in immunoprecipitation) with BAP31.

Figure 7.4 shows cross-linking and immunoprecipitation (with anti-GFP antibodies) of microsomes from COS-7 cells expressing SERCA-EGFP, PMCA-EGFP or calnexin-EGFP. This was carried out to ensure that the desired proteins were efficiently purified by the immunoprecipitation step. Anti-GFP antibodies were used in the blot shown in figure 7.4. The most prominent bands in lanes 4-9 represent the mouse IgG used in the immunoprecipitation, detected by the anti-mouse secondary antibody used in the blot. Under reducing conditions, IgG runs as heavy and light chains at approximately 25 kDa and 50 kDa respectively. Non-reducing conditions are unable to break the disulphide linkages connecting the heavy and light chains so a band is seen at approximately 150 kDa, the molecular weight of the two heavy and two light chains that make up the IgG molecule[170]. Immunoprecipitated SERCA-EGFP and PMCA-EGFP can be seen in lanes 4 and 5 respectively (figure 7.4). These proteins have been immunoprecipitated with anti-GFP antibodies and have been reduced so run at their predicted molecular weights. Calnexin-EGFP is not visible in lane 6, suggesting that the immunoprecipitation of this protein was not successful. The presence of EGFP-tagged SERCA, PMCA or calnexin cannot be established in lanes 7-9 (figure 7.4) due to the high abundance of IgG at approximately 150 kDa.

PMCA is localised to the plasma membrane and so will presumably only interact with BAP31 if BAP31 is involved in anterograde transport from the ER to the Golgi. Calnexin is localised exclusively to the ER and does not enter the ERGIC[28], so would not be expected to interact with BAP31 as it does not undergo transport in either direction between the ER and Golgi. The blot shown in figure 7.4 was reprobed with anti-BAP31 antibodies but no interaction was detected between BAP31 and SERCA, PMCA or calnexin.

A combination of cross-linking (to strengthen any low-affinity interactions between SERCA and potential retrieval receptors) and immunoprecipitation (to enrich the samples for EGFP-

tagged proteins) has been used here in an attempt to detect protein machinery that may be involved in the retrieval of SERCA to the ER. Cross-linking and immunoprecipitation both showed a potential interaction between SERCA and BAP31, but these results could not be repeated reliably. The large pool of EGFP-tagged SERCA present in transfected COS-7 cells compared to the relatively small population interacting with BAP31 or any other retrieval receptor at any one time could account for the unreliability of these results. Differences in expression levels of SERCA-EGFP from sample to sample would change the proportion of the protein being retrieved at any one time. However, it was hoped that the use of cross-linking in combination with immunoprecipitation would cause stabilisation of any interactions occurring at the point of cross-linking, increasing the chance of detection. Another explanation could simply be that BAP31 is showing non-specific interactions with SERCA and ΔF508 CFTR, although the absence of BAP31 coimmunoprecipitated with PMCA (figure 7.1) suggests that this interaction may show some specificity at least for ER localised proteins. More work is required here to determine if BAP31 shows a specific interaction with SERCA. Other techniques could be used to answer this question. The effect of BAP31 on SERCA localisation could be determined by using RNA interference to knockdown the expression of BAP31[144]. The presence of SERCA in the late Golgi or plasma membrane of BAP31 knockdown cells would indicate that it is important in the maintenance of the calcium pump in the ER. Although it has not been possible to demonstrate beyond reasonable doubt that there is an interaction between SERCA and BAP31, the use of other techniques in combination with the immunoprecipitation and cross-linking shown here may be able to shed some more light on this interesting possibility.

# 8. General discussion

## 8.1 Introduction

The localisation of SERCA in the ER is vital for the maintenance of high calcium concentrations in the organelle that are required for processes including protein trafficking and calcium signalling[15,33]. In addition to the ER, SERCA is also present in the ERGIC and cis-Golgi[33,94]. For this reason, retrieval from downstream compartments is likely to be the primary mechanism for the maintenance of the protein in the ER. Several different mechanisms have been described for ER retrieval of both soluble and membrane-spanning proteins. Some of these mechanisms rely on discreet consensus sequences such as the KDEL[90] and di-lysine motifs[109]. No canonical retrieval signals are conserved in SERCA so two possibilities therefore exist for its retrieval: either it is retrieved by a receptor such as Rer1p which appears not to require a particular sequence motif[130], or it is retrieved by a different and so far unknown mechanism. This investigation attempted to decipher the sequences and mechanisms involved in the retrieval of SERCA to the ER.

## 8.2 Searching for an ER retrieval signal in SERCA

In order to determine where in SERCA the sequence(s) mediating ER retrieval are located, a series of SERCA/PMCA chimeric calcium pumps were built, and their subcellular localisations determined (see chapter 3). This approach has been used before in the search for retrieval signals in SERCA. However, no precise conclusions have been drawn from previous investigations other than that the N-terminus of the protein is at least partially required for ER retrieval[94,146,147]. For this reason, the first chimeras built in this investigation were designed to dissect the N-terminus of SERCA to establish which (if any) regions of the N-terminus are required for ER localisation of the pump. By expressing chimeras with C-terminal EGFP tags in COS-7 cells, it was possible to determine the subcellular localisations of the proteins. It was not possible to determine whether the very N-terminal amino acids of SERCA are important for retrieval as both S/PNterm and P/SNterm (see figure 3.12, chapter 3) were located in the ER. However, the first and second transmembrane domains of SERCA (M1 and M2

respectively) were shown not to be required for ER localisation. Replacing either M1 (S/PM1), M2 (S/PM2) or both helices (S/PM1-2) of SERCA sequence with corresponding PMCA sequence did not disrupt the ER localisation of the protein. Consistent with this, replacing M1 (P/SM1), M2 (P/SM2) or both M1 and M2 (P/SM1-2) of PMCA sequence with that of SERCA did not cause redistribution of the plasma membrane pump to the ER.

The pump was then divided into three similar sized sections, and six constructs were built to scan the whole SERCA sequence for retrieval motifs. This highlighted the C-terminus of SERCA (residues 712-1001) as being required for ER localisation of the pump. Replacement of this section with corresponding PMCA sequence produced a plasma membrane localised pump (S/PM5-10), with the opposite construct (P/SM5-10) showing ER localisation. In order to elucidate which section(s) of the C-terminus of SERCA are required for the retrieval process, constructs were built in which the C-terminus was dissected further. Unfortunately, the ER localisation of all of these constructs rendered drawing any conclusions impossible. The localisations of the chimeras built in this study therefore suggest that there is sequence mediating retrieval within amino acids 712-1001 of SERCA, but where exactly, and what features of this sequence are important remains elusive.

Of the 22 chimeras constructed here, only 5 showed plasma membrane localisation in COS-7 cells. In several cases, both members of a mirror pair of constructs (containing opposite sections of SERCA and PMCA sequence) showed ER localisation. This is unexpected, as presumably one would contain the ER retrieval signal of SERCA and one would not. This could be explained by redundancy in the system, whereby both members of a pair of constructs could contain enough of the retrieval signal to be located in the ER. However, this does not explain all of the pairs of ER localised constructs. For example, both S/PNterm and P/SNterm are located in the ER but the localisation of S/PM5-10 in the plasma membrane demonstrates that the entire N-terminal section is not able to cause ER retrieval. In these cases, protein misfolding seems a more likely explanation for the ER localisation observed. Although SERCA and PMCA have approximately 30% sequence identity and share common structural and functional characteristics, they are both relatively large and complex proteins which presumably require many intramolecular interactions to fold correctly. Building chimeras of

these two, albeit highly related, calcium pumps may therefore still result in aberrant protein folding. In an attempt to reduce the propensity of chimeras to misfold, the two protein sequences were joined in conserved regions wherever possible throughout the investigation, but this proved to be no guarantee that correct folding would always occur.

If it were possible to detect which of the ER localised chimeras were misfolded, more information could be yielded from the results shown in chapter 3. For this reason, experiments were carried out in an attempt to detect misfolding in the chimeric constructs (described in chapter 4). A large amount of misfolded protein in the ER, such as could be caused by overexpression of misfolded SERCA/PMCA chimeras, is known to elicit the UPR[69,155]. The chaperone protein BiP is upregulated as part of the UPR[69]. This was confirmed in the system used here by treating cells with tunicamycin; a known inducer of the UPR[159]. This treatment resulted in a clear upregulation of BiP, as shown in chapter 4 (figure 4.1). It was not possible however, to detect initiation of the UPR in HeLa or COS-7 cells expressing any of the chimeric constructs when tested by western blotting. This could be because the transfection rate of the cells expressing the recombinant chimeras was sufficiently low as to cause dilution of any increase in BiP levels by untransfected cells. Immunofluorescence was then tested as a means to detect BiP increases in cells exhibiting the UPR. No difference in BiP (detected by anti-BiP antibodies and visualised using a fluorescent secondary antibody) was detected between transfected cells expressing the constructs tested, and neighbouring, untransfected cells. Furthermore, cells expressing the well characterised misfolded CFTR ΔF508 mutant, a protein which is known to elicit the UPR[158], showed no detectable BiP upregulation in immuofluorescence experiments.

Although it has not been possible to detect misfolding in SERCA/PMCA chimeras, this does not rule out the possibility that abberant folding of these proteins is occuring. In fact, the only positive result seen for protein misfolding (measured by an increase in BiP levels) was achieved by treating cells with tunicamycin. Even expression of the known misfolded protein CFTR ΔF508 did not show a detectable increase in BiP levels observed by immunofluorescence. Further experiments were carried out to locate the retrieval signal and attempts were made to circumvent the problem of misfolding. These experiments are

described in chapter 5. The tenth transmembrane domain (M10) of SERCA was selected for investigation in these experiments. Studies with the chimeras shown in chapter 3 have shown that the C-terminal section of SERCA is responsible for ER localisation of the protein. Structural studies suggest that M10 is probably the least dynamic of the ten transmembrane domains and is accessible from the bilayer[18]. If the ER retrieval signal of SERCA is located in a transmembrane domain (for example if it were recognised by Rer1p or a similar receptor) then M10 would be a logical candidate. M10 contains the conserved charged residue K972 which crystal structures suggest points out into the bilayer[18]. Rer1p has been shown to recognise charged or polar residues in transmembrane domains of proteins retrieved to the ER[128-130,171], so it is possible that this lysine residue in M10 of SERCA is involved in its ER localisation. This was tested by mutating K972 to phenylalanine (the residue in the corresponding position in M10 of PMCA) to produce the K972F mutant. This was shown by fluorescence microscopy to be located in the ER, suggesting that either K972 is not involved in ER retrieval or that this mutation causes misfolding of the protein.

The involvement of M10 in the ER localisation of SERCA was further tested by elongating the membrane-spanning helix with the addition of three leucine residues to produce the SERCA M10 3Leu construct. This technique has been used by other researchers on single-pass ER membrane proteins that are mis-targeted to the plasma membrane upon elongation of their membrane-spanning helices[28,74]. If M10 of SERCA is key to its ER retrieval, elongation (and the resulting change in position of residues within the membrane) could conceivably cause loss of retrieval. However, the M10 3Leu construct was localised to the ER and did not escape to the plasma membrane. This could be due to misfolding of the construct or that 3 leucine residues are not sufficient to cause a significant change in the transmembrane domain to disrupt retrieval. Clearly it could also be that the ER retrieval of SERCA relies on sequence elsewhere in the protein.

A CD8 reporter construct[163] has also been used in the search for retrieval signals in M10 of SERCA. Both SERCA and PMCA M10 sequences were used to replace the single transmembrane domain of the plasma membrane localised CD8 protein. Although M10 of SERCA caused the protein to be ER localised, the M10 sequence of PMCA had the same

effect, meaning that no conclusions can be drawn here. These results may simply reflect the fact that single transmembrane domains from complex polytopic membrane proteins are not able to stand alone in the bilayer without aggregating.

The chimeras and M10 constructs described in chapters 3 and 5 respectively, illustrate that manipulating a relatively large, polytopic membrane protein such as SERCA is problematic to say the least. Although it was not able to be shown in chapter 4, it is likely that some of these constructs are located in the ER as a result of misfolding. The chance of misfolding, combined with the use of ER localisation as a read out of signal-mediated retrieval could allow misleading conclusions to be drawn. Therefore only plasma membrane localised chimeras have been used to draw information on where in SERCA a retrieval signal may be found. Any pairs of opposite chimeras in which both members are ER localised have been disregarded in the search for the retrieval signal.

## 8.3 Identifying protein machinery responsible for ER retrieval of SERCA

Searching for the ER retrieval signal in SERCA using chimeras, M10 mutants and CD8 reporter constructs pointed to the importance of the C-terminus, but it was not possible to isolate any specific region of the protein required for ER localisation. The next stage in the investigation was to attempt to identify the protein machinery involved in the ER retrieval of SERCA. Aside from Rer1p and BAP31 (both discussed in chapter 1, section 1.6), no convincing candidate receptors exist for the retrieval process that proteins lacking canonical signals such as KDEL or the di-lysine motif undergo. As Rer1p has been shown to retrieve membrane proteins from the early Golgi to the ER in both yeast and mammalian cells[119,123-126,128,129], it was selected for further investigation as a candidate receptor for SERCA. As no commercial antibodies to human Rer1p were available at the start of this investigation, antibodies were raised (by Eurogentec) to two epitopes in human Rer1p. Chapter 6 describes characterisation of these antibodies. Although the predicted molecular weight of Rer1p is 23 kDa, the antibodies raised in this study detect a protein of 50 kDa in western blots of various mammalian cell lysates. However, three different heterologously expressed, tagged forms of Rer1p appeared at the correct predicted molecular weights in western blots with these

antibodies. This was confirmed in the case of two of these proteins which were also detected at the same sizes using an anti-GFP antibody.

Immunofluorescence was also used in the characterisation of these antibodies. The antibody was not able to detect endogenous Rer1p by immunofluorescence. This could be due to insufficient levels of endogenous Rer1p, coupled with the fact that the antibody was directed at a peptide rather than a conformational epitope in the protein. In cells expressing Rer1p-EGFP however, the antibody, visualised with a fluorescent secondary antibody, showed colocalisation with the Rer1p construct in transfected cells. The antibody was also able to detect His-Rer1p in transfected cells and illuminated a reticular pattern, typical of ER proteins, in these cells. Colocalisation studies showed that His-Rer1p (as detected by the antibody raised in this study) is present in both the ERGIC and trans-Golgi. This fits with previous reports that Rer1p is distributed throughout the ERGIC and Golgi[122]. His-Rer1p also showed significant, but not total, colocalisation with SERCA-EGFP. This is in agreement with what is known about the distribution of the two proteins[33,122]. The areas where Rer1p is present but SERCA is absent presumably represent the medial- and trans-Golgi. The results from this characterisation of the anti-Rer1p antibodies raised in this study are consistent with specific recognition of the Rer1p protein. Although the endogenous protein appears at 50 kDa and not the predicted molecular weight of 23 kDa, detection of tagged forms of the protein, coupled with immunofluorescence results, suggest that the antibody is specific for Rer1p.

Immunoprecipitation experiments were then carried out to determine if either Rer1p or BAP31 interact with SERCA, with a view to determining what protein machinery is responsible for this retrieval process. No interaction was found between SERCA and Rer1p. Tantalisingly however, BAP31 appeared to coimmunoprecipitate with SERCA and CFTR ΔF508 but not PMCA. This result could not be repeated, so cross-linking was employed to strengthen any interaction between SERCA and BAP31 before immunoprecipitation. The cross-linker DTBP was tested and was able to cause a shift of proteins to higher molecular weights as deteced by both anti-GFP and anti-BAP31 antibodies in western blots. A potential interaction was seen here between SERCA and BAP31, but again, this could not be reliably repeated. It is possible that these proteins interact, and if that is the case, BAP31 may be involved in the retrograde

traffic of SERCA to the ER. However, more work is required to verify this and to ensure that the potential interaction between BAP31 and SERCA is a specific one.

## 8.4 Future directions

The results shown here suggest that the ER retrieval signal of SERCA is located towards the C-terminus of the protein, but it has not been possible to determine exactly where in this section of the protein the signal lies. Due to the apparent propensity of these chimeric constructs to misfold and be retained in the ER, further work of this kind to find the retrieval signal is likely to be problematic. It may be necessary to use simpler proteins with fewer membrane-spanning helices that also undergo ER retrieval in order to continue the search for signals by making large changes to their sequences. SERCA could then be revisited and subtle mutations made to the protein, guided by new knowledge yielded from studying other proteins. ER retrieval by Rer1p appears not to require a specific consensus sequence as in the case of the KDEL system, but instead the receptor has been reported to recognise polar or charged residues in transmembrane domains[128,130]. Transmembrane domains of polytopic ion transporters, such as SERCA, exhibit a high prevelence of charged amino acids. Therefore searching for what would normally be considered unusual residues in membrane-spanning helices of proteins such as SERCA, with a view to discovering a retrieval signal, would undoubtedly be misleading.

A useful approach to this problem may be to first determine the protein machinery responsible for retrieval of SERCA to the ER. This may shed light on the sequences involved. Rer1p and BAP31 should first be tested, as they are logical candidates for this role. If they are both ruled out, a proteomics approach could be applied to search for relevant protein interactions. This can be done by immunoprecipitation of the protein of interest, resolution of the coimmunoprecipitated proteins using SDS-PAGE and identification by mass spectrometry[172]. This approach could be feasbily be used to find the protein machinery responsible for ER retrieval of SERCA. Immunoprecipitation and SDS-PAGE of SERCA and PMCA (or a plasma membrane chimera) would presumably lead to two different patterns of coimmunoprecipitated proteins. Those that are coimmunoprecipitated with SERCA (but not

PMCA or plasma membrane localised chimeras) may be involved in retrieval and could be identified and characterised. Other proteins interacting with the new candidate retrieval receptor(s) could then be determined and by comparing these proteins it may be possible to elucidate what features they have in common. This may be a way of not only identifying the protein machinery responsible for ER retrieval of SERCA, but also resolving which sections of SERCA sequence are required for ER localisation.

Yeast genetics has proved to be a powerful approach in the field of protein trafficking. For example, the KDEL receptor was characterised by transferring the ERD2 gene between different species of yeast, allowing an elegant demonstration that the protein it encodes specifically recognises an ER retrieval motif[98]. In addition, Rer1p was discovered by screening mutant yeast strains that were unable to retain the Sec12p protein in the ER[119]. It may be possible to identify other retrieval receptors in a similar way by observing the ER retention of other proteins in yeast. P-type calcium ATPases exist in the yeast Golgi (PMR1) and plasma membrane (PMC1)[173]. However, no ER localised homologue to SERCA has been described in yeast so carrying out screens on yeast mutants by looking for SERCA mistargeting is not possible. Despite this, screening of yeast mutants may still may be a valuable technique for finding other retreival receptors, mammalian homologues of which might be involved in SERCA trafficking.

RNA interference has become a staple technique for scientists in many fields, not least cell biology. By introducing short RNAs (complementary to a gene of interest) into cells, it is possible to acheive a targeted knockdown of a specific gene at the mRNA level[174]. RNA interference has been used to study the interaction of Rer1p with nicastrin and Pen2 (two components of the γ-secretase complex)[128,129]. A significant increase in plasma membrane localised Pen2 derived constructs was seen upon knockdown of Rer1p, implicating the receptor in the ER localisation of Pen2[129]. As well as studies on individual proteins, RNA interference has been used in higher throughput screening studies to investigate mechanisms of secretion. One study by Simpson *et al.* (2007)[175] demonstrated the power of this approach by selecting secretory pathway proteins of unknown function and using RNA interference to systematically knockdown each gene. The secretion of a fluorescently-tagged temperature

sensitive membrane protein was then monitored by fluorescence microscopy. The knockdown of several previously unidentified proteins was found to inhibit secretion of the marker protein[175]. It may be possible to knockdown a set of secretory pathway proteins and monitor mistargeting of SERCA to the late Golgi or plasma membrane. This could allow identification of the protein machinery which mediates the retrieval of SERCA to the ER.

## 8.5 Concluding remarks

The aim of the experiments conducted in this investigation was to determine the sequences and protein machinery involved in the retrieval of SERCA from the ERGIC or early Golgi to the ER. The subcellular localisations of the chimeric calcium pumps built in this study suggest that the ER retrieval signal lies between residues 712 and 1001 of SERCA. Chimeras built to dissect this C-terminal region further, resulted in ER localised chimeras from which conclusions could not be drawn. The protein machinery involved in the retrieval of SERCA to the ER remains elusive. There is a possibility that SERCA interacts with BAP31, which may mediate the retrieval process. Further work is required to determine whether this is a specific interaction, and whether BAP31 is required for maintenance of SERCA in the ER. The KDEL and di-lysine motif mediated retrieval processes are now relatively well characterised[176,177]. However, for proteins such as SERCA that lack these motifs there is still much to learn about the sequences and machinery that mediate their retrieval to the ER.

# Appendix

## Appendix 1: Oligonucleotides used in the production of SERCA/PMCA chimeras

PCR primers used in the construction of all SERCA/PMCA chimeras (discussed in chapter 3) are shown. Fwd and rev indicate forward and reverse primers respectively. The 5' and 3' ends of the primers are also indicated.

### Mutagenesis of PMCA3 to introduce a KpnI restriction site

Fwd 5′-ATGCTGCTCTCAGGTACCCATGTCATGGAAGGTTCTGG-3′

Rev 5′-CCTTCCATGACATGGGTACCTGAGAGCAGCATAGGATC-3′

### Mutagenesis of PMCA3 to introduce an AflII restriction site

Fwd 5′-AATGATGGACCAGCTCTTAAGAAGGCAGATGTGGGCTTC-3′

Rev 5′-CCCACATCTGCCTTCTTAAGAGCTGGTCCATCATTGGTG-3′

### S/PNterm

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-ACCAGGAGGTCTTCAAACTGTTCCCACACCAGCTGCAGG-3′

Fwd 5′-CTGCAGCTGGTGTGGGAACAGTTTGAAGACCTCCTGGTG-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

### S/PM1

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-GTCACGTCCTGCAGGGCTTCTATCACCAGCTCCCACAGG-3′

Fwd 5′-CCTGTGGGAGCTGGTGATAGAAGCCCTGCAGGACGTGAC-3′

Rev 5′-AAGGCAGTGATGGTCTCTCCCTCGTCTTCTGCCCCACCAG-3′

Fwd 5′-GGGGCAGAAGACGAGGGAGAGACCATCACTGCCTTCGTTG-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

### S/PM2

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CCAGCCTCGGCCTCTTCCCCTTCTTCAAACCAGG-3′

Fwd 5′-CCTGGTTTGAAGAAGGGGAAGAGGCCGAGGCTGG-3′

Rev 5′-ATATTCCTTAAGACCTCTGAACTGCTTTTCCTTGCTCCAG-3′

Fwd 5′-AAAGCAGTTCAGAGGTCTTAAGGAATATGAGCCCGAGATG-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

**P/SNterm**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-GTCACGTCCTGCAGGGCTTCTATCACCAGCTCCCACAGG-3′

Fwd 5′-CCTGTGGGAGCTGGTGATAGAAGCCCTGCAGGACGTGAC-3′

Rev 5′-CATCTTGGTACCTGAGAGCAGCATAGGATC-3′

**P/SM1**

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-ACCAGGAGGTCTTCAAACTGTTCCCACACCAGCTGCAGG-3′

Fwd 5′-CTGCAGCTGGTGTGGGAACAGTTTGAAGACCTCCTGGTG-3′

Rev 5′-CCAGCCTCGGCCTCTTCCCCTTCTTCAAACCAGG-3′

Fwd 5′-CCTGGTTTGAAGAAGGGGAAGAGGCCGAGGCTGG-3′

Rev 5′-CATCTTGGTACCTGAGAGCAGCATAGGATC-3′

**P/SM2**

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-AAGGCAGTGATGGTCTCTCCCTCGTCTTCTGCCCCACCAG-3′

Fwd 5′-GGGGCAGAAGACGAGGGAGAGACCATCACTGCCTTCGTTG-3′

Rev 5′-CTGCTCAATTCGGCTCTGAAGGGCTTCTATGGCGTTC-3′

Fwd 5′-GAACGCCATAGAAGCCCTTCAGAGCCGAATTGAGCAG-3′

Rev 5′-CATCTTGGTACCTGAGAGCAGCATAGGATC-3′

**S/PM1-2**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-GTCACGTCCTGCAGGGCTTCTATCACCAGCTCCCACAGG-3′

Fwd 5′-CCTGTGGGAGCTGGTGATAGAAGCCCTGCAGGACGTGAC-3′

Rev 5′-ATATTCCTTAAGACCTCTGAACTGCTTTTCCTTGCTCCAG-3′

Fwd 5′-AAAGCAGTTCAGAGGTCTTAAGGAATATGAGCCCGAGATG-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

**P/SM1-2**

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-ACCAGGAGGTCTTCAAACTGTTCCCACACCAGCTGCAGG-3′

Fwd 5′-CTGCAGCTGGTGTGGGAACAGTTTGAAGACCTCCTGGTG-3′

Rev 5′-CTGCTCAATTCGGCTCTGAAGGGCTTCTATGGCGTTC-3′

Fwd 5′-GAACGCCATAGAAGCCCTTCAGAGCCGAATTGAGCAG-3′

Rev 5′-CATCTTGGTACCTGAGAGCAGCATAGGATC-3′

**P/SNtermM1-2**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

**S/PNtermM1-2**

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-CATCTTGGTACCTGAGAGCAGCATAGGATC-3′

**S/PM3-4**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CCGCGATGTTGGTACCCGAG-3′

**P/SM3-4**

Fwd 5′-GCTTTTCTCGGGTACCAACATC-3′

Rev 5′-GTGCCAGATCCCATAGCTATGC-3′

**S/PM5-10**

Fwd 5′-GAGCAGCTTAAGAAGGCAGATGTGGGC-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

**P/SM5-10**

Fwd 5′-CAACGATGCCCCTGCCCTTAAG-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

**S/PM7-10**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CTTCATCATGGTGCGTGAGATCAGGGGCTCCTTGGGACTC-3′

Fwd 5′-GAGTCCCAAGGAGCCCCTGATCTCACGCACCATGATGAAG-3′

Rev 5′-AATCAGAAGCTTCGGAATACTCTCACCACTCGGATTGCCAGCAG-3′

**P/SM7-10**

Fwd 5′-GGGAAAGCTAGCGATGGGTGACATGGCGAACAG-3′

Rev 5′-GCCAGCCACTGATCAGAGGTTTGTCCCGGCCATATGG-3′

Fwd 5′-CCATATGGCCGGGACAAACCTCTGATCAGTGGCTGGC-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

**S/PM9-10**

Fwd 5′-GAGTAGCTTAAGAAGGCCGAGATCG-3′

Rev 5′-GATCTTTCGAGCATTGATTTCAGACAGGCTGTTGAGAGCAT-3′

Fwd 5′-ATGCTCTCAACAGCCTGTCTGAAATCAATGCTCGAAAGATC-3′

Rev 5′-AATCAGAAGCTTCGGAATACTCTCACCACTCGGATTGCCAGCAG-3′

**P/SM9-10**

Fwd 5′-CCAGCTCTTAAGAAGGCAGATGTG-3′

Rev 5′-CGCATCAAGGACTGGTTCTCATTGAAAAGCTGCATCATGAC-3′

Fwd 5′-GTCATGATGCAGCTTTTCAATGAGAACCAGTCCTTGATGCG-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

**SM1-2M9-10**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CTCCTCCTCCACTGCCTCCTCCGCCGTTCCGCTCATGCCAAACTC-3′

Fwd 5′-GGAGGCAGTGGAGGAGGAGGGTCTATCTGGCTGCTGGGCTCC-3′

Rev 5′-CTTTTGTTCAAGCTTACGACGTTC-3′

**SM1-2/PM9-10**

Fwd 5′-TCCTTCGCTAGCCACCATGGAAGCTGCTCACTCTAAGTC-3′

Rev 5′-CTCCTCCTCCACTGCCTCCTCCGCCGTTCCGCTCATGCCAAACTC-3′

Fwd 5′-GAGGCAGTGGAGGAGGAGGATCTAACCCCATCTTCTGTACCATTG-3′

Rev 5′-AATCAGAAGCTTCGGAATACTCTCACCACTCGGATTGCCAGCAG-3′

**P/S2bM5-11**

Fwd 5′-CTACACCTTAAGAAAGCTGAGATTGGCATTGCTATG-3′

Rev 5′-CTTTTGCTCAAGAGACCAGAACATATCGCTAAAGTTAGTG-3′

Fwd 5′-TATGTTCTGGTCTCTTGAGCAAAAGCTGATCTCTGAAGAG-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

**P/S2bM11**

Fwd 5′-GAGCAGCTTAAGAAGGCAGATGTGGGC-3′

Rev 5′-GTAGTTGCGGGCCACAAAAGCGATGACCTGTCCCCAGACC-3′

Fwd 5′-CTGGGGACAGGTCATCGCTTTTGTGGCCCGCAACTACCTG-3′

Rev 5′-AGAGATCAGCTTTTGCTCAGACCAGAACATATCGCTAAAG-3′

Fwd 5′-CGATATGTTCTGGTCTGAGCAAAAGCTGATCTCTGAAGAG-3′

Rev 5′-GATCTGTCTAGACTCGAGGGTTACTTGTACAGCTCGTCC-3′

## Appendix 2: Oligonucleotides used in the production of constructs to study the tenth transmembrane domain of SERCA

PCR primers used in the construction of SERCA and PMCA M10 constructs (discussed in chapter 5) are shown. Fwd and rev indicate forward and reverse primers respectively. The 5' and 3' ends of the primers are also indicated.

**SERCA K972F**

Fwd 5′-TGGCTGATGGTTCTGTTTATCTCTCTGCCAGTTATCGGTC-3′

Rev 5′-AACTGGCAGAGAGATAAACAGAACCATCAGCCATTGAGTC-3′

**SERCA M10 3Leu**

Fwd 5′-CTCTCTGCTGCTGCTGCCTGTTATCGGTCTGGACGAAATC-3′

Rev 5′-TAACAGGCAGCAGCAGCAGAGAGATCTTCAGAACCATCAG-3′

**CD8-EGFP**

Fwd 5′-GATCGTGCTAGCACCATGGCCTTACCAGTGACCGC-3′

Rev 5′-GATCCAAAGCTTGACGTATCTCGCCGAAAGG-3′

**CD8 SERCA M10**

Fwd 5′-GATCGTGCTAGCACCATGGCCTTACCAGTGACCGC-3′

Rev 5′-CAGAACCATCAGCCATTGAGTATCACAGGCGAAGTCCAGC-3′

Fwd 5′-GCTGGACTTCGCCTGTGATACTCAATGGCTGATGGTTCTG-3′

Rev 5′-TTAAGCCGCTTGCAGTAAAGTTTCAGGATTTCGTCCAGAC-3′

Fwd 5′-GTCTGGACGAAATCCTGAAACTTTACTGCAAGCGGCTTAA-3′

Rev 5′-GATCCAAAGCTTGACGTATCTCGCCGAAAGG-3′

### CD8 PMCA M10

Fwd 5′-GATCGTGCTAGCACCATGGCCTTACCAGTGACCGC-3′

Rev 5′-CACCAGAGCCACTGTTCTGTATCACAGGCGAAGTCCAGC-3′

Fwd 5′-GCTGGACTTCGCCTGTGATACAGAACAGTGGCTCTGGTG-3′

Rev 5′-CTTAAGCCGCTTACAGTAAAGAGCAATGACCTGTCCCCAGAC-3′

Fwd 5′-GTCTGGGGACAGGTCATTGCTCTTTACTGTAAGCGGCTTAAG-3′

Rev 5′-GATCCAAAGCTTGACGTATCTCGCCGAAAGG-3′

## Appendix 3: SERCA-EGFP and PMCA-EGFP full length sequences

The full-length sequences of the SERCA-EGFP (rabbit SERCA1) and PMCA-EGFP (rat PMCA3) constructs used are shown here. Relevant restriction sites are underlined in red. The start and stop codons are highlighted in yellow and the linker between the calcium pumps and EGFP is shown in blue.

### SERCA-EGFP

GCTAGCGAATTCGAGCTCCCGGGATCCATGGAAGCTGCTCACTCTAAGTCTACTGAAGAATGTCTGGCTTACTTC
GGTGTTTCTGAAACTACTGGTCTGACTCCAGACCAAGTTAAGCGACATCTAGAGAAATACGGCCACAATGAGCTT
CCTGCTGAGGAAGGGAAATCCCTGTGGGAGCTGGTGATAGAGCAGTTTGAAGACCTCCTGGTGCGGATTCTTCTG
CTGGCCGCCTGCATCTCCTTTGTGCTGGCCTGGTTTGAAGAAGGGGAAGAGACCATCACTGCCTTCGTTGAGCCC
TTTGTCATCCTCCTGATCCTCATTGCCAATGCCATCGTGAGAGTTTGGCATGAGCGGAACGCTGAGAACGCCATA
GAGGCGCTGAAGGAATATGAGCCCGAGATGGGGAAGGTGTACCGGGCTGACCGCAAGTCAGTGCAAAGGATCAAG
GCTCGGGACATCGTCCCCGGGGACATCGTGGAGGTGGCGGTTGGGGACAAAGTCCCTGCAGACATCCGCATCCTG
TCTATCAAGTCCACCACCCTCCGCGTGGACCAGTCCATCCTGACAGGCGAGTCCGTGTCCGTCATCAAGCACACG
GAGCCAGTCCCTGACCCGCGGGCTGTCAACCAGGACAAGAAGAACATGCTTTTCTCGGGTACCAACATCGCGGCC
GGCAAGGCCCTGGGCATCGTGGCCACCACCGGCGTGAGCACCGAGATCGGGAAGATCCGTGACCAGATGGCCGCC
ACGGAGCAGGACAAGACGCCGCTGCAGCAGAAGCTGGATGAGTTCGGGGAGCAGCTGTCCAAGGTCATCTCCCTC
ATCTGCGTGGCCGTGTGGCTTATCAACATCGGCCACTTCAACGACCCCGTCCACGGGGGCTCCTGGATCCGCGGT
GCCATCTACTACTTCAAGATCGCCGTGGCCTTGGCTGTGGCTGCGATCCCAGAAGGTCTTCCCGCTGTCATCACT
ACCTGCCTGGCCCTGGGCACCCGCCGGATGGCGAAGAAGAACGCCATCGTGAGGAGCCTGCCCTCTGTGGAGACC
CTGGGCTGCACCTCTGTCATCTGCTCTGACAAGACTGGCACCCTCACCACCAACCAGATGTCTGTGTGCAAGATG
TTCATCATCGACAAGGTGGACGGAGACTTCTGTTCGCTGAACGAGTTCTCCATCACCGGCTCCACCTACGCTCCA
GAGGGGGAGGTCCTGAAGAATGATAAACCCATCCGGTCAGGGCAGTTTGATGGGCTGGTGGAGCTGGCCACCATT
TGTGCCCTGTGCAATGATTCCTCCTTGGACTTCAATGAGACCAAAGGCGTCTATGAGAAGGTGGGTGAGGCCACG
GAGACGGCGCTCACCACTCTGGTGGAGAAGATGAATGTGTTCAACACGGAAGTTCGGAACCTCTCGAAGGTGGAG

AGAGCCAACGCCTGCAACTCGGTGATCCGCCAGCTCATGAAGAAAGAGTTCACCCTGGAGTTCTCCCGAGACAGG
AAGTCCATGTCTGTGTACTGTTCTCCAGCCAAATCTTCCCGCGCTGCTGTGGGCAACAAGATGTTTGTCAAGGGC
GCCCCCGAGGGGGTCATCGACCGCTGTAACTACGTGCGAGTCGGCACCACCCGGGTGCCCATGACTGGGCCGGTG
AAGGAGAAGATCCTCTCCGTGATCAAGGAGTGGGGCACCGGCCGGGACACGCTGCGCTGCCTGGCCCTGGCCACC
CGCGACACGCCGCCCAAGCGAGAGGAAATGGTGCTGGACGACTCCTCCCGGTTCATGGAGTACGAGACGGACCTG
ACGTTCGTGGGCGTCGTGGGCATGCTGGACCCGCCCCGCAAGGAGGTCATGGGCTCCATCCAGCTGTGCCGGGAC
GCCGGGATCCGTGTCATCATGATCACCGGCGACAACAAGGGCACGGCCATCGCCATCTGCCGCCGCATCGGCATC
TTTGGGGAGAACGAGGAGGTGGCAGACCGCGCCTACACCGGCCGCGAGTTTGACGACCTGCCCCTGGCCGAGCAG
CGGGAAGCCTGCCGCCGCGCCTGCTGCTTCGCGCGCGTGGAACCCTCCCACAAGTCCAAGATCGTGGAATACCTG
CAGTCCTACGATGAGATCACGGCCATGACAGGGGATGGCGTCAACGATGCCCCTGCCCTTAAGAAGGCCGAGATC
GGCATAGCTATGGGATCTGGCACCGCCGTGGCCAAGACAGCGTCTGAGATGGTGCTGGCGGACGACAACTTCTCC
ACCATCGTGGCCGCCGTGGAGGAGGGCCGCGCCATCTACAACAACATGAAGCAGTTCATCCGCTACCTCATCTCC
TCCAACGTGGGCGAGGTGGTCTGCATCTTCCTGACGGCCGCCTTGGGGCTGCCCGAGGCCCTGATCCCTGTGCAG
CTGCTGTGGGTGAACCTGGTGACGGACGGGCTCCCGGCCACAGCCCTGGGCTTCAACCCACCAGACCTGGACATC
ATGGACCGGCCCCCCCGGAGTCCCAAGGAGCCCCTGATCAGTGGCTGGCTCTTCTTCCGCTACATGGCCATCGGG
GGCTATGTGGGTGCAGCCACCGTGGGAGCCGCTGCCTGGTGGTTCATGTATGCGGAGGATGGGCCGGGTGTCACC
TACCACCAGCTGACCCACTTCATGCAGTGCACCGAGGACCACCCTCACTTTGAGGGTCTGGACTGTGAGATCTTT
GAGGCCCCAGAGCCCATGACCATGGCCTTGTCTGTGCTGGTGACCATCGAGATGTGCAATGCTCTCAACAGCCTG
TCCGAGAACCAGTCCTTGATGCGGATGCCGCCCTGGGTGAACATCTGGCTGCTGGGCTCCATCTGCCTGTCCATG
TCCCTCCACTTCCTCATCCTCTACGTCGACCCACTGCCAATGATCTTCAAGCTGAAGGCTCTGGACCTGACTCAA
TGGCTGATGGTTCTGAAGATCTCTCTGCCAGTTATCGGTCTGGACGAAATCCTGAAGTTCATCGCTCGTAACTAC
CTGGAAGACCCAGAAGACGAACGTCGTAAGCTTGAACAAAAGCTGATCTCTGAAGAGGACCTACCGGTCGCCACC
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC
CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC
ACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTAC
CCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC
TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAAC
GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGC
AGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCAC
TACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTG
ACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAACTCGAG

**PMCA-EGFP** (before addition of KpnI site)

GCTAGCCTCGAGGATGGGTGACATGGCGAACAGTTCCATTGAGTTCCACCCCAAACCCCAGCAGCAGCGGGAAGT
GCCTCATGTGGGTGGCTTTGGATGCACGCTGGCAGAACTACGCAGCCTCATGGAGCTCCGAGGTGCTGAGGCACT
GCAGAAGATCCAAGAAGCCTATGGGGATGTCAGTGGGCTGTGTAGGAGACTAAAGACCTCACCTACTGAAGGCCT
GGCAGACAACACCAATGACTTGGAGAAACGCAGGCAGATCTATGGGCAGAACTTCATCCCTCCAAAGCAGCCCAA

GACCTTCCTGCAGCTGGTGTGGGAAGCCCTGCAGGACGTGACTCTCATCATCCTGGAGGTGGCTGCTATCGTCTC
CCTGGGCCTCTCCTTCTATGCACCACCTGGAGAGGAAAGTGAAGCCTGTGGGAATGTGTCTGGTGGGGCAGAAGA
CGAAGGAGAGGCCGAGGCTGGCTGGATAGAGGGGGCTGCCATCCTACTCTCTGTCATCTGTGTGGTGCTGGTCAC
AGCCTTCAATGACTGGAGCAAGGAAAAGCAGTTCCGAGGTCTTCAGAGCCGAATTGAGCAGGAACAGAAGTTTAC
TGTCATCCGAAATGGGCAGCTCCTCCAGGTCCCTGTGGCAGCCCTGGTGGTAGGGGACATTGCCCAGGTCAAATA
CGGAGATCTTCTGCCTGCCGATGGTGTGCTCATCCAAGGCAATGACCTCAAGATCGACGAGAGCTCCCTGACCGG
CGAGTCGGACCATGTGCGCAAATCAGCAGACAAAGATCCTATGCTGCTCTCAGGCACTCATGTCATGGAAGGTTC
TGGAAGAATGGTAGTAACAGCTGTTGGTGTGAACTCCCAGACAGGCATCATCTTTACATTGCTTGGGGCAGGTGG
AGAGGAGGAGGAGAAGAAAGACAAGAAAGCTAAGAAGCAGGATGGGGCTGTTGCCATGGAAATGCAGCCCCTGAA
GAGTGCCGAGGGTGGGGAAATGGAGGAGCGGGAAAAGAAGAAAGCCAACGTACCCAAGAAGGAGAAGTCAGTCCT
GCAAGGGAAGCTCACAAAACTGGCTGTGCAGATTGGGAAAGCAGGATTGGTGATGTCTGCTATCACTGTCATCAT
TCTGGTCCTCTACTTTGTGATTGAGACCTTCGTTGTGGATGGCCGGGTGTGGCTGGCAGAGTGCACACCAGTGTA
TGTGCAGTACTTTGTGAAGTTTTTCATTATTGGAGTCACTGTGTTGGTTGTGGCTGTCCCTGAGGGCCTGCCTCT
TGCTGTTACTATCTCCTTGGCTTACTCTGTTAAGAAAATGATGAAGGACAATAACCTGGTACGCCACCTGGATGC
CTGTGAGACCATGGGCAATGCCACAGCCATCTGTTCTGACAAGACAGGCACACTCACCACCAACCGTATGACAGT
GGTTCAGTCCTACCTAGGAGACACCCACTACAAAGAGATTCCAGCTCCCAGCGCCCTGACCCCCAAGATCCTCGA
CCTTCTGGTTCATGCCATCTCCATCAACAGTGCCTACACCACCAAAATTCTACCTCCAGAGAAAGAAGGCGCTCT
CCCACGCCAAGTGGGCAACAAAACAGAGTGTGCTCTTTTGGGCTTCATCTTGGACCTGAAACGTGACTTCCAACC
TGTACGGGAACAGATACCAGAAGATCAGCTTTACAAAGTGTACACCTTCAACTCAGTTCGCAAGTCCATGAGCAC
AGTTATCCGAATGCCTGATGGTGGCTTCCGCCTCTTCAGCAAGGGAGCCTCAGAGATCCTGCTCAAAAAGTGTAC
AAACATCTTAAACAGCAATGGTGAACTCCGAGGATTTCGTCCTCGGGACCGGGATGACATGGTAAAGAAGATCAT
TGAGCCTATGGCTTGTGATGGCCTCCGCACCATATGCATCGCCTACAGGGACTTCTCTGCTATCCAGGAACCGGA
CTGGGACAATGAGAATGAGGTGGTGGGTGACCTTACCTGCATAGCTGTCGTGGGCATCGAGGACCCTGTGCGACC
TGAGGTCCCTGAAGCCATTCGAAAATGCCAGCGTGCTGGCATTACAGTCCGTATGGTAACTGGAGATAACATCAA
CACTGCCCGGGCTATTGCAGCTAAGTGTGGCATCATCCAGCCAGGGGAGGATTTCCTGTGCCTGGAGGGGAAGGA
ATTCAACAGAAGGATTCGAAATGAGAAAGGCGAGATAGAACAGGAGAGGCTGGACAAGGTGTGGCCCAAGCTTCG
GGTGCTTGCCCGGTCATCTCCCACTGATAAACATACTCTGGTCAAAGGCATAATTGACAGCACAACTGGTGAGCA
GCGGCAGGTGGTGGCTGTGACCGGGGATGGCACCAATGATGGACCAGCCCTTAAGAAGGCAGATGTGGGCTTCGC
CATGGGCATCGCAGGCACTGATGTGGCCAAGGAGGCCTCTGACATCATTCTGACTGATGACAACTTCACCAGCAT
TGTCAAGGCGGTCATGTGGGGCCGCAATGTCTATGACAGCATTTCCAAGTTCCTGCAGTTTCAGTTGACAGTCAA
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GCGGAAGCCATATGGCCGGGACAAGCCTCTCATCTCACGCACCATGATGAAGAACATCCTTGGACATGCTGTTTA
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CAGCCAGCTCAAGTGCCTGAAGGAAGCAGGGCATGGGCCTGGGAAGGATGAGATGACTGATGAAGAGTTGGCGGA
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CACCAGCGCTGCTGGCAATCCGAGTGGTGAAAGCATTCCGCTCGAGCAAAAGCTGATCTCTGAAGAGGACCTACC
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GGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATCTAGA

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