University of Southampton

The Targeting of Phospholamban and Sarcolipin to the Endo / Sarcoplasmic Reticulum

By

John Butler

Department of Biochemistry
University of Southampton
UK

February 2005

University of Southampton

Abstract

FACULTY OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

Doctor of Philosophy

The Targeting of Phospholamban and Sarcolipin to the Endo / Sarcoplasmic Reticulum

By John Butler

Phospholamban and sarcolipin are two sarcoplasmic reticulum resident C-terminally anchored transmembrane proteins that are involved in the modulation of sarcoplasmic reticulum calcium ATPases (SERCAs). Very little is known however, about the targeting of these proteins to the sarcoplasmic reticulum and how they are maintained in that cellular location. To examine the targeting of phospholamban and sarcolipin they were tagged with EGFP so that the trafficking of these proteins within the cell could be observed by fluorescence microscopy. Using antibodies against known cellular markers it was shown that both phospholamban and sarcolipin exit the endoplasmic reticulum and enter the ERGIC compartment but do not proceed to the trans-Golgi. This indicates that these proteins are maintained in the endoplasmic reticulum mainly by a process of retrieval and not retention. In addition mutagenesis of the transmembrane domains of phospholamban and sarcolipin led to a loss of the retention signal indicating that the transmembrane domains play a major role in the retention process. Mutagenesis of one of the positively charged residues flanking the transmembrane domain resulted in a inversion of topology of sarcolipin demonstrating that these flanking residues are involved in orientating this tail anchored protein during its insertion into the endoplasmic reticulum.

Acknowledgements

At last it is complete! I just want to say thank you to everybody who helped me to get here.

The biggest thanks has to go to my supervisor Dr. Malcolm East for all the help, support and knowledge that he has given me over the last 3 years. Another huge thanks has to go to Dr. Matt Cuttle for the help with the confocal microscopy.

I figure I should also mention a few people who have made me laugh and chuckle and laugh until I can laugh no more as well as help out with some of the molecular biology, you guys have made this experience a very pleasant one. So thank you in no particular order to Sanjay, Andy, Jo, Simon, Liz, Gordon, Steve, Nicky, Karen, Mich, Rob, Trish, Jeab, Phedra, Sam, Aimee, Ian, Verity, Abi, Leighton, Emma, Charlie and Lucy (Sorry if I have forgotten anyone).

I would also like to thank Barbara for all her technical assistance and guidance especially at the beginning of my PhD. I would also like to make a special mention to Loraine, Bridgette and Dianne in the office(s) for all their help over the last 3 years.

I should just mention a few people outside of Boldrewood who have kept me amused and provided endless humour and entertainment whilst doing my PhD. So thank you to Gav, Hodgy, Stav, OB1, Harriet, Kev, Wayne, Steve D, Kat, and all the many others you know who you are!!!

I am most indebted to my family who have supported me and pushed me at the right times to get on with it! Special thanks to Mum, Dad, Darren and Granddad for well just being there and not asking too many complicated questions about what it is that I actually do.

I dedicate this thesis to the memory of my Nan who sadly died in 1998

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
APS	Ammonium persulphate
BSA	Bovine Serum Albumin
Ca^{2+} ATPase	Calcium and Magnesium activated ATPase
COP I and COP II	Coat proteins I and II
DTT	Dithiothrietol
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green-fluorescent protein
ER	Endoplasmic reticulum
ERGIC	ER/Golgi intermediate compartment
GFP	Green fluorescent protein
Kb	Kilo base
KDa	Kilo Dalton
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Triton X-100 (0.01%)
PCR	Polymerase chain reaction
PLB	Phospholamban
PMCA	Plasma membrane Ca^{2+} ATPase
SDS	Sodium dodecyl sulphate
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SLN	Sarcolipin
SR	Sarcoplasmic reticulum

SRP	Signal-recognition particle
SRPR	Signal-recognition particle receptor
SDW	Sterile distilled water
TAE	Tris-acetate EDTA buffer
TEMED	N,N,N',N'- Tetramethylethylenediamine
TGN	Trans-Golgi network
Tris	Tris(hydroxymethyl)aminomethane
T-tubule	Transverse tubule
ARF	ADP ribosylation factor
GEF	Guanine nucleotide exchange factor
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate

Contents

Chapter one introduction

Chapter Three:

Localisation and orientation studies of sarcolipin and phospholamban EGFP fusion proteins expressed in COS 7 cells.

List of figures and tables

Figures

Tables

1.1 Introduction

Eukaryotic cells contain a number of specialised organelles (Toole and Toole, 1995). Proteins synthesised by the ribosomes in the cytoplasm need to be targeted to these organelles in order to maintain them (Alberts *et al.*, 1994). This thesis deals with the targeting of two proteins phospholamban and sarcolipin to the endoplasmic reticulum of cells. Both phospholamban and sarcolipin play a very important role in Ca^{2+} signalling (MacLennan and Kranias, 2003). These two proteins are different from many other endoplasmic reticulum membrane spanning proteins in that they are C-terminally anchored. Phospholamban and sarcolipin are also very short proteins and because of this their targeting to the endoplasmic reticulum is quite different from that of the majority of endoplasmic reticulum membrane spanning proteins. The difference lies in the targeting of phospholamban and sarcolipin to the endoplasmic reticulum. Phospholamban and sarcolipin are not co-translationally inserted into the endoplasmic reticulum (Keenan *et al.*, 2001). They appear to use a post-translational insertion route involving cytoplasmic chaperones (Wattenberg and Lithgow, 2001). Cross-linking studies by Abell *et al.*, (2003) and Abell *et al.*, (2004) suggests that the signal recognition particle (SRP) may be involved in the insertion of C-terminally anchored membrane proteins like phospholamban and sarcolipin. The functions of phospholamban and sarcolipin have been widely studied, see review (East, 2000). However the mechanism(s) by which these proteins are directed to and maintained in the endoplasmic reticulum has not been addressed. The focus of this thesis is to study the targeting and maintenance of phospholamban and sarcolipin in the endoplasmic reticulum.

1.1.1 Phospholamban and sarcolipin regulators of Ca^{2+} ATPases

Phospholamban and its homologue sarcolipin are endogenous regulators of calcium ATPases located in the sarcoplasmic reticulum of muscle cells (MacLennan and Kranias, 2003). The sarcoplasmic reticulum plays a pivotal role in the contraction and relaxation of muscle cells. This is achieved by regulating intracellular calcium levels. The calcium pumps located in the sarcoplasmic reticulum (SR) are the proteins primarily responsible for the reaccumulation of calcium into the sarcoplasmic reticulum after muscle contraction. As the calcium levels in the sarcoplasm fall the muscle returns to its relaxed state. Phospholamban and sarcolipin are structurally very similar proteins and they both interact with calcium ATPases in a similar way (MacLennan and Kranias, 2003). Their primary role is to inhibit these pumps and thereby reduce the amount of calcium pumped. Phospholamban is mainly expressed in cardiac muscle along with the calcium pump SERCA2a (Toyofuku *et al.*, 1993). SERCA2a is not only responsible for cardiac muscle relaxation but also is the major determinant of the amount of calcium stored in the sarcoplasmic reticulum lumen. The amount of calcium stored in the sarcoplasmic reticulum directly influences the strength of cardiac contraction since calcium release from the sarcoplasmic reticulum is responsible for triggering contraction (MacLennan and Kranias, 2003). Phospholamban regulates SERCA2a by direct protein-protein interactions (Hutter *et al.*, 2002). Chen *et al.*, (2003) performed cross-linking studies where residues 27 and 30 of phospholamban were mutated from asparagine to cysteine. These residues cross-linked to lysine 328 and cysteine 318 of SERCA2a respectively. Cross-linking with phospholamban was favoured with SERCA2a in the E2 ATP bound state whereas cross-linking was abolished with micro molar levels of Ca^{2+} and by the

calcium pump inhibitor thapsigargin. This study indicated that phospholamban appears to interact favourably with SERCA2a in the Ca^{2+}-free, E2 state, with a strong preference for the nucleotide-bound, E2 state. Phospholamban's interaction with SERCA2a lowers the pumps apparent affinity for calcium and thus reduces calcium pumping. Phosphorylation of phospholamban (which occurs during β- adrenergic stimulation) and the varying calcium concentration are 2 major factors controlling the degree of inhibition caused by phospholamban. There are also reports that sarcolipin may enhance phospholamban's inhibitory effects on SERCA by stabilizing the inhibitory complex formed between phospholamban and SERCA (Asahi *et al.*, 2002). Sarcolipin also seems to be important in fast twitch skeletal muscle where it is co-expressed with SERCA1a. Sarcolipin regulates both SERCA1a and SERCA2a by lowering calcium affinity and reducing V_{max} (Odermatt *et al.*, 1998). Sarcolipins inhibition is not controlled by phosphorylation, as there are no sites for phosphorylation to occur. The mechanism(s) by which the influence of sarcolipin is controlled is not known, but presumably levels of sarcolipin may be controlled by the levels of transcription, translation or protein removal (Odermatt *et al.*, 1998).

1.1.2 The structure of cardiac muscle

The myocytes are the cells responsible for cardiac contraction. The myocyte, a highly specialized cell, is made up of many membrane bound organelles (see figure 1). It is surrounded by a basement membrane made up of collagen fibres, glycoproteins, laminin, fibronectin and proteoglycans (Walker and Spinale, 1999). The basement membrane has

2 main functions; it provides an initial barrier that influences the exchange of macromolecules between the myocyte and the extra cellular matrix and it provides an interface for myocyte adhesion (Walker and Spinale, 1999).

The next boundary is the sarcolemma or plasma membrane. The sarcolemma forms 2 specialized structures, the intercalated disks and the transverse tubules (Alberts *et al.*, 1994). Transverse tubules are invaginations of the sarcolemma into the myocyte, which forms a barrier between extracellular and intracellular sides. These transverse tubules contain L-type Ca^{2+} channels (dihydropyridine receptors) that are voltage sensitive. The intercalated disks allow a pathway of low resistance for the rapid conduction of an action potential between the cardiac myocytes. The disks also provide a strong mechanical linkage between myocytes.

The sarcoplasmic reticulum is an intracellular membrane network highly specialized for Ca^{2+} handling. This membrane forms specialized structural regions within the myocyte in close proximity with the sarcolemma, particularly the transverse tubule system. The sarcoplasmic reticulum is the primary source for Ca^{2+} in excitation – contraction coupling and contains 3 important components for calcium homeostasis: the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2), the regulatory protein of SERCA phospholamban and the Ca^{2+} release channel (ryanodine receptor) (Stokes, 1997; Voss *et al.*, 1994).

All mammalian hearts are said to be myogenic, that is, the heart contraction is initiated from within the heart muscle itself rather than from an external nervous impulse (Toole and Toole, 1995). The initial stimulus for the human heartbeat originates in a group of histologically different cardiac muscle cells known as the sino-atrial node

(Toole and Toole, 1995). The sino-atrial node is located in the wall of the right atrium near where the vena cava enters the heart. The action potential is released across both atria causing them to contract more or less at the same time. The wave of excitation reaches a similar group of cells called the atrio ventricular node that lays between the two atria. To allow blood to be forced upwards into the arteries, the ventricles of the heart need to contract from the apex of the heart upwards. This is achieved as the wave of excitation is conducted from the atrio ventricular node along Purkinje fibres collectively known as the bundle of His. The Purkinje fibres conduct the impulse to the apex of the heart causing the two ventricles to contract simultaneously. The contraction starts at the apex of the heart and is transmitted upwards along the Purkinje tissue forcing blood into the arteries (see figure 2).

As the wave of depolarisation reaches the cell the depolarisation travels down the transverse tubules (Walker and Spinale, 1999). There the voltage sensitive L-type Ca^{2+} channel (dihydropyridine receptor) opens allowing Ca^{2+} to enter the myocyte. This small amount of Ca^{2+} is not enough to trigger cellular contraction (Lamb, 2000) but instead causes the opening of adjacent ryanodine receptors of the sarcoplasmic reticulum (Stokes and Wagenknecht, 2000). Such discrete openings give rise to localized regions of calcium release sometimes referred to as calcium sparks (Marx *et al.*, 2000). This mechanism whereby the dihydropyradine receptor allows low levels of extra cellular Ca^{2+} to enter the cell which in turn triggers the ryanodine receptors to open is termed calcium induced calcium release (Stokes and Wagenknecht, 2000; MacLennan, 2000). The transverse tubule and the ryanodine receptor of the sarcoplasmic reticulum are spatially organized in to triad junctions; that are visible by electron microscopy (Stokes and Wagenknecht,

2000). The sum of the calcium sparks results in a huge increase in the cytoplasmic Ca^{2+} concentration that is the key signal for muscle contraction.

1.1.3 Muscle contraction

Muscle contraction itself is primarily controlled by 2 proteins troponin and tropomyosin (Alberts *et al.*, 1994). These 2 proteins associate closely with the actin filaments of the muscle fibre and act as molecular switches. These proteins act as switches and only allow muscle contraction to occur when the Ca^{2+} concentration reaches a high enough level. Tropomyosin is a rod shaped molecule that binds to the actin helix. Troponin is a complex made up of 3 polypeptides troponins T, I and C named for their tropomyosin binding, inhibitory and calcium binding activities. Subunits I and C form a globular head region and subunit T forms a long tail region that binds to tropomyosin. It is this subunit that is thought to be responsible for binding the whole complex to the thin actin filament. Troponin C is the Ca^{2+} sensing subunit and can bind up to 4 Ca^{2+} ions. Once all 4 binding sites are occupied inhibition between actin and myosin is reduced allowing contraction to occur (Alberts *et al.*, 1994). Troponin I is responsible for causing tropomyosin molecules to bind to actin in such a position that occupies the binding sites for myosin heads thus contraction cannot occur. When elevated Ca^{2+} levels result troponin C undergoes a conformational change which in turn is transmitted to troponin T causing tropomyosin to move enough for myosin heads to bind to their once blocked sites. Once the myosin heads can bind muscle contraction can occur. The actual process of muscle contraction

relies on the hydrolysis of ATP to power the interactions of the myosin heads with the actin filament.

After muscle contraction has taken place an equally important process must occur relaxation of the muscle. Relaxation of the muscle occurs when the level of calcium in the cytoplasm decreases to resting levels. Ca^{2+} may be extruded from the cell across the sarcolemma into the extra cellular space, but most of it is pumped back into the sarcoplasmic reticulum stores. Relaxation within muscle cells is mainly dependent upon SERCA2a, which is a member of the P-type ATPase family (Carafoli, 1994). For each molecule of ATP hydrolysed, 2 ions of Ca^{2+} are transported back into the sarcoplasmic reticulum. The function of SERCA2a and the regulatory state of phospholamban significantly influences the active relaxation process within the myocyte. Other proteins involved in the removal of Ca^{2+} from the cytosolic compartment include the Na^{+} / Ca^{2+} exchanger. This exchanger has a low affinity for Ca^{2+} but high capacity. For each Ca^{2+} removed 3 Na^{+} ions are exchanged. The sarcolemmal Ca^{2+} ATPase and additional Ca^{2+} binding proteins including calmodulin and calsequestrin also play a role in Ca^{2+} removal. The complex formed by binding of cytosolic Ca^{2+} to calmodulin can activate the sarcolemmal Ca^{2+} ATPase to extrude cytosolic Ca^{2+} (Feher and Lebolt, 1990; Sheu *et al.*, 1986; Walker and Spinale, 1999). Calsequestrin, located in the endoplasmic reticulum can bind up to 40 Ca^{2+} ions (Wang *et al.*, 1998). This has the effect of increasing the Ca^{2+} storage capacity of the endoplasmic reticulum and also means that more Ca^{2+} can be removed from the cytosol (Feher and Lebolt, 1990).

1.1.4 Phospholamban structure

Phospholamban has 52 amino acid residues, corresponding to a molecular weight of approximately 6.1 kDa (Buck *et al.*, 2003). The amino terminal is acetylated (Fujii *et al.*, 1987). Circular dichromism indicates that phospholamban has a structure which is 78% α-helical and 22% β-sheet (Simmerman *et al.*, 1989; Tatulian *et al.*, 1995). Phospholamban also appears to be highly conserved between species with the chicken form having 85% homology to its mammalian counterparts (see figure 3) (Arkin *et al.*, 1997).

Structural information on phospholamban comes mainly from 4 sources nuclear magnetic resonance (NMR) (Ahmed *et al.*, 2000; Buck *et al.*, 2003), circular dichromism (Simmerman *et al.*, 1989), mutational studies (Toyoshima *et al.*, 2003; MacLennan and Kranias, 2003) and fourier transform infra red spectroscopy (FTIR) (Tatulian *et al.*, 1995). NMR structures of phospholamban show it to consist of two helices spanning residues 4-16 and 21-49. These helices are connected by a short β turn (Mortishire-Smith *et al.*, 1998; Pollesello *et al.*, 1999; Lamberth *et al.*, 2000). Phospholamban can be divided into 2 distinct domains firstly a cytosolic hydrophilic domain (methionine 1 to arginine 25) containing two sites of phosphorylation (serine 16 and Threonine 17). The second domain is a membrane spanning hydrophobic stretch (glutamine 26 to leucine 52). The hydrophilic cytoplasmic domain can be further subdivided into 2 subdomains (see figure 4). Domain 1A (methionine 1 to proline 21) forms an amphipathic α helix broken at proline 21. Domain 1B (asparagine 22 to asparagine 30) could act as a hinge between the cytoplasmic and membrane spanning regions which may allow a change in

the angle between the 2 domains upon phosphorylation of phospholamban (Simmerman *et al.*, 1989).

Phospholamban shows a strong tendency to aggregate into homopentamers. The interactions between the phospholamban monomers are so strong that pentamers still form even in sodium dodecyl sulphate (SDS). Only upon boiling the protein in this detergent do the pentamers monomerise. This can be seen using SDS polyacrylamide gel electrophoresis (PAGE) where using different temperatures prior to separation reveal dimers, trimers and tetramers (Fujii *et al.*, 1986). Phosphorylation of phospholamban pentamers by protein kinase A at serine 16 shows pentameric species with up to 5 phosphates indicating that all phospholamban monomers are capable of being phosphorylated in the pentameric form. Phospholamban can also be phosphorylated at threonine 17 by Ca^{2+}/calmodulin dependent protein kinase (Colyer and Wang, 1991; Wegener *et al.*, 1989). These kinases are responsible for the modulation of the interaction between phospholamban and calcium pump, which will be discussed later. Oligomerisation of phospholamban is probably driven by the hydrophobic transmembranous domain 2, as tryptic digests of phospholamban which remove the cytoplasmic region of phospholamban still yield pentamer fragments (Wegener *et al.*, 1986). The pentameric structure of phospholamban is stabilised by cysteine residues in the transmembrane region. Mutagenesis of cysteine residues 36, 41 and 46 have shown that these residues stabilise the formation of pentamers (Fujii *et al.*, 1986). The most important of these cysteine residues was residue 46 which when removed reduced pentamer formation. The cysteine residues are thought to exist as free SH groups rather than disulphide bonds and it is hydrogen bonding between these cysteine residues that

stabilises pentamer formation (Simmerman *et al.*, 1986). Young *et al.*, (1989) investigated the oligomeric state of phospholamban in cardiac sarcoplasmic reticulum. However, only monomeric and dimeric forms of phospholamban were found with no evidence of pentameric species.

Phospholamban is expressed mainly in cardiac muscle where its role is to regulate SERCA2a. Phospholamban is expressed at lower levels in smooth muscle and some types of slow twitch skeletal muscle (Lalli *et al.*, 1997; Slack *et al.*, 1997). Aortic and bladder tissues deficient in phospholamban were less responsive to added agonists compared to wild type muscle (Lalli *et al.*, 1997; Nobe *et al.*, 2000). Phospholamban is also present in vascular endothelium and here it seems to regulate the endothelium-dependent relaxation of the aorta (Sutliff *et al.*, 2000). In soleus muscles removal of phospholamban was associated with significant increases in relaxation rates, without any effects upon contractility. So it appears that phospholamban's role in non-cardiac muscle appears to be involved with regulation of contractility.

1.1.5 Calcium pumps (SERCAs)

Calcium pumps are the proteins primarily responsible for the relaxation of muscle cells (Toyoshima *et al.*, 2000), the sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCAs) being the most important in cardiac and skeletal muscle relaxation (Walker and Spinale, 1999). The sarcolemmal Ca^{2+} ATPase (PMCA) though responsible for pumping Ca^{2+} out of the cell has only a minor role to play in muscle relaxation (Walker and Spinale, 1999). Both SERCAs and PMCAs are members of the P-type ATPase family of ion pumps; so

called due to phosphorylation of an aspartate residue during the catalytic cycle. The energy required to pump Ca^{2+} against an electrochemical potential gradients is supplied by the hydrolysis of the terminal phosphate bond of ATP (Lee and East, 2001).

Original structural information on SERCA came from many fields but the most dramatic breakthrough came with the crystal structure of SERCA1a in the E1 conformation (Toyoshima *et al.*, 2000) followed by the crystal structure of this pump in the E2 conformation (Toyoshima and Nomura, 2002). The crystal data obtained enabled the examination of the calcium pump in its two conformational extremes. This was followed by crystal data showing SERCA1a cytoplasmic head groups and transmembranous regions in different positions during the catalytic cycle for a detailed explanation see (Toyoshima and Mizutani, 2004). These structures have provided insight into the mechanisms of Ca^{2+} pumping (see figures 6A and 6B); see (Toyoshima and Nomura, 2002) for a detailed explanation. In actual fact there are 3 different genes encoding for 3 very closely related SERCAs (Moller *et al.*, 1996). Differential splicing of these primary transcripts gives rise to at least 7 SERCA isoforms. However the general structure of the pumps appears to be the same. Studying the crystal structure of SERCA1a shows the pump to have 3 cytoplasmic domains and a membrane-spanning domain. The cytoplasmic domains are labelled N domain (nucleotide binding domain), P domain (phosphorylation domain) and an A domain (actuator domain) (see figure 5). The N domain contains the ATP binding site. Residues critical in maintaining this site are phenylalanine 487, lysine 515 and lysine 492 these residues are thought to form a positively charged pocket in the N domain that the negatively charged phosphates of ATP will bind to. The ATP binding site is approximately 20-30 Å away from aspartate 351,

which becomes phosphorylated in the catalytic cycle. This means a conformational change must occur to bring the N domain and the P domain into close proximity (Mcintosh *et al.*, 1992). All 3 domains are linked by flexible loop regions, which allow the coming together of the 2 domains so that phosphorylation can occur.

The M4, M5, M6 and M8 helices surround the two Ca^{2+} binding sites. The two sites are at similar heights with respect to the membrane and are approximately 5.7 Å apart (Toyoshima *et al.*, 2000). Site 1 is located between M5 and M6 helices with residues from M8 contributing. Crystallographic images show the oxygen atoms from residues asparagine 768, glutamate 771, threonine 799, aspartate 800 and glutamate 908 to contribute to Ca^{2+} binding. Site 2 is formed by the main chain carbonyl groups from the M4 helix. Oxygen atoms from residues valine 304, alanine 305 and isoleucine 307 are mainly responsible for the Ca^{2+} binding. The calcium pump is known to have 2 conformations. The E1 conformation has a high affinity for binding calcium from the cytoplasm. Once Ca^{2+} has bound so does ATP. Phosphorylation of aspartate 351 then follows causing a change in the conformation of the pump from E1 to E2. The E2 conformation has a lower affinity for Ca^{2+} and the binding sites now face the lumen of the sarcoplasmic reticulum. The Ca^{2+} is thus released into the lumen of the sarcoplasmic reticulum.

The pumping mechanism of the calcium pump requires significant conformational changes within the pump (Moller *et al.*, 1996; Toyoshima and Nomura, 2002) (See figure 7 A and 7 B for calcium pump conformations). The P1 and P2 helices in the P domain contact the M6-M7 loop linking phosphorylation to Ca^{2+} binding (Lee and East, 2001). Phosphorylation of aspartate 351 leads to movement in the P1 and P2 helices. The reason

for this is that aspartate 351 is ionically bonded to lysine 684, which is linked directly to helix P1. Once phosphorylation takes place the ionic bond is broken moving the P1-P2 loop. This in turn contacts the M6-M7 loop and causes them to move (Lee and East, 2001). The shift in structure results in the movement of the calcium binding sites from facing the cytoplasm to facing the lumen of the sarcoplasmic reticulum (E1 conformation converts to the E2 conformation). Once this occurs Ca^{2+} can be released in the lumen of the sarcoplasmic reticulum as the E2 conformation has less affinity for the calcium ions. Pumping is complete with the hydrolysis of the phosphate from aspartate 351 allowing the pump to return to its original E1 conformation. Both phospholamban and sarcolipin play roles in modifying the way in which the calcium pump functions and as a result can alter muscle contraction and relaxation.

1.1.6 Phospholamban interactions with the calcium pump

The first evidence of a physical interaction between phospholamban and SERCA2a came from cross-linking studies. Lysine 3 in phospholamban domain 1a can be cross-linked to lysine 397 and 400 of the N domain of the calcium pump (James *et al.*, 1989). These interactions between phospholamban and SERCA can be disrupted either by elevation of the Ca^{2+} concentration or by phosphorylation of phospholamban at serine 16 and threonine 17 (James *et al.*, 1989). In the cycle of Ca^{2+} pumping several inter convertible phosphorylated and unphosphorylated conformations of SERCA have been defined (see figure 6). The important conformation of SERCA for phospholamban inhibition is E_2. Phospholamban asserts its inhibitory effect by binding to SERCA2a in its E_2

conformation and prolonging the time that SERCA2a spends in this conformation (Cantilina *et al.*, 1993). Either phosphorylation of phospholamban or the conformational changes of SERCA2a upon Ca^{2+} binding and progression from E2 to the E1.2 Ca^{2+} state cause dissociation of phospholamban from SERCA. Mutagenesis studies have been used to study the interaction of SERCA1a and SERCA2a with phospholamban in more detail. SERCA1a and SERCA2a have been used interchangeably in both the mutagenic and structural studies as phospholamban has been reported to inhibit both isoforms equally (Asahi *et al.*, 1999; Toyoshima *et al.*, 2003). Phospholamban interaction with SERCA is preferentially studied using an assay which measures Ca^{2+} dependence of Ca^{2+} transport in isolated sarcoplasmic reticulum vesicles (Kirchberger *et al.*, 1972). By varying the Ca^{2+} concentration over four orders of magnitude and calculating the rate of ATP-dependent Ca^{2+} transport relative to the maximal rate observed at 10 μM Ca^{2+} (V_{MAX}), the apparent affinity of SERCA for Ca^{2+} can be determined (MacLennan and Kranias, 2003). In early studies the addition of cAMP and PKA increased both the rate and the extent of Ca^{2+} uptake in cardiac sarcoplasmic vesicles (Kirchberger *et al.*, 1972; Katz, 1998). The effect of PKA and cAMP was to cause phosphorylation of phospholamban and thus cause dissociation of phospholamban from SERCA thus removing its inhibitory effect. Kinetic analysis showed that the principal effect of unphosphorylated phospholamban association with SERCA was to diminish the apparent affinity of SERCA for Ca^{2+}, with little effect on V_{MAX} at non-limiting ATP and Ca^{2+} concentrations. Kimura *et al.*, (1998) designed and built phospholamban domain 1 / transmembrane cytochrome b5 chimeras. It was demonstrated using these chimeras that the transmembrane region of phospholamban was responsible for inhibition of SERCA2a. The cytoplasmic regions of phospholamban in

this report seemed to regulate the inhibition rather than add to it. However, (Hughes *et al.*, (1994) and Hughes *et al.*, (1996) showed that the hydrophilic cytoplasmic domain of phospholamban seemed to play an inhibitory role on SERCA so this remains controversial.

Toyoshima *et al.*, (2003) performed cross-linking studies between phospholamban and SERCA1a. This provided reference points for molecular modelling of the phospholamban and SERCA interaction (see figure 8A and 8B). In the E2 conformation of SERCA1a a groove is formed in the lipid-facing surface of the transmembrane domain. The carboxyl terminal transmembrane helix of phospholamban can fit into this groove and form interaction sites with amino acids in transmembrane helices M2, M4, M6 and M9 of SERCA1a. Phospholamban is prevented from continuing as a helix into the cytoplasm by the M4 helix of SERCA. Instead phospholamban is thought to unwind and form a second amino terminal helix. This can fit into a groove in the cytosolic nucleotide-binding domain of SERCA1a. Phospholamban would be stabilised in this position by hydrophobic interactions and an ionic bond (MacLennan and Kranias, 2003). The unstructured section of phospholamban is unwound; this would enable kinases to access the phosphorylation sites on phospholamban. Further unwinding of phospholamban at the carboxyl end of the protein would enable phosphorylation sites at serine 16 and threonine 17 to become accessible. As SERCA1a moves to the E1 $2Ca^{2+}$ conformation the M2 helix moves pushing out phospholamban. This disruption to the phospholamban-SERCA1a interaction terminates the ability of phospholamban to inhibit SERCA. It is also thought that movement of the N domain could cause dissociation of

phospholamban from SERCA once the strong interactions are disrupted. Phospholamban may leave SERCA and arrange itself into a pool of homo pentamers in the sarcoplasmic reticulum membrane.

1.1.7 Sarcolipin structure and function

Sarcolipin has 31 amino acid residues, corresponding to a molecular weight of approximately 3.7 kDa. Sarcolipin co-purifies with the fast twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase (SERCA1) (Asahi *et al.*, 2002). Sarcolipin's 31 amino acid residues share considerable homology to phospholamban (Odermatt *et al.*, 1997). This homology with phospholamban suggests that the first 7 hydrophilic amino acid residues are cytoplasmic. The next 19 amino acid residues are hydrophobic and probably form a single transmembrane helix, which spans the sarcoplasmic reticulum membrane. The last 5 residues are hydrophilic and thought to be lumenal. Figure 4 shows a comparison of sarcolipin and phospholamban structure. It shows how the two proteins are thought to span the membrane of the sarcoplasmic reticulum. Identical residues are shown in red and conservative substitutions are shown in green. It can be easily noted that the membrane spanning regions show high levels of similarity. NMR structures of sarcolipin have been generated by (Mascioni *et al.*, 2002) (see figure 9). The NMR structure shows the 16 lowest energy structures superimposed on one another. The C_α atoms from residues 9 – 27 are shown. In this structure it can be seen that the cytoplasmic N-terminus is very flexible along with the lumenal C-terminus. The α-helical probable transmembrane region however appears to be much more ordered as it transverses the

sarcoplasmic reticulum membrane. Mascioni *et al.*, (2002) also carried out NMR experiments on (SLN-M_{32}DYKDDDDK$_{40}$) a modified version of sarcolipin which has been tagged to give it a highly negative charge at the C-terminus. This tagged protein is reported to have a super inhibitory effect on SERCA1 (Odermatt *et al.*, 1998). This mutant was found to adopt the same structure as wild type sarcolipin. This indicates that perhaps the lumenal part of sarcolipin may play a role in inhibition of SERCA1.

Sarcolipin is not only expressed in fast twitch skeletal muscle but to a lesser extent in slow twitch muscle and at even lower levels in cardiac muscle (Odermatt *et al.*, 1997). Recently however there are reports that sarcolipin expression in the heart is confined to the atria where the level is similar to that seen in fast twitch skeletal muscle (Gayan-Ramirez *et al.*, 2000; Minamisawa *et al.*, 2003b). Sarcolipin interacts with SERCA1 to modulate its activity (Odermatt *et al.*, 1997; Asahi *et al.*, 2002). It has been found that sarcolipin regulates SERCA1a by lowering Ca^{2+} affinity and increasing the V_{max} at high Ca^{2+} concentrations (Odermatt *et al.*, 1998). In addition sarcolipin is able to inhibit SERCA2 by inducing a super inhibitory effect on phospholamban. This is achieved by sarcolipin binding to phospholamban and preventing it from oligomerising into pentamers. This results in more of the monomeric form of phospholamban available for inhibition (Asahi *et al.*, 2002). Co-immuno precipitation studies carried out by Asahi *et al.*, (2003) using alanine substituted single amino acid mutants of phospholamban or sarcolipin determined that the binding site of SERCA1a with sarcolipin is the same as with phospholamban. The modelling suggested that the cavity is formed by transmembrane helices M2, M4, M6 and M9 (figure 8A, B). SERCA1a in the E_2 conformation can accommodate both phospholamban and sarcolipin helices; however

phospholamban has a stronger affinity for SERCA than sarcolipin. Sarcolipin may however have additional interactions with the SERCA phospholamban complex and help in stabilizing this complex. Sarcolipin can therefore inhibit SERCA by direct interaction or through stabilising phospholamban's interaction with SERCA2a (Asahi *et al.*, 2003). Sarcolipin's role in the sarcoplasmic reticulum of skeletal muscle is still unclear, although there are reports of sarcolipin causing slippage and leak increase by the Ca^{2+} ATPase of the sarcoplasmic reticulum (Smith *et al.*, 2002). Sarcolipin could have a role in the sarcoplasmic reticulum by promoting non-shivering thermogenesis to occur (Block *et al.*, 1994; Block, 1994). In animals lacking brown adipose tissue the main source of heat production via non-shivering thermogenesis is the hydrolysis of ATP by SERCA1a of skeletal muscle (Block *et al.*, 1994). Ca^{2+} ion transport by SERCA is not 100% efficient; any energy not used to pump Ca^{2+} is given of as heat. Sarcolipin is reported to reduce pumping efficiency by increasing slippage and leak (Smith *et al.*, 2002). If the pump's efficiency at pumping Ca^{2+} ions is decreased then the energy usually available for pumping is given off as heat instead. This idea of sarcolipin being important in non-shivering thermogenesis could also explain its distribution in different muscles. For example if certain muscles were more important in non-shivering thermogenesis then a higher level of expression would expect to be seen in these muscles. It has been postulated that sarcolipin mutations / over expression might account for muscle relaxation defects (Odermatt *et al.*, 1997; Tupling *et al.*, 2002). Brody disease is a genetic disease whereby an exercise-induced impairment of muscle relaxation occurs. The disease is usually caused by mutations in the SERCA1 gene but some individuals with normal SERCA1 genes and normal SERCA1 expression still developed the disease. A

search for mutations in the sarcolipin gene was launched, as this gene was perceived as a possible cause of non-SERCA related Brody disease (Odermatt *et al.*, 1997). No mutations in the coding, splicing or promoter sequences were found in the sarcolipin genes of patients with non-SERCA related Brody disease (Odermatt *et al.*, 1997). Alterations in sarcolipin expression levels in these patients could not be ruled out. However, it is not possible to assay sarcolipin expression due to its size and hydrophobicity. (There are no antibodies to detect native sarcolipin). The possibility also exists that the expression levels of sarcolipin may be affected by mutations outside of the coding regions.

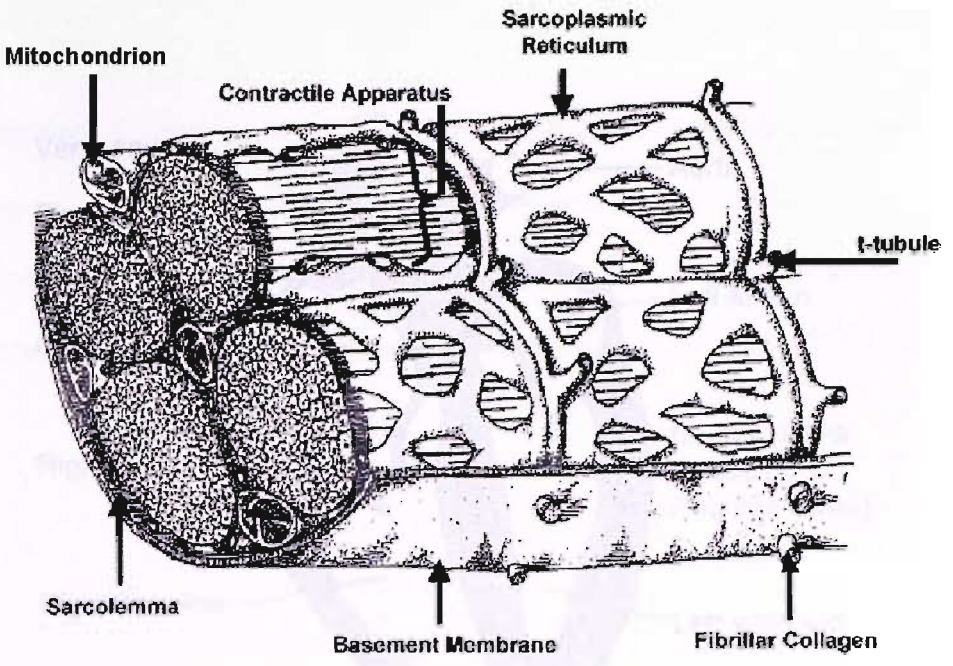

Figure 1

The structure of a human cardiac myocyte

This diagram shows the basic structure of a cardiac muscle cell. The sarcoplasmic reticulum can clearly be seen as a specialised organelle surrounding the contractile apparatus. The sarcoplasmic reticulum wraps around the actin and myosin filaments in very close proximity. This allows quick release and re-uptake of Ca^{2+} ions for muscle contraction and relaxation. The t-tubules can be seen penetrating the sarcolemma these are important in allowing the wave of depolarisation into the muscle and causing myocyte contraction. Modified from Walker and Spinale (1999).

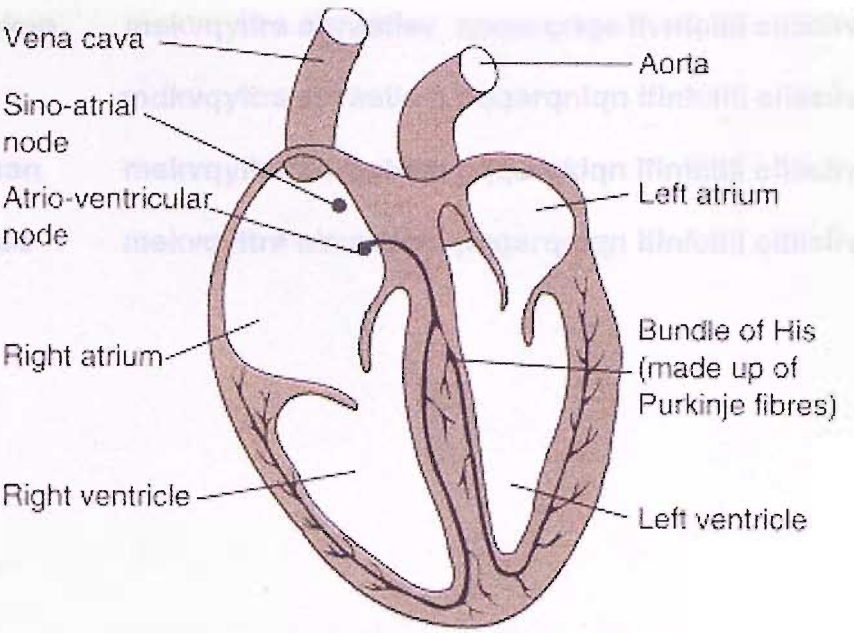

Figure 2

The structure of the mammalian heart

The diagram shows the main feature of the mammalian heart. The sino-atrial node is a specialised group of cells that are electrically active and are responsible for initiating depolarisation of the heart. The wave of depolarisation spreads across the two atria causing contraction filling the ventricles with blood. The atrio-ventricular node is activated once the depolarisation has reached this point. The atrio-ventricular node then relays the electrical stimulation to the ventricles of the heart via the Purkinje fibres. This then causes the ventricles to contract, forcing blood to the lungs and to the rest of the body. Taken from Toole and Toole (1995).

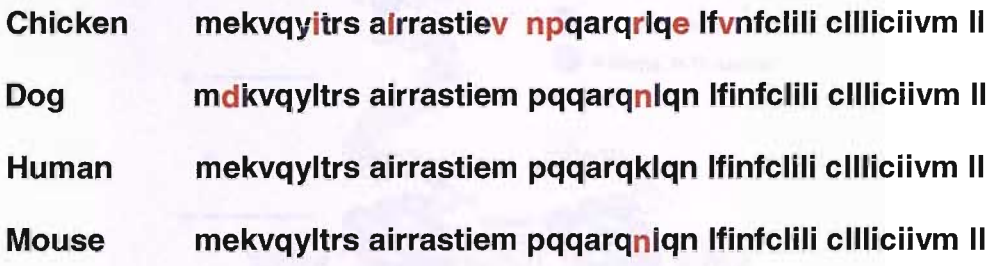

Figure 3

Sequence comparisons for phospholamban from 4 different species

The sequence comparisons between mouse and human only differ by one amino acid residue. The chicken and human also show high levels of sequence identity showing that the protein is highly conserved in higher vertebrates (Arkin *et al.*, 1997). Amino acid residues shown in red are non-conserved residues. The data obtained for this figure were downloaded from www.ncbi.nlm.nih.gov (the NCBI protein data bank).

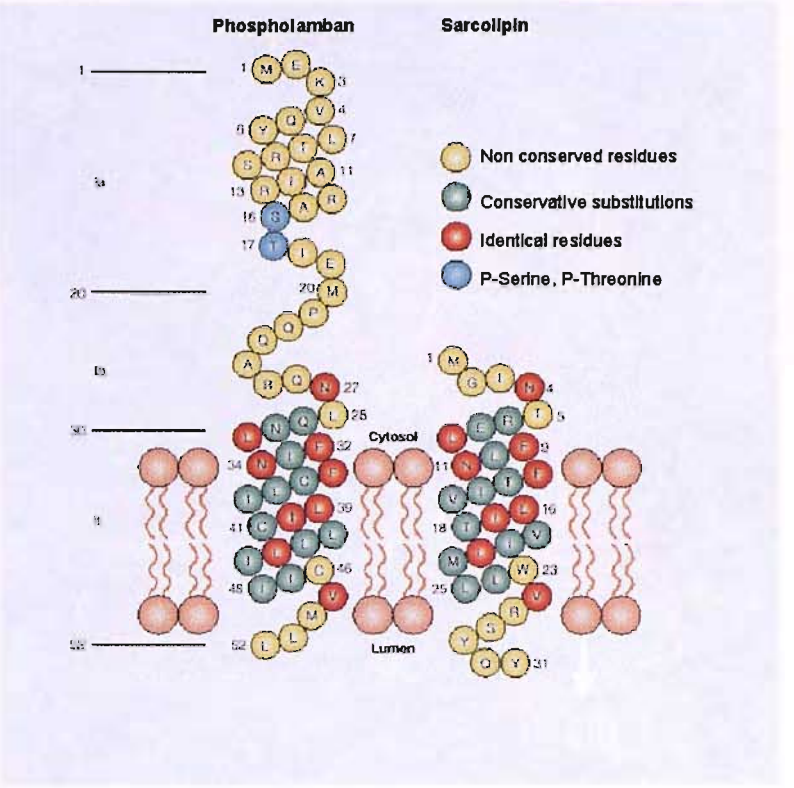

Figure 4

Schematic representations of phospholamban and sarcolipin

This diagram shows the organisation of phospholamban and sarcolipin in the sarcoplasmic reticulum membrane. Identical amino acid residues are shown in red and conservative substitutions in green. The two structures can be seen to be very similar. Both proteins can be divided into 2 main domains a cytoplasmic domain and a membrane-spanning domain. Phospholamban has a relatively large cytoplasmic region in comparison with sarcolipin this may be important in recognition of the phosphorylatable residues serine 16 and threonine 17. Once phospholamban has been phosphorylated SERCA inhibition is removed. The transmembrane regions of the two proteins are very similar in structure and amino acid homology. Sarcolipin when compared with phospholamban has 1 difference in that it appears to have a short lumenal tail. This diagram was taken from MacLennan and Kranias (2003).

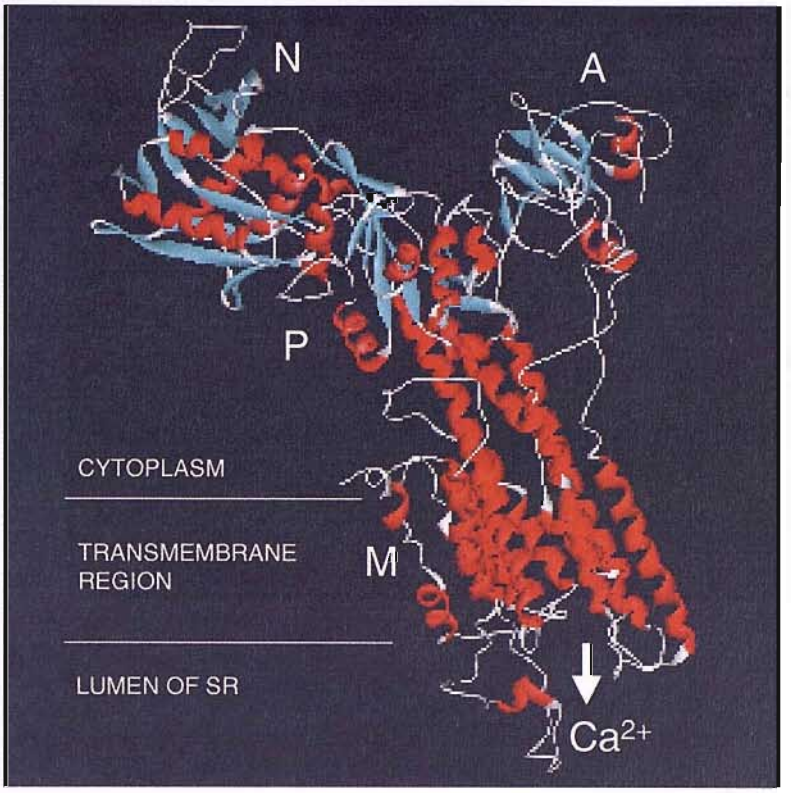

Figure 5

The crystal structure of SERCA1a

The diagram shows the crystal structure of SERCA1a and the arrangement of the 3 cytoplasmic domains. The N domain (nucleotide binding domain) is the region of the pump where ATP binds. The P domain (phosphorylation domain) is the region of the protein where phosphorylation of aspartate 351 takes place and converts the conformation of the protein. The actuator domains (A domain) function appears to be unclear although mutation and proteolytic digest of this region uncouples ATP hydrolysis from Ca^{2+} transport indicating its involvement in energy transfer from the P and N domains see (Moller *et al.*, 1996). The diagram was taken from Toyoshima *et al.*, (2000) (The figure was produced from the coordinates of the PDB file 1 EUL).

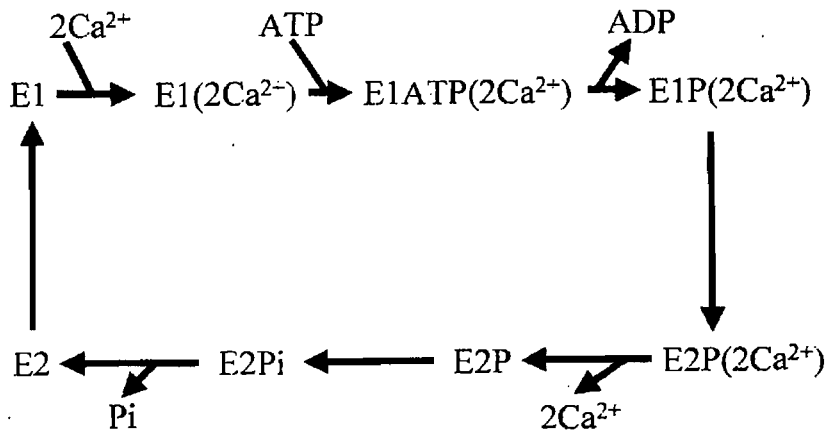

Figure 6

The catalytic cycle of Ca^{2+} pumping by SERCAs

The diagram shows the various stages in the catalytic cycle of SERCA. Firstly 2 Ca^{2+} ions bind to their binding sites in the transmembrane region of the E1 conformation of the pump. ATP binds and phosphorylation of aspartate 351 occurs forming E1P.(2 Ca^{2+}). The pump then changes conformation to the E2 form and the Ca^{2+} ions are released into the lumen of the sarcoplasmic reticulum. Hydrolysis of the phosphate group then occurs to yield E2 + Pi. The pump is then ready for ATP and Ca^{2+} ions to bind again for the next catalytic cycle. This diagram was taken from East (2000).

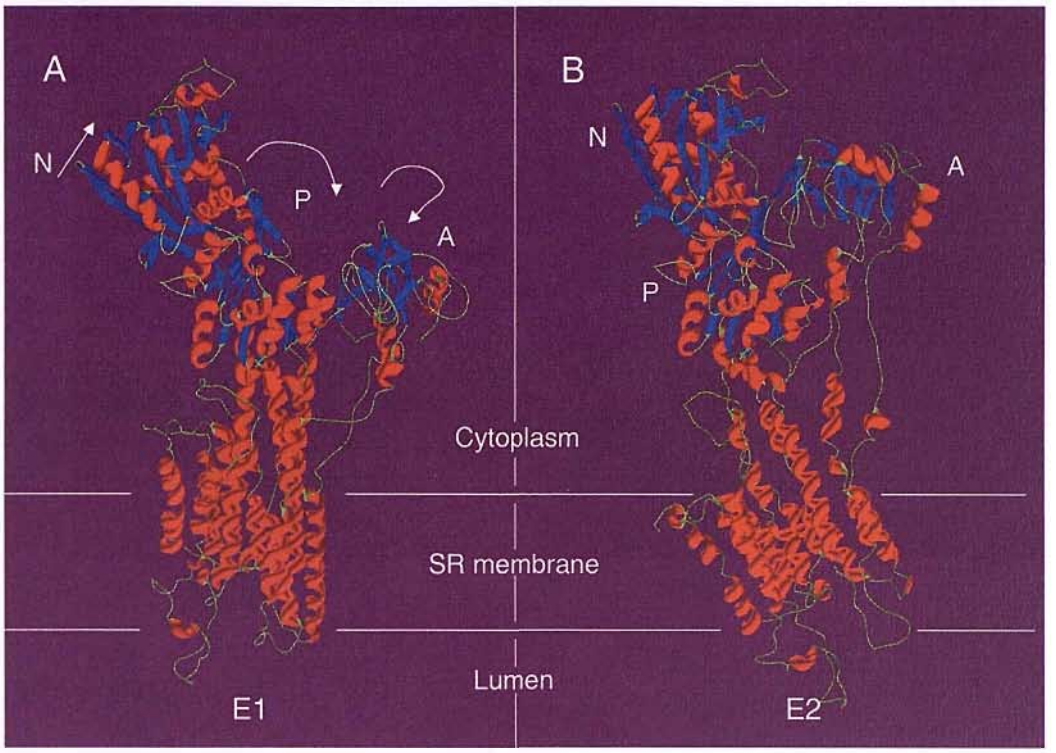

Figure 7

A comparison of the E1 and E2 conformations of SERCA1

The diagram shows two crystals structures of SERCA1 in two different conformations. The pump converts between these two conformations when pumping 2 Ca^{2+} ions from the cytoplasm of the cell into the lumen of the sarcoplasmic reticulum. The white arrows in figure 7 A show the movements of the nucleotide binding domain (N), phosphorylation domain (P), and the actuator domain (A). It is also clear that the transmembrane helices of the pump undergo a significant rearrangement. The diagram was modified from Toyoshima and Nomura (2002) (The figure was produced from the coordinates of the PDB file 1SU4 and 1KJU).

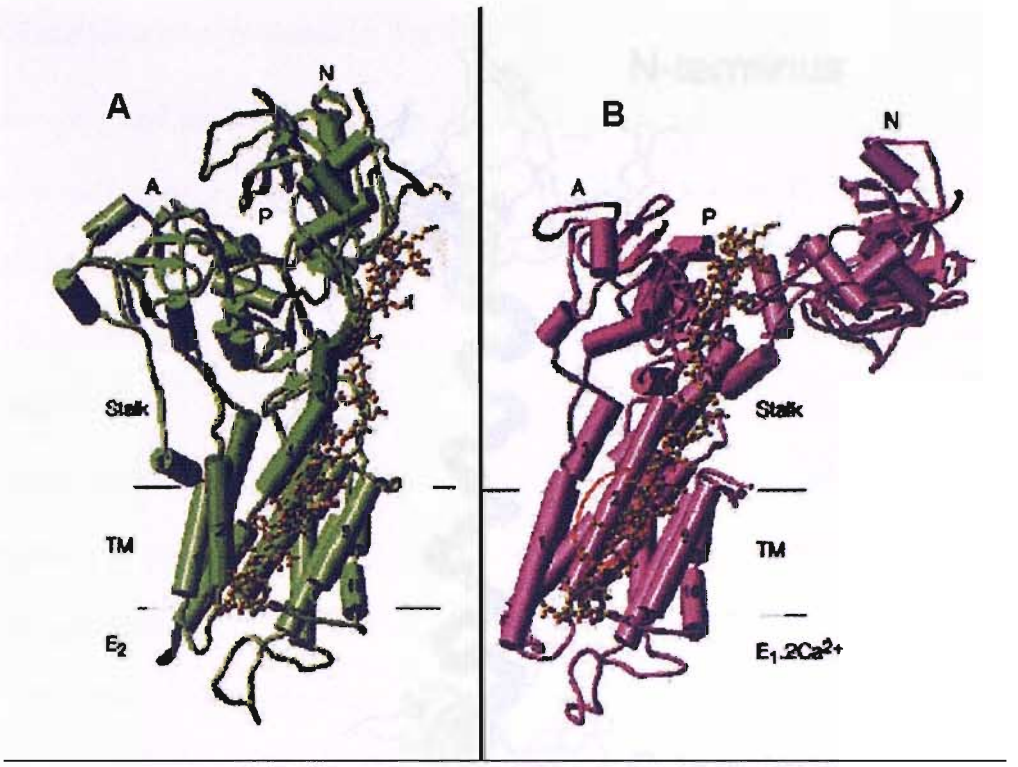

Figure 8 A and 8 B

A structural model of SERCA1 showing the putative interaction with phospholamban

Phospholamban is depicted as a ball and stick model bound to SERCA1a in its E2 conformation (8 A) and in its E1 conformation (8 B). The phospholamban molecule appears to fit in to a grove formed between helices 4 and 9 with its cytoplasmic domain interacting with the N domain of SERCA1a. When the pump is converted to the E1 conformation helix 2 moves across pushing phospholamban out of the grove formed previously by helices 4 and 9. The N domain of SERCA1a also moves removing interaction between itself and phospholamban. Due to the loss of interaction between phospholamban and SERCA1a the inhibitory effect is lost. This diagram was taken from MacLennan and Kranias (2003).

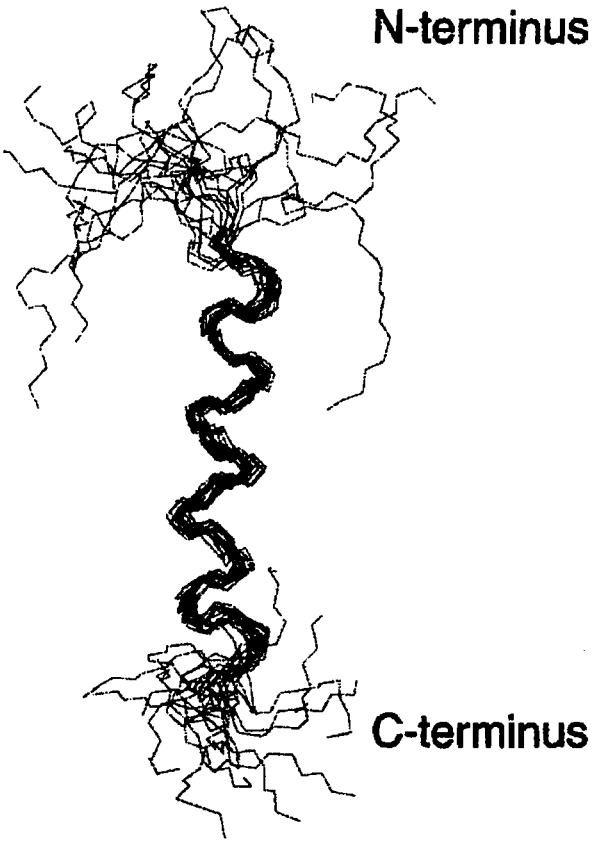

Figure 9

Sarcolipin structure deduced from NMR spectroscopy

The diagram shows the 16 lowest energy conformations that the protein may adopt superimposed on one another. The transmembrane region of sarcolipin appears to be relatively immobile. However, the cytoplasmic and lumenal tails seem to move more freely. Taken from Mascioni *et al.*, (2002).

1.1.8 Introduction to protein sorting

The secretory and endocytic pathways of the eukaryotic cells consist of many multiple compartments, each containing a unique set of protein and lipids. Specific transport mechanisms are needed to deliver the correct proteins and lipids to the correct location in order to ensure that the identity, and hence function, of individual compartments are maintained (Alberts *et al.*, 1994). Proteins contain signals in their molecular structure that target them to the correct location. The organelles of the secretory pathway are involved in the sorting of proteins to a variety of intracellular membrane compartments and the cell surface (see figure 10).

Proteins that are transported within the secretory pathway are either secreted from the cell, bound to the plasma membrane, sorted in to lysosomes, or are retained in any of the compartments in the secretory pathway as resident proteins. The endoplasmic reticulum is the first organelle that a secretory protein is likely to be inserted into. Here it is checked to ensure it is folded correctly before initial glycosylation may take place and then packaged into transport intermediates or vesicles see review (Ellgaard and Helenius, 2003). The material then moves into endoplasmic reticulum exit sites and on to the Golgi apparatus. Secretory proteins are transported through the Golgi cisternae until they reach the trans-Golgi network (TGN). At the TGN proteins are sorted according to their final destinations.

The TGN is also important in the endocytic pathway, as this is where newly synthesised proteins can meet proteins coming in to the cell from the plasma membrane. Molecules can be taken in from the cell surface and internalised into endocytic vesicles; they are then transported to early endosomes (Alberts *et al.*, 1994). Some proteins in

these endosomes are recycled back to the plasma membrane. These include receptors such as low-density lipoprotein receptor and the transferin receptor. Others are transported to lysosomes for degradation and some are targeted to the TGN. Thus it can be seen that the TGN and early endosomes can be considered as 2 sorting stations, for proteins on their way out of the cell or their way in to the cell.

Protein transport in the secretory pathway is a multi step process involving the generation of transport vesicles that have defined sets of cargo. These vesicles themselves are highly specific for their destination membrane ensuring that proteins end up in the correct compartments. The transport of proteins involves a complex array of protein lipid interactions. To understand the molecular mechanisms involved in targeting it is important to understand the organelles involved and the general flow of material through the pathways between these organelles (Alberts *et al.*, 1994). In this introduction the components of the secretory pathway will be described and then the molecular interaction between the components will be examined.

1.1.9 Transport of newly synthesized proteins to the endoplasmic reticulum

All protein synthesis begins on cytoplasmically located ribosomes. From here proteins can be released in the cytoplasm or they can be directed to the endoplasmic reticulum. Most proteins are targeted to the endoplasmic reticulum by a signal sequence located on the N-terminus of the peptide (Keenan *et al.*, 2001). The N-terminus protrudes from the

ribosome first, displaying the signal sequence (Walter and Johnson, 1994). To this exposed signal sequence a ribonucleic acid protein complex called the signal recognition particle (SRP) binds, causing the ribosome to go into elongation arrest. SRP then targets the ribosome protein complex to the endoplasmic reticulum. The SRP interacts with its receptor (SRPR) located in the membrane of the endoplasmic reticulum and the docking of SRP with its receptor triggers the formation of a protein pore called a translocon (Fons *et al.*, 2003). This pore provides an opening into the lumen of the endoplasmic reticulum. Once the formation of the translocon is complete translation of the protein can continue. The protein in the process of synthesis has now two options. It can go through the pore formed by the translocon and become a lumenal located protein of the endoplasmic reticulum or it can be shuffled through a side gate of the translocon and become a transmembrane protein. The fate of the protein at this stage is usually coded for by various signals within the peptide chain of the protein see review (Keenan *et al.*, 2001). Within the lumen of the endoplasmic reticulum N-terminal signal sequences of soluble proteins are cleaved by a signal peptidase (Martoglio and Dobberstein, 1998). There are also a number of chaperones that bind to the polypeptide chain and aid in protein folding. These chaperones include Bip (Zaitseva *et al.*, 1999), the lectins calnexin and calreticulin (Zhang *et al.*, 1997) and protein disulphide isomerase. Chaperones not only assist in the folding of the peptide chain but they can prevent aggregation and ensure that the correct disulphide bonds form. The chaperones are also important in quality control, directing incorrectly folded proteins out of the endoplasmic reticulum through the translocon to the proteosome for degradation (Ellgaard and Helenius, 2003).

The majority of plasma membrane and secretory proteins are glycosylated. N-linked oligosaccharides are added to the growing polypeptide chain as it enters the endoplasmic reticulum (Kornfeld and Kornfeld, 1985). Trimming of the oligosaccharides occurs by the action of glucosidases I and II as well as ER-α-mannosidase. This glycosylation is important in the correct folding of certain proteins and also aids in the recognition of some proteins by chaperones such as calreticulin and calnexin (Hammond and Helenius, 1995). Proteins are thought to be exported from the ER at specialised exit sites called transitional elements or transitional endoplasmic reticulum. The transitional elements are areas of endoplasmic reticulum that are free of ribosomes. They are usually adjacent to the centre region of the cis-Golgi but can also be found through out the endoplasmic reticulum in lower densities (Klumperman, 2000). These transitional elements are specialised domains for the production of transport vesicles. The vesicles can fuse together to form a network of vesicular tubular clusters (VTCs) (Bannykh *et al.*, 1996). This region is some times referred to as the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Schweizer *et al.*, 1991). The ERGIC is an important compartment for protein sorting. It sorts proteins that are normally resident in the endoplasmic reticulum, but that have escaped to the ERGIC into COP I vesicles for retrograde transport (returning them to the endoplasmic reticulum) as well as sorting proteins into delivery vesicles for the cis-Golgi (Warren and Mellman, 1999). Vesicles leave the ERGIC along microtubules to which they are attached by a molecular motor and an adapter protein. Cytoplasmic dynein complex 1 (CD1) is thought to be the motor responsible for the transport of vesicles from the endoplasmic reticulum to the Golgi apparatus see review (Allan *et al.*, 2002). Bicaudial D 2 (BICD 2) may be an adapter

protein that associates with cytoplasmic dynein complex 1 (Hoogenraad *et al.*, 2001). BICD 2 may be required to link the vesicles to the motor in order that they can move along the microtubules. Once vesicles are attached they track along the microtubules until they reach the cis-Golgi. Here they fuse and become part of the Golgi stack. Once in the Golgi, the proteins are exposed to a large number of glycosidases and glycosyltransferases. These enzymes modify the N-linked $Man_8GlcNAc_2$ sugar moieties of the glycoproteins that have arrived from the endoplasmic reticulum. The glycosylation reactions that take place vary from cell to cell and are exceptionally diverse. See (Narasimhan *et al.*, 1985) and (Kornfeld and Kornfeld, 1985) for a detailed description of glycosylation within the Golgi. It is unclear why such diverse glycosylation systems have evolved (Alberts *et al.*, 1994). The complexity may be related to the fact the glycosylation of protein serves several functions. Glycosylation can play a role in protein targeting. This is apparent in directing proteins to lysosomes (Rouille *et al.*, 2000) and in directing proteins to the correct membrane in polarised cells (Muth and Caplan, 2003). Glycosylation may also play a role in protecting proteins from proteolysis (Alberts *et al.*, 1994). Glycoproteins are also involved in cell adhesion and mucus formation (Toole and Toole, 1995). These two processes are especially important in the digestive and respiratory tracts.

1.2.0 The cytoskeleton

The cytoskeleton plays a major role in directing the movement of material around the cell. It is important in the maintenance of organelle location and the efficient transport of

vesicles throughout the cell (Lippincott-Schwartz, 1998). The cytoskeleton provides a transport network. This network is made up of filamentous tracks along which membrane transport carriers can be guided as they move from one compartment to another. The filaments are made up from microtubules, actin and intermediate filaments. These fibres run out from a perinuclear microtubule organizing centre (MTOC) they are polarized with the plus ends at the MTOC and their negative ends are located at the periphery of the cell. The Golgi is located adjacent to the MTOC through association with the negative ends of the microtubules (LippincottSchwartz and Cole, 1995). Efficient long distance transport in the cell is dependent on microtubules (Kamal and Goldstein, 2000). For example transport from the endoplasmic reticulum to the Golgi as well as transport from the Golgi to the plasma membrane is directed via microtubular tracks (Hirschberg *et al.*, 1997; Scales *et al.*, 1997).

The actin cytoskeleton also plays a role and recent data indicate that some membrane trafficking utilizes both microtubules and actin filaments in the same journey (Goode *et al.*, 2000). Many molecular motors have evolved to pull cargo around the cell. The best characterised of these seems to be kinesin and dynein. Kinesin and dynein can attach directly to cargo although sometimes adaptor or linker proteins are needed (Tai *et al.*, 1999; Goode *et al.*, 2000). Both actin and microtubule motor proteins with their linkers are thought to form large complexes to facilitate transport of cargo around the cell see review (Allan *et al.*, 2002).

1.2.1 Transport from the endoplasmic reticulum to the Golgi apparatus

Secretory protein leaving the endoplasmic reticulum is packaged into vesicles with a specialized coat protein called COP II (Barlowe, 1998). Whether the process by which material leaves the endoplasmic reticulum is part of a bulk flow process or whether it is selectively packaged has been the subject of heated debate see (Kuehn and Schekman, 1997; Warren and Mellman, 1999). According to the bulk flow hypothesis cargo is simply packaged into vesicles and is concentrated as it moves through the Golgi. Certain proteins may then be retained within specific compartment or recycled from upstream compartments. This involves specific targeting signals. If proteins lack these signals they will most probably be secreted or become inserted into the plasma membrane (Weiland *et al.*, 1987). The selective transport hypothesis predicts that proteins are concentrated during export from the endoplasmic reticulum (Kuehn and Schekman, 1997) (see figure 11) evidence for this will be discussed in (section 1.3.2). In this model the proteins for forward trafficking contain signals that preferentially cause them to package into secretory vesicles so they will predominantly move forward. In reality both mechanisms are probably at work in the endoplasmic reticulum. Such an arrangement may be advantageous because abundant proteins can be transported via bulk flow where as rare proteins may be selectively transported to the correct location (Warren and Mellman, 1999). After departure of the COP II coated vesicle uncoating occurs and the vesicles fuse to form the ERGIC compartment (Aridor *et al.*, 1995). Another coat protein COP I is at work at the ERGIC; here it forms vesicles which return escaped endoplasmic reticulum proteins back to this compartment (Scales *et al.*, 1997). The ERGIC is a very dynamic

compartment and can move as discrete packages along the microtubules of the cell to the Golgi where it fuses to form the cis-Golgi network (Lippincott-Schwartz *et al.*, 2000).

1.2.2 Transport of material through the Golgi

Two models have been proposed to describe trafficking through the Golgi apparatus. These models are referred to as vesicular transport and cisternal maturation. For a review see (Allan *et al.*, 2002).

In the vesicular transport model the Golgi stacks are static compartments in which retro and anterograde vesicular transport takes place between the stacks. The vesicles are solely responsible for transporting cargo from one cisternae to the next. This model accounts for the different distributions of Golgi enzymes throughout the stack, with each compartment containing a unique set of proteins. Evidence to support this model comes from the findings that COP I vesicles were found to bud from Golgi membranes in vitro and found to surround Golgi stacks in vivo. Treatments that prevented COP I vesicle formation were found to inhibit anterograde transport (Rothman and Orci, 1992). However, difficulties arose with this model when it was found that COP I vesicles mediate retrograde transport from the Golgi to the endoplasmic reticulum (Cosson and Letourneur, 1994). Later studies showed that COP I vesicles are responsible for retrograde transport through out the secretory pathway (Cole *et al.*, 1998). It is clear that these COP I vesicles perform retrograde transport and because of this it is awkward to

conceive a single model that would hold these vesicles responsible for the retro and anterograde movement of material.

In the cisternal maturation model one stack moves forward to form the next stack carrying the anterograde cargo as they go. Cisternae are formed by the fusion of vesicles from the ERGIC compartment at the cis face of the stack. Studies showing de novo formation of cis-Golgi strongly supports this model (Presley *et al.*, 1997). One problem with this model was that it was hard to see how gradients of enzymes could be maintained by the Golgi stacks especially the glycosyltransferases which are concentrated in specific compartments (Pelham, 1998). However with the discovery of retrograde transport it meant there was a mechanism to achieve gradients of specific proteins across the Golgi. In this cisternal maturation model cisternae mature with the selective removal and additions of proteins as they progress along the stack. The main problem with this model is that it cannot explain the formation of the TGN. The TGN is a compartment that is in steady state it contains a unique set of resident proteins and behaves differently from the Golgi stack in many respects. It is hard to see with the cisternal maturation model how a compartment could keep its unique set of resident proteins as the whole stack moves forward (Griffiths, 2000).

It is likely that both types of transport are occurring in the Golgi as neither model can fully explain the way material moves through the stack (Pelham and Rothman, 2000). One proposal is that large proteins and aggregates too large for vesicles may move through the Golgi in maturing cisternae where as vesicles could provide a so called fast

track transport system for cargo (Orci *et al.*, 1997). Electron microscopic evidence suggests that distinct populations of COP I vesicles budding from the Golgi stack carry either retrograde or anterograde directed cargo (Orci *et al.*, 2000). Orci *et al.*, (2000) have proposed that one population of COP I vesicles which have the Golgi SNARE GOS-28 may percolate over the whole Golgi providing movement up and down the Golgi. Molecular tethers observed linking COP I vesicles to Golgi membranes would constrain the vesicles limiting vesicle fusion only with adjacent cisternae (Orci *et al.*, 1998). This type of movement would result in a net movement of material through the Golgi as new material is continually being entered at the cis face and material is constantly being removed at the trans face. This random movement of vesicles across the Golgi stack would overcome the requirement for multiple sets of SNARE complexes (Orci *et al.*, 2000). Another population of COP I vesicles could carry retrograde proteins (Pelham and Rothman, 2000). This would provide a mechanism to segregate cargo from resident Golgi enzymes into fast and slow moving carriers.

1.2.3 Transport from the trans-Golgi network

The TGN is the last major sorting station for many proteins, it is here that many proteins will be packaged into their last vesicle and delivered to their final destination. The proteins that make it this far in the secretory pathway often end up in the plasma membrane, secretory granules, lysosomes, endosomes or undergo retrograde transport to deliver them back to an earlier compartment. Mechanisms for sorting to these different compartments are slowly being unravelled and appear to be generally signal dependent

(Keller and Simons, 1997). Transport from the TGN was initially thought to occur in small vesicles (Rothman and Wieland, 1996). However studies with live cells expressing GFP-fusion proteins have shown that some transport occurs in larger tubulovesicular structures (Toomre *et al.*, 1999). This is controversial because it has been suggested that high levels of expression of GFP constructs may affect normal trafficking events (Girotti and Banting, 1996). Further evidence of these larger tubulovesicular structures comes from (Lippincott-Schwartz *et al.*, 2000; Stephens and Pepperkok, 2001). Both groups suggest that larger tubulovesicular structures appear to be used for transport over longer distances, mainly from the endoplasmic reticulum to the Golgi and also from the TGN to the plasma membrane. The reason for these discoveries only being made recently is due to improvements in live cell imaging techniques. Although these large tubulovesicular structures can be seen it is still the smaller vesicles that seem to be the predominant way that material is moved around the cell (Stephens and Pepperkok, 2001).

Proteins destined for the plasma membrane are usually delivered there by default but these can then become endocytosed into endosomes. In polarized cells transcystosis can occur where proteins are delivered to either the apical or basolateral membranes endocytosed and then delivered to the target membrane (Mostov *et al.*, 2000). Generally speaking proteins end up in the plasma membrane when they have no specialized signals directing them anywhere else, hence this is called the default pathway. Proteins destined for regulated secretion aggregate into immature secretory granules and bud off the TGN. Clathrin coats form on these vesicles, and they migrate into the cytoplasm. When the correct signalling events occur the mature secretory granules will fuse with the plasma membrane and liberate their contents (Kelly *et al.*, 1987). Lysosomal enzymes are

transported from the TGN in special vesicles. Most proteins destined for lysosomes carry a mannose 6-phosphate (M6P) sugar moiety that is recognized by M6P receptors in the Golgi. The M6P receptors along with cargo are then packaged into vesicles with specialized protein coats that then deliver the proteins to lysosomes via endosomes. The receptors are then recycled from the endosome back to the Golgi for reuse (Rouille *et al.*, 2000). For a more detailed description of protein trafficking to lysosomes see the review by (Hunziker and Geuze, 1996).

1.2.4 Molecular mechanisms of protein sorting and transport

This area is so vast and constantly being updated. As a result only the main protein components will be discussed here along with the more recent models of transport. When considering transport it is important to remember each compartment has to be at equilibrium with material entering and leaving the compartment. Otherwise some organelles would decline in size while others become much larger. An elaborate set of proteins ensures that trafficking is controlled and coordinated in a balanced manner and the major players in this process will be discussed here.

1.2.5 The role of COP I in vesicular trafficking

COP I coated vesicles are responsible for retrograde transport from the Golgi to the endoplasmic reticulum as well as transport between Golgi cisternae. Purification of the COP I proteins revealed a complex made up of 7 subunits (Waters *et al.*, 1991).

Abbreviation	Size KD	Nomenclature
α	160KD	Sec33p
β	110KD	Sec26p
β-	98KD	Sec27p
γ	61KD	Sec21
δ	36KD	Ret2p
ε	35KD	Sec28p
ζ	20KD	Ret3p

Table 1

A table showing the abbreviations for the COP I subunits

The table shows the size and the names of the proteins that make up the COP I coated vesicles. This diagram was modified from www.bch.bris.ac.uk/staff/stephens/.

α, β-, and ε subunits can form binding domains for di-lysine retrieval motifs that will be discussed later. The subunits come together in a stepwise manner from different subcomplexes. This assembly is blocked by Brefeldin A (Chardin and McCormick, 1999). COP I vesicle budding is initiated when a membrane bound guanine nucleotide exchange factor (GEF) catalyses the exchange of GDP for GTP by ARF 1 (ADP ribosylation factor 1) (Horng and Tan, 2004; Kremer *et al.*, 2004). Membrane bound ARF-GTP recruits pre assembled COP I complexes, which causes the membrane to deform and this is the beginning of vesicle formation (see figure 12) (Orci *et al.*, 1993). The minimum requirements for the formation of COP I vesicles are the coatamer

proteins, ARF 1 and GTP. There is evidence however that vesicle biogenesis *in vivo* requires another protein called p24 (Bremser *et al.*, 1999).

The p24 family of proteins are transmembrane proteins that are responsible for promoting membrane association of the coatamer complex with the membrane and hence vesicle formation. The cytoplasmic tail region of p24 interacts directly with COP I via its C-terminal KKXX sequence this is the region responsible for enhancing COP I binding to the membrane (Bremser *et al.*, 1999; Fiedler *et al.*, 1996). It was initially believed that p24 may have a second role in selecting lumenal proteins for transport in COP I vesicles via direct interaction of its lumenal region with certain domains on lumenal proteins (Fiedler *et al.*, 1996). However, it is now thought that p24 is involved in withholding resident proteins from entering COP I vesicles for transport (Kaiser, 2000; Springer *et al.*, 2000). The vesicles need to uncoat when they meet the correct compartment. This occurs with the help of another protein called ARF GAP (GTPase activating protein). ARF GAP is responsible for enhancing ARFs ability to hydrolyse GTP. Once this occurs the coatamer falls away and the vesicles are able to fuse with the aid of other proteins.

1.2.6 COP II

COP II coatamer is mainly responsible for anterograde transport between the endoplasmic reticulum and the Golgi apparatus. COP II was discovered using yeast mutants with defects in secretion (Kaiser and Schekman, 1990). These mutants revealed four genes that were essential for transport from the endoplasmic reticulum to the Golgi. These genes were SEC12, SEC13, SEC16 and SEC23. It is the products of these genes

that are responsible for COP II coat biogenesis (Kaiser and Schekman, 1990). The COP II coat itself is composed of 2 heterodimeric complexes Sec23p/24p and Sec13p/31p (Barlowe *et al.*, 1994). Sar1p is a small GTPase that is activated by Sec12p (see figure 13). Sec12p acts as the GTP exchange factor (GEF) for Sar1p as it catalyses the exchange of GDP for GTP that in turn activates Sar1p. The activated Sar1p then binds to the endoplasmic reticulum membrane and recruits Sec23p/24p. This in turn causes Sec13p/31p to bind (Schekman and Orci, 1996). The coming together of the various components then initiates vesicle formation by deforming the membrane (Barlowe *et al.*, 1994). COP II uncoating is caused by GTP hydrolysis. Sec23p acts as the GAP for Sar1p, although it is worth noting that Sec13p/31p components increase the rate of GTPase activities by 10 fold when in combination with Sec23p (Antonny *et al.*, 2001).

Three additional proteins appear to be important in COP II vesicle formation *in vivo* Sec7p, Sec16p and Ypt1p; they appear to be important in ensuring that the vesicles fuse only with the Golgi apparatus (Schekman and Orci, 1996). It was initially thought that COP II coatamers themselves were important in cargo selection within the endoplasmic reticulum (Kuehn and Schekman, 1997). However, it is now thought that cargo selection and vesicle biogenesis are distinct processes (Nishimura and Balch, 1997; Nishimura *et al.*, 1999; Yeung *et al.*, 1995). There are exceptions to this as receptors such as Erv29p seem to concentrate in COP II vesicles from yeast (Belden and Barlowe, 2001). This receptor then appears to interact with pro-α-factor a soluble lumenal protein and concentrate it in the COP II vesicles for transport. This is an example of specific cargo selection (Belden and Barlowe, 2001). SNARE proteins such as Bet1p and Bos1p seem to play a role in COP II formation and have been shown to interact with Sar1p and

Sec23p/24p. Their role may be vesicle biogenesis and vesicle targeting (Springer and Schekman, 1998).

1.2.7 Clathrin

Clathrin coated vesicles are important in the transport of material from the TGN to the plasma membrane as well as for material being brought into the cell by endosomes. Clathrin is made up from two chains, one light chain and one heavy chain. They form triskelions that can polymerise to form polygonal lattices. This in turn causes curvature of the membrane and initiates vesicle formation (Marsh and McMahon, 1999). The clathrin coat associates with the membrane via an adaptor protein complex (AP) (Schmid, 1997). The AP is made up from 4 adaptin molecules. There are in fact many different types of adaptins that can come together to form several classes of (AP); for structural information see (Robinson and Bonifacino, 2001). Adaptor proteins are thought to act as linkers that link cargo proteins with the clathrin cage. Clustered cargo with attached adaptors recruits clathrin that provides a cage for the membrane to anchor to. As the cage becomes more spherical the vesicle begins to form and this is called a clathrin coated pit (Marsh and McMahon, 1999). The actual process of severing the vesicle requires a protein called dynamin. Dynamin forms a collar like structure around the vesicle at the point where it is still attached to the membrane. Dynamin has GTPase activity and the hydrolysis of this GTP provides the energy to sever the vesicle from the membrane (Vanderbliek *et al.*, 1993). After budding of the vesicles they often deliver the cargo to an endosome. Uncoating of the vesicle takes place with the use of two proteins, auxillin, which in turn

recruits heat shock cognate 70 (Hsc70) that is thought to cause the disassembly of the clathrin cage in an ATP dependent manner. Once uncoating has occurred fusion can take place (Waters and Hughson, 2000).

1.2.8 Vesicle targeting and fusion

Vesicle docking and fusion occurs primarily in three steps. Firstly the vesicle is tethered to the target membrane by so called tethering complexes (Pfeffer, 1999). Secondly, if the target membrane and the vesicles contain compatible SNAREs, they will interact docking the vesicle on to the target membrane. Finally the SNAREs bring the membranes close together and promote membrane fusion (Jahn and Sudhof, 1999). After fusion has occurred the SNAREs need to be separated and recycled back to their donor compartment so that more vesicles can be targeted correctly. As has already been discussed in (section 1.2.0) the vesicles themselves are moved around the cell by interactions with microtubules (Lippincott-Schwartz, 1998). This probably gives them a general direction to follow so that they are trafficked to the appropriate area of the cell.

1.2.9 SNAREs

Soluble NSF attachment protein receptors (SNAREs) are extremely important proteins in the targeting and fusion of vesicles (Weber *et al.*, 1998a). SNAREs were identified through both genetic and biochemical analysis of components responsible for membrane fusion (Novick *et al.*, 1981; Nichols and Pelham, 1998). SNAREs have an array of other

proteins that interact with them. One component is an ATPase that is sensitive to N-ethymaleimide (NEM) this is called NEM sensitive factor (NSF) (Horsnell *et al.*, 2002). Another important protein is soluble NSF attachment protein or SNAP (Clary *et al.*, 1990). The cytoplasmic tails of SNAREs interact with one another to form stable four helix bundles referred to as SNARE pins (Weber *et al.*, 1998b). The formation of a SNARE pin brings the two membranes close together this allows membrane fusion between vesicle and target membranes. SNAREs are divided into two types either vesicle SNAREs (v-SNAREs) or target SNAREs (t-SNAREs). V-SNAREs interact with t-SNAREs on the target membrane to form structures referred to as trans-SNARE pairs. After fusion of the vesicle, driven by the trans-SNARE pairs, the SNAREs form a different structure referred to as cis-SNARE pairs as they are now on the same membrane. NSF and SNAP act together to disrupt these cis-SNARE complexes. NSF binds to assembled SNARE complexes via SNAP and hydrolysis of ATP results in disassembly of the SNARE complex (see figure 14) (Sollner *et al.*, 1993). After disassembly occurs the v-SNAREs are transported back to the donor compartment for reuse. Sec1 proteins are also very important in regulating vesicle fusion. They bind to t-SNAREs and regulate SNARE pin formation. If the SNAREs are not compatible then Sec1 prevents a SNARE complex formation (Pevsner *et al.*, 1994; Bryant *et al.*, 2001). This prevents inappropriate vesicular fusion. SNARE complexes play a huge role in vesicle fusion as they act in a targeting role and as a driving force for vesicle fusion. There must however be additional levels of regulation as SNAREs are always circulating between donor and receiver compartments and yet vesicle fusion only occurs between vesicles that are at the target compartment.

1.3.0 RAB proteins

Rab proteins are small Ras like GTPases that act before the SNARE proteins. They act in the initial tethering of vesicles to target membranes. Rab proteins act to recruit other effector proteins involved with the initial attachment of vesicles to membranes this brings the SNAREs close enough to interact (Waters and Pfeffer, 1999). Different organelles tend to have their own set of Rab proteins. This means they are not interchangeable between different compartments.

Rab's cycle between the cytoplasm and the membrane. They are regulated by guanine nucleotide exchange factors (GEFs). These GEFs are capable of stimulating Rab's to exchange their GDP for GTP, which in turn switches the protein into an active state (Chavrier and Goud, 1999). GTPase activating proteins (GAPs) cause Rab's intrinsic GTPase activity to increase resulting in the hydrolysis of GTP and hence switching Rab off. Two further proteins have a role in Rab's ability to bind to the membrane and hence its activation. GDP dissociation inhibitor (GDI) extracts GDP-Rab from the membrane and helps maintain it in an inactive form. While GDI displacement factor (GDF) displaces GDI and recruits Rab to membranes (Gonzalez and Scheller, 1999). Rabs are thought to be controlled by numerous factors see (Gonzalez and Scheller, 1999) for more details. There are even reports of Rabs being involved with the transport of vesicles via the cytoskeleton therefore it is possible that Rabs may regulate vesicle fusion at a number of levels (Armstrong, 2000; Woodman, 1998).

1.3.1 Tethers

Activated Rab proteins recruit tethers to link vesicles to membranes. Tethers act to form a bridge between the vesicle and the target membrane before SNARE protein complexes form. Tethering proteins tend to be of two distinct types long coiled coil proteins or large multimeric complexes (Pfeffer, 1999). Tethers are not essential for vesicular transport but they do increase the efficiency of vesicular fusion by bringing vesicles and there target membranes close together so that SNARE complexes can form and drive fusion. There now seems to be a number of tethers emerging in the secretory pathway and many different organelles seem to have multiple sets to aid vesicular fusion (Pfeffer, 1999).

1.3.2 Cargo selection

Newly synthesized secretory and plasma membrane proteins can be concentrated as they travel along the secretory pathway. Most models proposed to account for concentrating proteins in particular compartments centre around bulk flow out of the endoplasmic reticulum followed by selective retrieval to return resident endoplasmic reticulum proteins (Pfeffer and Rothman, 1987). A refinement of this model in which endoplasmic reticulum resident proteins are held back by a physical means such as oligomerisation fails to account for why resident proteins which have retention / retrieval signals removed are slowly exported out of the endoplasmic reticulum. It is unlikely that such proteins contain export motifs therefore it is reasonable to assume that a low level export pathway exists (Teasdale and Jackson, 1996). Strong evidence now exists that some cargo is actually selectively extracted from the endoplasmic reticulum. Evidence for this comes

from studies with yeast expressing a secretory protein called pre pro-α-factor. This protein is found to be enriched in vesicles budding from the endoplasmic reticulum compared with levels expected by bulk flow mechanisms (Barlowe *et al.*, 1994). Mizuno and Singer, (1994) carried out immuno-electron microscopy with VSV-G protein in transfected fibroblasts. The protein again was found to be concentrated in budding vesicles indicating that selective cargo packaging may be taking place. Findings such as these have led to people trying to identify cargo receptors capable of selective packaging of cargo.

There are a few candidates for such receptors. ERGIC-53 cycles between the endoplasmic reticulum and the Golgi and it also has lectin binding properties. This means that the lumenal domain could in theory bind proteins which have mannose residues attached (Arar *et al.*, 1995). ERGIC-53 could act as a cargo receptor for packaging secretory proteins that are usually glycosylated into budding vesicles. Once such glycosylated proteins reach the Golgi mannose is usually removed by trimming enzymes leaving ERGIC-53 to recycle back while the secretory protein remains to travel along the rest of the pathway (Teasdale and Jackson, 1996).

Some theories hypothesise that cargo selection in vesicles depends upon transmembrane proteins that can interact with soluble lumenal proteins via so called receptor domains. Some transmembrane proteins selected for exit from the endoplasmic reticulum are known to have diacidic sorting motifs on their cytoplasmic side that are thought to interact with Cop II subunits such as Sec23p/24p. This interaction leads to the incorporation of such transmembrane proteins into endoplasmic reticulum donor vesicles

(Miller *et al.*, 2002). Thus leading to these proteins preferentially entering the secretory pathway (see figure 11).

1.3.3 Retention and retrieval signals

As has been discussed the general flow of material in the endomembrane system is towards the cell surface, however certain organelles need to maintain certain proteins in order to carry out their specific functions. Proteins that are localized and kept within a specific compartment are called resident proteins. In order to maintain a protein within a compartment two methods can be envisaged retention and retrieval. Retention is a process whereby proteins are prevented from leaving a compartment; may be they are sequestered away from trafficking vesicles. Retrieval by contrast is a process whereby proteins that exit a compartment in which they are resident are recognized in a later compartment and returned to the compartment in which they are resident. It is possible, in certain cases that both processes operate together to maintain a protein in a specific organelle. In order for proteins to be maintained in a specific compartment retention / retrieval signals are needed so that the protein can be recognized as a resident protein for a specific organelle.

1.3.4 Retention and retrieval sequences responsible for endoplasmic reticulum localisation

KDEL / HDEL

Many soluble endoplasmic reticulum proteins contain the carboxy-terminal tetra peptide, KDEL or HDEL which is necessary for retrieval from the Golgi (Munro and Pelham, 1987; Pelham, 1997). The KDEL / HDEL sequence is recognized by a receptor in the Golgi called Erd2p (Semenza *et al.*, 1990). The Erd2p receptor binds to proteins that have escaped the endoplasmic reticulum and entered the slightly acidic environment of the Golgi. Erd2p then recycles the protein back to the endoplasmic reticulum and releases it. Deletion of KDEL / HDEL from endoplasmic reticulum localized proteins results in their leakage from the endoplasmic reticulum in to the secretory pathway (Munro and Pelham, 1987). If the tetrapeptide is attached to the C-terminus of normally secreted proteins such as lysozyme then endoplasmic reticulum localisation can result (Munro and Pelham, 1987). Experiments with calreticulin revealed that retention and retrieval both play a role in maintaining this protein in the endoplasmic reticulum. A mutant form of calreticulin was expressed in COS cells which lacked a Ca^{2+}-binding domain that also appeared to be important for its retention, but still had a KDEL sequence for retrieval. This mutant form of calreticulin was found to leak from the endoplasmic reticulum and be secreted at a much higher rate, at 18% of total calreticulin compared to 1-2% for wild type calreticulin. This showed that retention for this protein was important for its localisation. A further mutation to the (Ca^{2+}-binding domain deficient calreticulin) removing the KDEL sequence increased the secretion to 29% thus it appears that both mechanisms are

important for the maintenance of calreticulin in the endoplasmic reticulum (Sonnichsen *et al.*, 1994). Erd2p is packaged into COP I coated vesicles in the Golgi, which will in turn return proteins that are bound to the receptor back to the endoplasmic reticulum. Like many other transmembrane proteins Erd2p contains a di-lysine signal (Lewis and Pelham, 1990). This K(X)KXX consensus sequence at the C-termini interacts with COP I coat proteins (Cosson and Letourneur, 1994). This means Erd2p binds KDEL tagged proteins and interacts with COP I via its di-lysine motif to return these escaped proteins. The di-lysine motif itself may act in other proteins as a retention signal.

Di-lysine motifs

Chimeras containing KKAA tags have been shown to be actively retained in the endoplasmic reticulum (Andersson *et al.*, 1999). Even though COP I binds KKAA in vitro, the endoplasmic reticulum retention signal is not dependent on COP I because retention of KKAA tagged chimeras was not affected in a ε-COP deficient cell line (Andersson *et al.*, 1999). Unlike the KDEL sequence the di-lysine sequence is not as conserved although mutation of the lysine residues to arginine or histidine destroys residency in the endoplasmic reticulum (Jackson *et al.*, 1990). Di-lysine retrieval seems to occur predominantly from the ERGIC compartment. There are reports that some di-lysine bearing proteins have been retrieved from medial and trans-Golgi compartments. This was seen as some proteins were exposed to glycoprotein processing enzymes which are only found this far on in the trafficking route (Jackson *et al.*, 1993; Martire *et al.*, 1996). The di-lysine tagged proteins seem to be retrieved by COP I vesicles from these compartments. The evidence that COP I is indeed the coat proteins responsible for

returning di-lysine tagged proteins to the endoplasmic reticulum is overwhelming (Pelham, 1994).

Di-arginine motifs for type II membrane proteins

Type II transmembrane proteins have a di-arginine motif for endoplasmic reticulum localisation the arginine residues are usually located at positions +2 to +5 of the amino acid sequence usually side by side or separated by one amino acid (Schutze *et al.*, 1994). Addition of this di-arginine signal to a plasma membrane type II membrane protein results in their localisation to the endoplasmic reticulum. The XXRR motif has been recognized as the motif responsible for localizing Ii p33 isoform of invariant chain to the endoplasmic reticulum. Using this protein it was found that two lysine residues could not substitute for the di-arginine motif. If the di-arginine sequence is transplanted onto the human transferin receptor or *N*-acetylglucosylaminyltransferase (type II proteins) usually located on the cell surface and in the Golgi stack then these proteins are relocated to the endoplasmic reticulum. Similar endoplasmic reticulum localisation sequences exist on TRAM and p63 (Schutze *et al.*, 1994). TRAM has eight transmembrane spanning domains and both its amino and carboxyl termini are located in the cytoplasm. Both cytoplasmic domains carry endoplasmic reticulum targeting motifs, suggesting this protein may be located in the endoplasmic reticulum by both di-lysine and di-arginine residues.

Rer 1p

Rer1p is a 188 amino acid residue peptide containing 4 transmembrane domains, the protein is also well conserved among species (Boehm *et al.*, 1997). Mutation of Rer1p caused mis-targeting of Sec12p to the TGN (Nishikawa and Nakano, 1993), suggesting that this might be a receptor candidate. Following this discovery it was found that other endoplasmic reticulum membrane proteins including Sed4p, Sec71p, Sec63p and Mns1p use the Rer1p receptor retrieval mechanism (Sato *et al.*, 1996a; Sato *et al.*, 1997). An important observation is that not all of these proteins have the same topology as Sec12p (Sec12p is a type 2 transmembrane protein) (Nakano *et al.*, 1988), Sec71 is type 3 (Feldheim *et al.*, 1993) and Sec63p spans the membrane 3 times (Sadler *et al.*, 1989) (see figure 15 for protein classification). Rer1p is however able to recognize these proteins and direct them back to the endoplasmic reticulum (Sato *et al.*, 1997). The Rer1p receptor seems to be able to direct these leaked proteins into Cop I vesicles which shuttle the escaped proteins back to the endoplasmic reticulum (Boehm *et al.*, 1997; Sato *et al.*, 1997). There is evidence to suggest that Rer1p is able to recognize retrieval signals in the transmembrane domains of various proteins (Letourneur and Cosson, 1998; Sato *et al.*, 2001).

1.3.5 Golgi localisation signals

A number of targeting signals have been identified for resident Golgi proteins. However the actual mechanisms of localisation remain relatively elusive. There is a large array of different proteins that are resident in the Golgi apparatus. The Golgi apparatus is also a

diverse organelle containing a number of discrete compartments. Due to the complexity of the Golgi apparatus a number of mechanisms may have evolved to ensure the correct sorting of Golgi resident proteins. As appears to be the case for the endoplasmic reticulum both retention and retrieval mechanisms appear to operate in the Golgi. TGN 38 a Golgi resident protein contains a tyrosine based motif (SDYQRL). This sequence is needed for the internalisation of this protein from the plasma membrane to the TGN (Bos *et al.*, 1993; Wong and Hong, 1993). This sequence interacts with the protein AP2, that is necessary for the formation of clathrin coated vesicles carrying material from the plasma membrane (Roquemore and Banting, 1998). The transmembrane domain of TGN 38 also appears to act as Golgi localisation signal and may function in Golgi retention (Ponnambalam *et al.*, 1994).

The endoprotease furin has two separate signals within its cytoplasmic tail, one signal for retention and one for retrieval. A tyrosine based motif (YKGL) is necessary for endocytosis, while an acidic cluster containing two serine residues is required for its retention (Jones *et al.*, 1995). Thus multiple signals are involved in Golgi localisation. An additional mechanism that may be important in maintaining Golgi resident proteins relates to the formation of a distinct population of lipids that have similar hydrocarbon tail chain lengths and hence a different thickness forms in the membrane in certain regions. These lipids are thought to preferentially interact with one another to form rafts in the membrane (Simons and Ikonen, 1997). Proteins preferentially partition into these rafts if they have similar length transmembrane regions. Certain proteins may not partition into these rafts that exit the TGN. Such a mechanism could act to retain specific proteins in the TGN (Munro, 1995).

1.3.6 Phospholamban and sarcolipin targeting and retention

As discussed previously phospholamban and sarcolipin are short C-terminally anchored proteins. This means they are unable to be inserted into the endoplasmic reticulum co-translationally as they have already left the ribosome before any possible signal sequence could protrude for the SRP to interact with. This means that endoplasmic reticulum insertion has to be post-translational. It is likely that phospholamban and sarcolipin interact with cytoplasmic chaperones that prevent interactions with other proteins or membranes. Such chaperones may be important for directing the proteins to the endoplasmic reticulum so they can be inserted appropriately, presumably by some sort of insertion machinery. There may be significant overlap with this machinery and the usual components of the membrane protein insertion pathway (Abell *et al.*, 2003). The insertion machinery may indeed still be translocon like components or even the translocon. Once in the endoplasmic reticulum phospholamban and sarcolipin are maintained there but they have none of the characterised retention or retrieval signals within their sequence. This thesis will examine what residues are important in endoplasmic reticulum targeting and the maintenance of phospholamban and sarcolipin in this compartment. Also addressed is whether retention or retrieval is the major process responsible for maintaining them in the endoplasmic reticulum. It could of course be a combination of both retention and retrieval that maintains these two proteins in the endoplasmic reticulum. Rer1p may play a role in the retrieval of phospholamban and sarcolipin. This receptor appears to interact with the transmembrane region of proteins to effect retrieval. Particular attention will be given to the role of the transmembrane domains in the retrieval process.

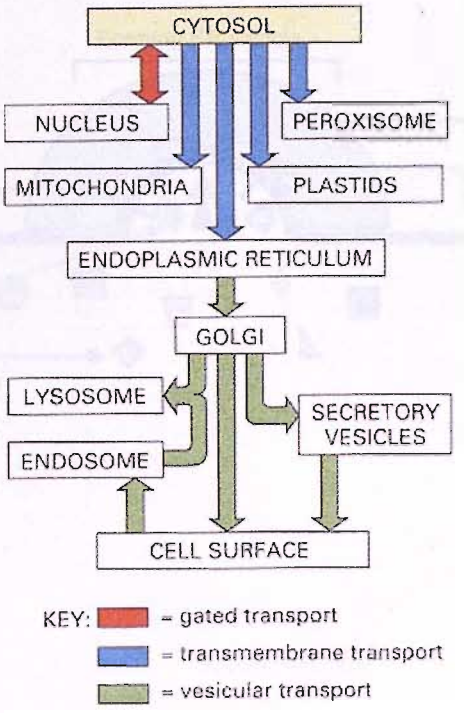

Figure 10

A flow diagram showing the trafficking of proteins through the cells secretory pathway

All proteins start synthesis on cytoplasmicly located ribosomes. From here they are targeted to all sorts of compartments to carry out their specific role. The mistargeting of a protein for example a pump or ion channel could have a devastating effect and so trafficking within cells is highly organised and stringently regulated. Taken from Alberts *et al.*, (1994).

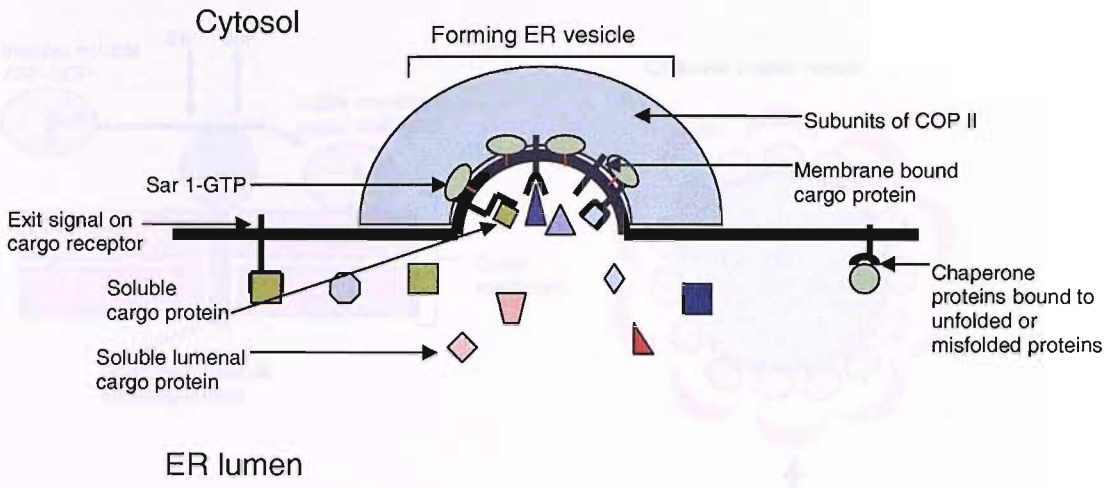

Figure 11

Selective transport from the endoplasmic reticulum

This diagram shows the formation of a vesicle that is about to bud from the endoplasmic reticulum. The activated Sar1p protein recruits COP II coat protein to the membrane, which in turn causes, the curving of the membrane into a forming vesicle structure. Within the forming vesicle there are specific transmembrane receptors that are capable of recognising soluble lumenal cargo proteins or even other transmembrane proteins. These can then be selectively packaged in to the secretory vesicle. The vesicle eventually buds from the endoplasmic reticulum and traffics to the ERGIC compartment.

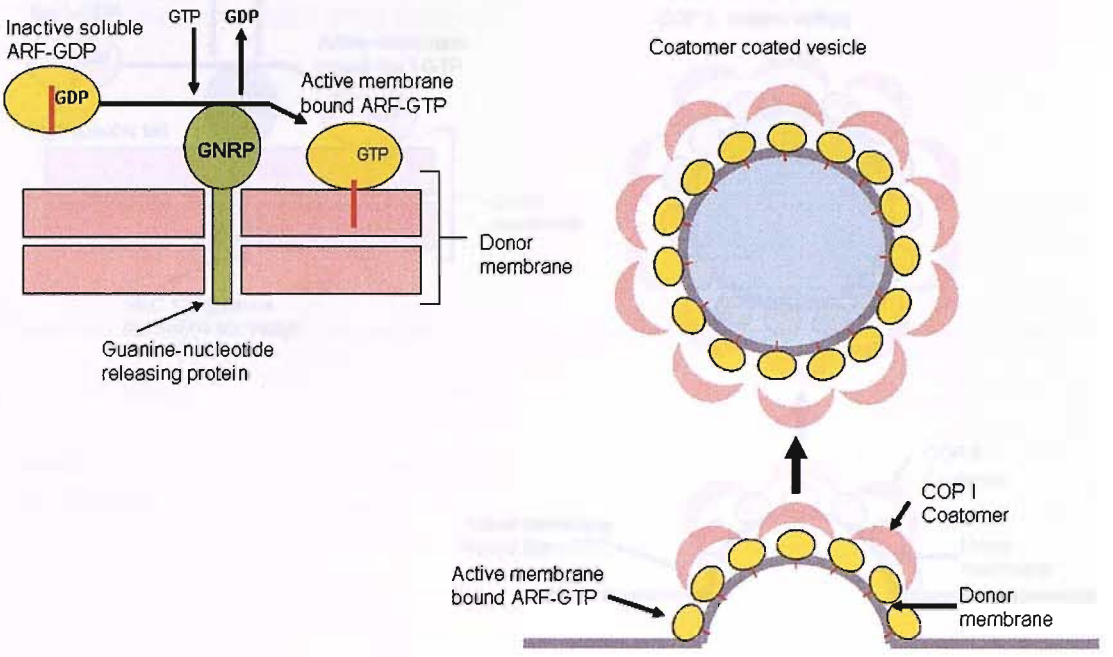

Figure 12

The activation of ARF 1 recruits COP I coatamer proteins to the membrane

The inactive ARF 1 protein has a hydrophobic tail tucked up inside its protein structure. Once ARF 1 has exchanged GDP for GTP it becomes activated and the hydrophobic tail becomes exposed. The tail is responsible for ARF 1 insertion and binding to the cytoplasmic side of the Golgi apparatus membrane. ARF 1 then attracts COP I coatamer proteins. The coatamer proteins are then responsible for causing curvature of the membrane and the beginning of vesicle formation.

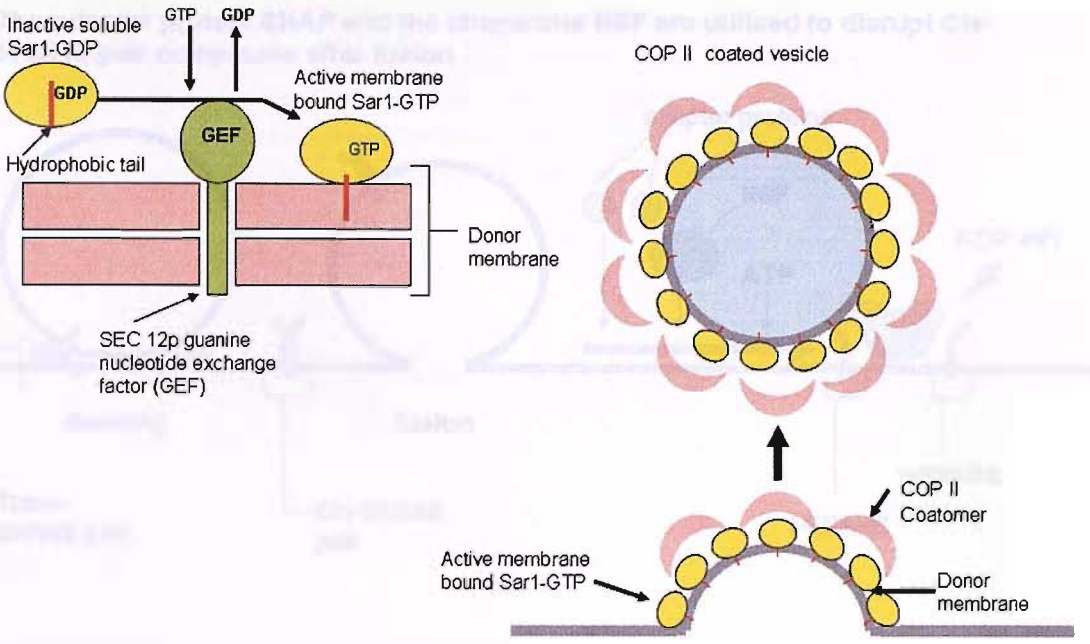

Figure 13

Sar1 activation and endoplasmic reticulum vesicle biogenesis

Sar1 is a small GTPase that is activated by Sec12p. Once GDP has been exchanged for GTP Sar1 enters an active state. Once activated Sar1 binds to the endoplasmic reticulum membrane via its hydrophobic tail. Sar1 then begins to recruit Sec23p/24p coatamer proteins, once bound these then recruit Sec13p/31p. The coming together of these coatamer proteins causes curvature of the membrane and initiates vesicle biogenesis. COP II uncoating is caused by GTP hydrolysis by Sar1. Sec23p acts as a GTPase activating protein for Sar1. This GTPase activity increases 10 fold when Sec23p is in association with Sec13p/31p.

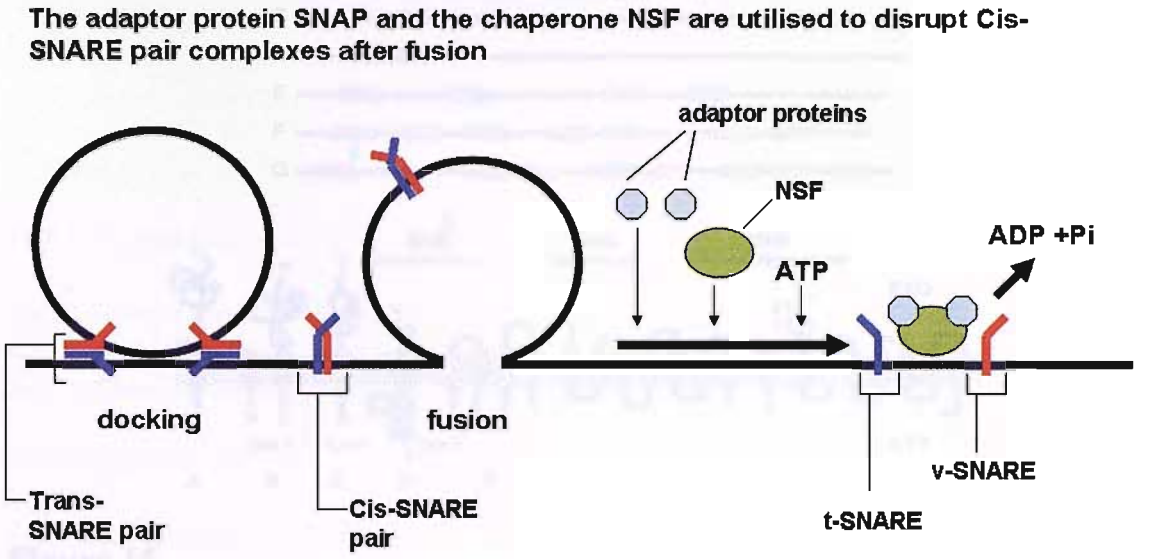

Figure 14

SNARE disassembly

The cytoplasmic tails of SNAREs interact with each other to form stable four-helix bundles referred to as SNARE pins. The formation of these SNARE pins brings the vesicle membrane and target membrane close enough for fusion to occur. If the v-SNARE and t-SNARE are compatible with each other they form a structure called a trans-SNARE pairs. After the fusion of the two membranes the SNAREs adopt a new structure called a cis-SNARE pairs as they are now on the same membrane. NSF and SNAP act together to disrupt these cis-SNARE pairs. NSF binds to these assembled SNARE complexes via SNAP. The hydrolysis of ATP results in the disassembly of the cis-SNARE complex and the SNAREs are recycled for use again.

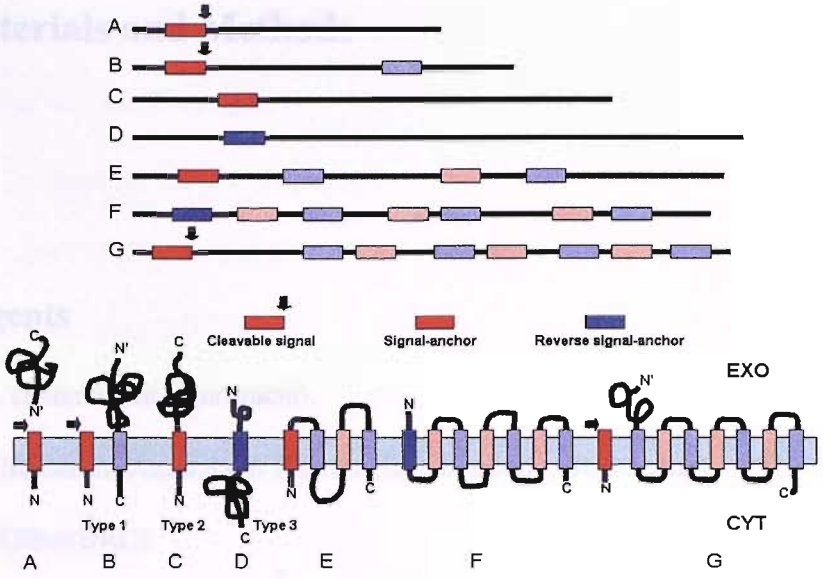

Figure 15

Nomenclature used to describe transmembrane proteins

Three types of signals initiate co-translational protein topogenisis. Cleavable signal sequences (red with arrow head indicating signal peptidase cleavable site) and uncleavable signal anchor sequences (red without arrow head) induce translocation of the C-terminal sequence and assume an N_{cyt}/C_{exo} orientation. Reverse signal anchors (dark blue) insert with the opposite N_{exo}/C_{cyt} orientation and translocate their N-terminus. More complex topologies are produced by the combination of the signal with additional transmembrane sequences inserting in alternating orientations. It is easy to see how more complex transmembrane proteins can be organised within cellular membranes. The diagram also depicts the 3 main types of protein orientation referred to as type 1, type 2 and type 3. Type 1 proteins are inserted via their C-terminus after the removal of a cleavable signal sequence. Type 2 proteins are inserted via their N-terminus with an uncleavable anchor sequence. Type 3 proteins are inserted via their C-terminus with a reverse signal anchor sequence. This figure was modified from Higy *et al.*, (2004).

2.1.0 Materials and Methods

2.1.1 Reagents

Agarose NA (Amersham Pharmacia).

Antibodies (Serotech, Amershem Pharmacia, Chemicon, Stressgene).

Bacto agar (GibcoBRL).

Dimethyl Sulphoxide DMSO, sterile-cell culture grade (Sigma).

Dubelcos Modified Eagles Medium – DMEM (GibcoBRL).

Dried skimmed milk powder (Marvel).

Easy gel acrylamide/bisacrylamide mix (Scotlab).

ECL western blotting detecting kit (Pierce).

Foetal bovine serum – FBS (GibcoBRL).

Fugene 6 transfection reagent (Roche).

Fungizone (GibcoBRL).

Gentamycin (GibcoBRL).

Hank's Balanced Salt Solution – HBSS (GibcoBRL).

LB broth EZ mix (Promega).

Nycodenz carbohydrate gradient (Calbiochem).

Protease inhibitor cocktail (Sigma).

Restriction enzymes (Promega, New England Bioscience).

Trypsin EDTA 10X (GibcoBRL).

Wizard™ DNA clean-up kit (Promega).

Wizard™ Miniprep kit (Promega).

Wizard™ PCR clean-up kit (Promega).

Oligonucleotides (MWG-Biotech).

100bp DNA ladder (GibcoBRL).

1Kilobase DNA ladder (GibcoBRL).

2.1.2 Equipment

0.2 μm disposable filters (Scheicher and Schuell).

13 mm x 2mm coverslips (Biorad).

14 mm diameter sterile tissue culture plates (Greiner).

Electroporation cuvettes (Biorad).

Microcentrifuge tubes 1.9ml (Eppendorf).

Microcentrifuge tubes 0.5ml (Eppendorf).

Sterile 20 ml syringes (Becton Dickinson).

Sterile 50 ml centrifuge tubes (Falcon).

Sterile Disposable 3 ml pipettes (Alpha laboratories).

Sterile 20ml sealable tubes (Bibby Sterilin).

Tissue culture flasks and dishes (Falcon).

2.1.3 Methods

2.1.4 Glycerol stocks

All bacterial clones were stored as glycerol stocks. These were made by adding 1 ml of bacterial culture to 0.5 mls of sterile glycerol in 2 ml cryotubes. The glycerol stocks were then stored at -80 ^{0}C.

2.1.5 Growth media

All media were sterilised using an autoclave that raised the temperature to 126 ^{0}C for 30 minutes.

2.1.6 LB broth

Luria broth was made by adding 6.2 g of LB broth EZ mix (Promega) to 300 mls of water. The pH was then checked and set to pH 7 before autoclaving.

2.1.7 LB Amp

To sterilised LB broth that had cooled below 50 ^{0}C ampicillin was added 180 µl of a 100 mg/ml aqueous solution.

2.1.8 Culture plates

LB agar was produced by adding 4.5 g of agar to 300 mls of LB broth. This was then autoclaved. Once the medium had cooled below 50 ^{o}C ampicillin was added 180 µl of a 100 mg/ml aqueous solution and the medium was poured into 6 cm sterile Petri dishes. The petridishes were then left to set for 15 minutes before drying at 37 ^{o}C.

2.1.9 SOB media

SOB media was produced by dissolving tryptone (20 g), yeast extract (5 g) and NaCl (0.5 g) in 1 L of distilled water. The solution was then sterilised by autoclaving.

2.2.0 SOC media

To the SOB media 20mM glucose was added through a sterile syringe and 0.2 µm sterile filter.

2.2.1 DNA techniques

2.2.2 Vector DNA

All constructs were cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen) (see figure 17). The vector contains a multiple cloning site, a bacterial origin of replication this enables the growth of the plasmid in bacterial cells, and 2 promoters T7 and CMV these enable the expression of cloned genes in a mammalian cells. The vector contains two antibiotic selectable genes; ampicillin for selection in bacterial cells and neomycin for selection in eukaryotic cells. COS cells are an SV 40 transformed cell line which means they express the large T-antigen. This recognises the SV 40 origin of replication on the vector and results in the replication of the vectorial DNA.

2.2.3 Small scale DNA purification

5 ml aliquots of LBamp were inoculated with single colonies of DH5-α strain of *E.coli* containing the construct of interest. These cultures were then incubated for 16 hours at 37 ^{0}C, shaking at 200 rpm to allow the bacteria to grow. Plasmid DNA was extracted using the Promega Wizard^{TM} Miniprep Kit, in accordance with the instructions supplied with the kit.

2.2.4 Agarose gels

For a 1% agarose gel 1g of agarose was dissolved in 100 mls of 1x TAE buffer (40 mM Tris HCl, 40 mM glacial acetic acid, 1 mM EDTA) by heating in the microwave for 2 minutes. To the molten agarose 5 µl of ethidium bromide (10 mg/ml) was added. Gel loading buffer (30% glycerol in 1 x TAE with 3 mg/ml bromophenol blue) was added at the ratio of 1 µl for every 5 µl of DNA solution before the samples were loaded onto the agarose gel. The gel was then electrophoresed at 100 V for approximately 30 minutes. The DNA in the gel was then visualised using a UVP GDS 5000 illuminator and camera. For the resolution of smaller DNA fragments 2% agarose gels were sometimes used.

2.2.5 Extraction of DNA from agarose gels (Freeze-Squeeze)

The agarose gel containing the seperated DNA fragments was placed on a transilluminator to visualise the fragments. The required fragment(s) was cut out from the gel with a sterile scalpel blade. The excised fragment containing the DNA was placed into a 0.5 ml centrifuge tube with siliconised glass wool forming a plug at the bottom of the tube. The tube was snap frozen in liquid nitrogen for 5 minutes. The tube then had a hole pierced in the bottom using a 25-gauge syringe needle and was then placed inside a 1.9 ml centrifuge tube. The 1.9 ml tube containing the 0.5 ml tube was placed into a microfuge and spun at 6000 rpm for several minutes. This centrifugation was repeated several times until no more liquid could be collected. The liquid that was collected was treated with a Promega Wizard™ DNA clean up kit to extract the DNA from the impurities that came from the gel.

2.2.6 Restriction digests

Restriction digests were either carried out as test digests to identify positive clones or as large digests to cut enough plasmid DNA for ligation reactions. For a test digest typically 1 μg of DNA was cut in a volume of 10 μl with the appropriate restriction endonucleases and the buffer recommended for the enzyme. The test digests were incubated at the recommended temperature (usually 37 ^{0}C) for 1 hr. The large digests in which fragements for ligation reactions were isolated usually involved the digestion of 7 μg of plasmid DNA with the appropriate restriction endonucleases and recommended buffer. These digests were incubated for 4 hrs at the appropriate temperature.

2.2.7 Ligation reactions

After digestion of the DNA, the cut fragments required were separated by electrophoresis using a 1% agarose gel. The DNA in the gel slice was then extracted by the Freeze-squeeze method (section 2.2.5). Fragments not requiring gel separation were isolated directly by the use of Promega Wizard™ DNA clean up kit. Ligations were performed using 200 ng of vector at a ratio of vector : insert ranging from 5:1 – 1:1. Ligations were performed with T4 DNA ligase (Promega) at 16 ^{0}C overnight following the manufacturer's protocol.

2.2.8 Transformation of *E.coli* DH5-α cells by electroporation

DH5-α strain of *E.coli* were electroporated with DNA from ligations in the following way:

The ligation reactions were dialysed by floating 0.025 μm VS disk filters (Milipore) on the surface of 20 mls of 10% glycerol solution and then placing a droplet of the reaction mix onto the top surface of the filter. The dialysis was carried out for approximately 20 minutes to remove enough salt from the solution so that electroporation could take place. 5 μl of DNA was taken from the dialysed ligation reactions and mixed with 60 μl of electro-competent cells. This solution was left mixing on ice for 1 minute. The DNA / cell mix was placed into a 2 mm electroporation cuvette and placed in the Biorad State model electroporator. The DNA / cell mix was then given a high voltage electric pulse (2.5 kV) as specified in the manufacturer's handbook. After the shock the DNA / cell mix was rapidly resuspended in 1 ml SOC media. The medium was then placed in an incubator at 37 ^{0}C and shaken for 30 minutes. The bacterial suspension was then spread onto LBamp plates to select transformed colonies of bacteria. Ampicillin resistant bacterial colonies were then used to isolate plasmid DNA so that diagnostic restriction digests could be performed to identify the bacterial colonies containing the required insert.

2.2.9 PCR amplification of DNA

PCR reactions were set up in 0.5 ml thin walled microfuge tubes containing a total volume of 100 µl.

PCR reaction mix:

81.2 µl	Sterile distilled water
10 µl	Pfu polymerase buffer
0.8 µl	dNTP mix (25 mM each)
1.0 µl	DNA template 100 ng/µl
2.5 µl	Primer # 1 (100 ng/ µl)
2.5 µl	Primer # 2 (100 ng/ µl)
1.0 µl	Pfu polymerase (2.5 U/µl) (Promega)

The Pfu polymerase is always added last to reduce the possibility of denaturation of the enzyme.

The tubes were then placed in a MJ Research PTC-200 thermal cycler and the thermocycling regime shown in table 2 was carried out to amplify the required DNA sequence.

Typical PCR cycle:

Stage	Cycles	Temperature	Length of time
1	1	96°C	45 seconds
2	30	96°C Primer Tm-5°C 72°C	45 seconds 45 seconds 2 minutes/kb
3	1	72°C	10 minutes

Table 2

A table to show the typical cycling parameters for a polymerase chain reaction.

2.3.0 DNA Sequencing

MWG Biotech performed DNA sequencing. Approximately 7 μg of DNA was provided along with the appropriate template primers. The sequencing was performed using ABI capillary sequencers and the results in the form of electropherograms were returned. Normally 700 bp of sequence could be interpreted from the electropherogram.

2.3.1 Preparation of electro competent DH5-α cells

A single colony of DH5-α *E.coli* was used to inoculate 50 mls of LB media and left to shake in an incubator at 37 ^{0}C, 200 rpm for 16 hours. The resulting culture was then split

into two 1 L flasks each containing 500 mls of LB media. This was then placed to shake at 37 ^{0}C 200 rpm until culture density achieved an absorption of 0.5 at 600 nm. The flasks were then placed on ice for 5 minutes. The cultures were then centrifuged in a Sorval RC 28s ultra centrifuge at 4000 g for 20 minutes at 4 ^{0}C. The pellets were then resuspended in 1 L ice cold sterile distilled water. After a second centrifugation step at 4000 g the pellets were resuspended in 500 mls of sterile distilled water, centrifuged again at 4000g and resuspended in 20 mls 10% glycerol this was then centrifuged again at 4000 g. finally the pellet was resuspended in 3 mls of 10% glycerol solution and stored as 60 µl aliquots at -80 ^{0}C after snap freezing in liquid nitrogen.

2.3.2 Polyacrylamide gels for SDS-PAGE

A discontinuous buffer system was used for SDS-PAGE, with a separating gel set onto a resolving gel. Two different percentage gels were made depending upon the size of the protein of interest. The gel mixes were made up as follows.

Running gel

	10%	15%
Protogel (30% acrylamide 0.8% Bis-acrylamide)	6.6 mls	9.9 mls
Tris HCl 1.5 M pH 8.8	3.4 mls	3.4 mls
SDS 10%	0.2 mls	0.2 mls
APS 25%	0.24 mls	0.24 mls
TEMED	10 µl	10 µl
SDW	9.56 mls	6.26 mls

Stacking gel

Protogel (30% acrylamide 0.8% Bis-acrylamide)	0.6 mls
Tris HCl 3.6 M pH 9.3	1.6 mls
SDS 10%	40 µl
APS 25%	20 µl
TEMED	4 µl
SDW	1.7 mls

Samples were dissolved in sample buffer and loaded onto the gel. The gel wells would hold up to 40 µl of sample. The gels were normally run at 45 mA for 45 minutes.

2.3.3 SDS Polyacrylamide gel transfer

Proteins were transferred from SDS-PAGE gels onto nitrocellulose (Hybond^{TM}-C; Amersham Biosciences) as outlined by (Colyer *et al.*, 1989). The gel was equilibrated for 10 minutes in a transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3). A wetted sheet of nitrocellulose paper was laid on top of the gel and trapped air removed. This was then sandwiched between 3M filter paper (Whatman) and nylon scouring pad supports and placed in the transfer apparatus with the nitrocellulose paper on the anodal side. Electrotransfer was performed at 100 V, 500 mA for 2 hours at room temperature. After that the nitrocellulose could be dried and stored or the immunoblotting procedure could be started. Non-specific binding sites on the nitrocellulose were blocked overnight in PBS 0.05% tween (PBST) supplemented with 5% milk powder before western blotting commenced.

2.3.4 Western blotting procedure

The nitrocellulose membrane was washed in PBST 3 times for 5 minutes each to remove the blocking agent. The primary antibody was diluted to a working concentration in 5 mls of PBST and was then applied to the membrane and incubated for 1 hr with gentle agitation. The unbound primary antibody was then removed by washing 3 times with PBST for 5 minutes. The secondary antibody was then diluted and incubated with the membrane as outlined for the primary antibody. The membrane was again washed 3 times to remove unbound secondary antibody. The membrane was then incubated with

Supersignal Western Dura ECL detection kit (Pierce) as instructed by the manufacturer. The signals were then detected using a Biorad Versa Doc detection system.

2.3.5 Mowiol mountant

2.4 g of mowiol (Calbiochem) was added to 6 mls of glycerol and 6 mls of water. This mixture was stirred continuously for 3 hrs. 12 mls (0.2 M) Tris HCl was then added to the mixture and the pH was adjusted to 8.5 the mixture was then stirred for a further 10 minutes at 50 ^{0}C. Insoluble material was removed by centrifugation at 5000 g for 15 minutes. The supernatant was collected and antibleaching agent (Citifluor) was added to give a final concentration of 0.1%. 500 µl aliquots of the mowiol mountant were then frozen at –20 ^{0}C.

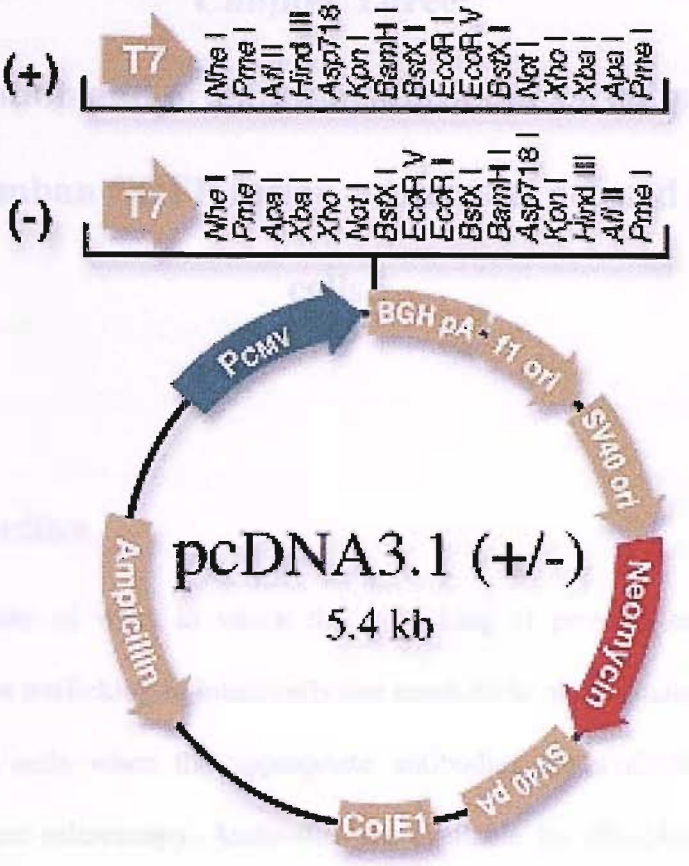

Figure 16

pcDNA3.1 (+) the vector used for COS cell expression

The diagram shows the main features of the vector used in these studies. The vector has a CMV promoter (232-819bp) that causes high-level expression in mammalian cell lines. The vector encodes bacterial origins of replication at the pUC ori site to allow multiple copies of the plasmid to be produced by *E. coli* cells. An ampicillin resistant gene is present which allows selection in *E.coli*. The plasmid also has an SV40 origin of replication, which allows DNA replication in SV40 transformed cells such as COS 7 cells. This diagram was taken from www.invitrogen.com/contents/sfs/manuals

Chapter Three:

Localisation and orientation studies of sarcolipin and phospholamban EGFP fusion proteins expressed in COS 7 cells.

3.0.0 Introduction

There are a number of ways in which the trafficking of proteins can be followed. However, to follow trafficking in intact cells one needs to be able visualise the protein of interest. In fixed cells when the appropriate antibodies are available one can use immunofluorescence microscopy. Antibodies are available for phospholamban (Suzuki and Wang, 1986; Li *et al.*, 1990) but not for sarcolipin. Sarcolipin is small and highly hydrophobic. This means it is unlikely to be a good immunogen, but more importantly it has virtually no hydrophilic domains to which an antibody could bind when it is in association with a membrane. Sarcolipin has been visualised by attaching a commercial epitope (FLAG epitope NH_2-MDYKDDDDK-M^1) (IBI) to its N-termini (Odermatt *et al.*, 1998). The use of immunofluorescence has one major draw back in that for visualisation of internal antigens it cannot be used to follow trafficking in living cells. In the study presented here constructs were designed so that sarcolipin and phospholamban were tagged at their N-termini with the fluorophore enhanced green fluorescent protein (EGFP). When a protein is tagged with a marker it is necessary to ensure that the protein of interest still behaves as the native protein. In this chapter the aim is to demonstrate that

when both phospholamban and sarcolipin are tagged with EGFP they still are targeted to the endoplasmic reticulum and that they still adopt the correct orientation within this membrane.

3.1.0 Green fluorescent protein

GFP was discovered in 1962 after being isolated from the Jellyfish *Aequora Victoria.* The protein was seen to have good potential as a tool for studying the localisation and trafficking of proteins. However, it was only really when molecular biology techniques became available that this proteins properties could be exploited in the production of fusion proteins. The fluorophore present in GFP is created by the nucleophilic attack by one of its amino acid residues on an adjacent residue. This means that the fluorescence seen requires no cofactors or activating enzymes hence the protein can be expressed in most cell types and used as a fluorescent tag (Tsien, 1998). At present there are approximately five variants of GFP with different spectral properties, which enables the simultaneous study of more than one protein in the same cell.

3.1.1 Methods

3.1.2 Tagging of phospholamban and sarcolipin with EGFP

The first step in tagging phospholamban and sarcolipin was to ligate EGFP DNA coding sequence into pcDNA3.1 (+). The plasmid pcDNA3.1 (+) was digested with endonucleases Hind III and Kpn I. The cut vector was then isolated using a Promega Wizard™ clean up kit ready for the EGFP sequence to be ligated in. The sequence coding for EGFP was amplified from pEGFP-N1 vector (Clonetech) using the polymerase chain reaction. Oligonucleotides were designed in such a way that endonuclease sites Hind III and Kpn I were added on to the ends of the sequence. The oligonucleotides also allowed the removal of the stop codon from EGFP so that phospholamban and sarcolipin coding sequence could be ligated downstream of the EGFP sequence in subsequent steps. The following oligonucleotides were used for the amplification of the EGFP coding sequence. The restriction sites in the oligonucleotides have been shown. Hind III is indicated in red and Kpn I is shown in blue. (EGFP FWD 5'-GAT TCT AAG CTT ACC ATG GTG AGC AAG GGC GA-3') and (EGFP REV 5'-ACA GTT GGT ACC CTT GTA CAG CTC GTC CAT GC-3').

Following this pcDNA3.1 (+) and EGFP sequences were analysed by separation on a 1% agarose gel to check the size and concentration of the DNA fragments before setting up a ligation reaction (see figure 17).

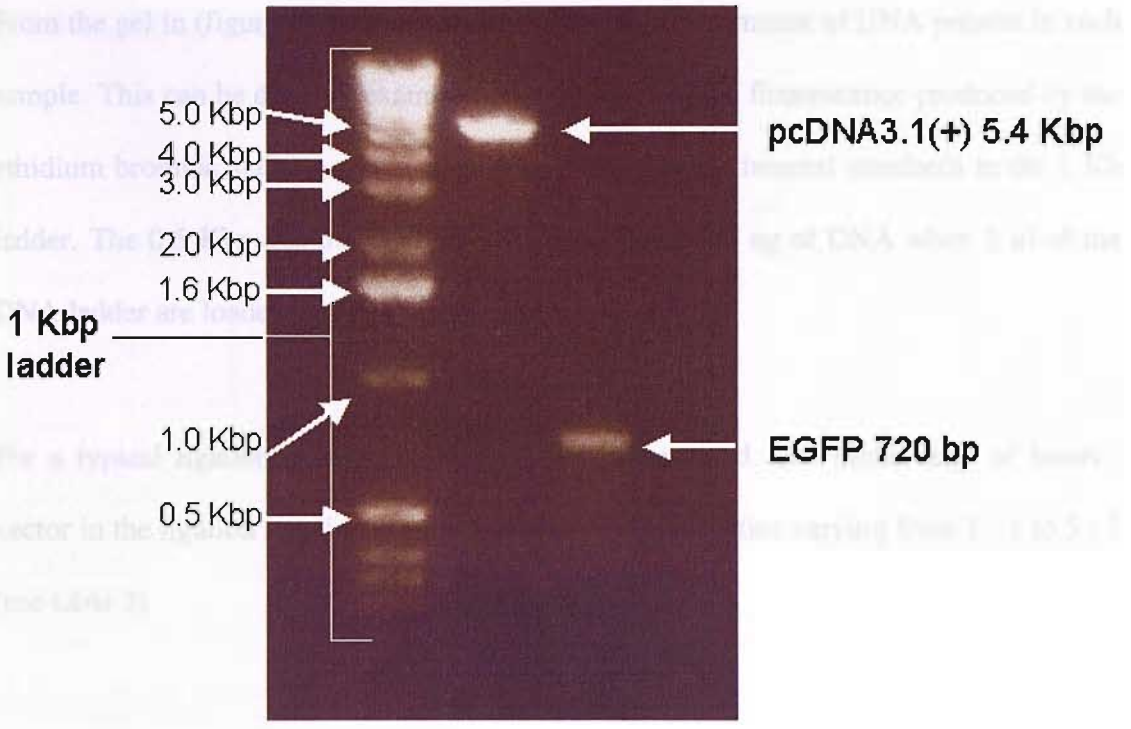

Figure 17

Cloning of pcDNA3.1 (+) EGFP

An agarose gel used to evaluate sequences encoding restriction digested pcDNA3.1 (+) and EGFP. The gel shows 5 µl of pcDNA3.1 (+) digested with Hind III and Kpn I electrophoresed along side 5 µl of EGFP coding sequence produced from the polymerase chain reaction, purified using Promega Wizard™ clean up kit and digested with the same endonucleases as pcDNA3.1 (+). Both DNA fragments were electrophoresed along side 2 µl of a 1 Kbp ladder (GibcoBRL). The gel was electrophoresed at 110 V for 45 minutes and viewed using a UVP GDS 5000 illuminator and camera.

From the gel in (figure 17) it was possible to estimate the amount of DNA present in each sample. This can be done by examining the intensity of the fluorescence produced by the ethidium bromide staining of the 2 samples relative to the internal standards in the 1 Kb ladder. The 0.5 Kbp and the 1.6 Kbp markers contain 200 ng of DNA when 2 μl of the DNA ladder are loaded and electrophoresed on the gel.

For a typical ligation reaction 200 ng of vector was used. The molar ratio of insert : vector in the ligation reaction was normally attempted at ratios varying from 1 : 1 to 5 : 1 (see table 3)

3.1.3 Ligation conditions for EGFP and pcDNA3.1 (+)

	Ligation 1	Ligation 2	Ligation 3	Ligation 4
Vector (80 ng/µl)	2.5µl	2.5µl	2.5µl	2.5µl
Insert (30 ng/µl)	1µl	2µl	5µl	----
T4 Buffer	2µl	2µl	2µl	2µl
SDW	12.5µl	11.5µl	8.5µl	15.5µl
T4 DNA ligase (3 U/µl)	2µl	2µl	2µl	----
Total	20µl	20µl	20µl	20µl

Table 3

A table to show the ligation conditions for EGFP and pcDNA3.1 (+)

The typical conditions used to ligate PCR generated inserts (720 bp) with pcDNA3.1 (+) (5.4 Kbp). The concentration of vector and insert were approximated by comparing the fluorescence of aliquots of vector and insert with standard markers separated on agarose gels and stained with ethidium bromide. The ratios of vector : insert was varied from a molar ratio of 1 : 1 to 5 : 1 (Ligations 1 – 3). Ligation number 4 includes no insert and acts as a negative control.

After an overnight ligation reaction *E.coli* DH5-α cells were transformed with the DNA and transformants were selected on Lbamp plates. The negative control included in the ligation (ligation in table 3) provides an initial guide as to whether the ligation has been successful. Growing colonies were selected from cells transformed from (Ligations 1 – 3); their plasmid DNA extracted using the Promega Wizard™ Miniprep Kit and the DNA analysed for the presence of a 720 bp fragment in pcDNA3.1 (+) following a restriction digest with restriction endonucleases Hind III and Kpn I.

3.1.4 Production of the coding sequence for sarcolipin

The sequence of the rabbit sarcolipin gene was obtained from the EMBL data base (www.ebi.ac.uk/tools/) (Accession number U96091). As sarcolipin is a short protein the DNA coding sequence was synthesised directly from 6 overlapping oligonucleotides prepared by MWG-Biotech (see table 4 below)

SLN upper 23mer	5'-AAT TCC ATG GAA AGG TCT ACT CG-3'
SLN middle upper 40	5'-AGA GCT GTG TCT GAA CTT TAC CGT TGT CCT GAT CAC AGT C-3'
SLN end upper 40	5'-ATC CTT ATT TGG CTA CTA GTG CGG TCT TAC CAG TAC TGA T-3'
SLN lower 40mer	5'-GTA AAG TTC AGA CAC AGC TCT CGA GTA GAC CTT TCC ATG G-3'
SLN middle lower 40mer	5'-CAC TAG TAG CCA AAT AAG GAT GAC TGT GAT CAG CAG AAC G-3'
SLN end lower 23mer	5'-CTA GAT CAG TAC TGG TAA GAC CG-3'

Table 4

A table to show the oligonucleotides used in the synthesis of the sarcolipin coding sequence.

The oligonucleotides were annealed to produce the following coding sequence for sarcolipin.

```
5'AATTCCATGGAAAGGTCTACTCGAGAGCTGTGTCTGAACTTTACCGT
   3' GGTACCTTTCCAGATGAGCTCTCGACACAGACTTGAAATGGCA

 TGTCCTGATCACAGTCATCCTTATTTGGCTACTAGTGCGGTCTTACCAG
 ACAGGACTAGTGTCAGTAGGAATAAACCGATGATCACGCCAGAATGGTC

TACTGAT 3'
ATGACTAGATC 5'
```

The sarcolipin sequence was constructed so that following annealing the double stranded DNA would have sticky ends compatible with the vector digested with EcoR I and Xba I. This meant that once the vector DNA was digested with these restriction endonucleases the sarcolipin coding sequence could be ligated directly in to the cut vector.

The oligonucleotides were at a concentration of 100 pmol/µl. 2 µl of each of the 6 oligonucleotides was taken and added to 345 µl of sterile distilled water. The water was heated to 95 ^{0}C for 10 minutes this was allowed to cool slowly until the temperature reached 20 ^{0}C this gave the oligonucleotides time to melt and anneal in the correct order. A 1 : 10 dilution was made after annealing the oligonucleotides so the concentration was 0.056 pmol/µl and therefore the sarcolipin coding sequence could be ligated into pcDNA3.1 (+). Ligation reactions were set up with 200 ng of vector and a range of concentrations for sarcolipin insert as outlined in (table 3).

3.1.5 Introduction of a Kozak consensus sequence and making sure the coding sequences were in frame

For efficient translation of the fusion protein the following sequence is required around the start codon ACCAUGG (Kozak, 1986). This sequence indicates to the ribosome that the methionine codon underlined in the Kozak consensus sequence is the correct start codon of the protein. When the EGFP coding sequence was ligated into pcDNA3.1 (+) between the restriction sites Hind III and Kpn I a Kozak consensus sequence was added to the start codon to ensure this was recognised by the ribosome as the start codon of the EGFP fusion proteins. It was also necessary to adjust the sequence of the inserted phospholamban and sarcolipin coding sequence to ensure that they were in frame with the EGFP coding sequence and that no Kozak consensus sequences were generated around the start of their inserted sequences. This ensures that there is no competition

between conflicting Kozak consensus sequences that might lead to untagged phospholamban or sarcolipin.

3.1.6 Production of the phospholamban gene

The phospholamban coding sequence was created by a process similar to the annealing of the sarcolipin gene. Oligonucleotides were annealed together to form a double stranded coding sequence for phospholamban. All oligonucleotides apart from PLB U1 and PLB L5 were phosphorylated on the 5' ends to allow them to be ligated together. The oligonucleotides were ligated together with T_4 DNA ligase (Promega) overnight at 16 ^{0}C.

PLB U1	5'-AAT TCC ATC GAT AAA GTC CA-3'
PLB U2	5'-ATA CCT CAC TAG ATC TGC CAT TAG AAG AGC TTC AAC-3'
PLB U3	5'-CAT CGA AAT GCC TCA ACA AGC CCG TCA AAA TCT GCA-3'
PLB U4	5'-AAA CCT ATT CAT CAA TTT CTG TCT CAT TTT AAT ATG T-3'
PLB U5	5'-CTC TTG TTG ATC TGT ATT ATT GTG ATG CTT CTC TAA T-3'
PLB L1	5'-ATG GAC GAT CTA GTG AGG TAT TGG ACT TTA TCG ATG G-3'
PLB L2	5'-GGC TTG TTG AGG CAT TTC GAT GGT TGA AGC TCT TCT A-3'
PLB L3	5'-ACA GAA ATT GAT GAA TAG GTT TTG CAG ATT TTG ACG-3'
PLB L4	5'-AAT AAT ACA GAT CAA CAA GAG ACA TAT TAA AAT GAG-3'
PLB L5	5'-CTA GAT TAG AGA AGC ATC AC-3'

Table 5

A table to show the oligonucleotides used to create the phospholamban coding sequence.

After the annealing of the oligonucleotides and after several unsuccessful attempts to ligate the phospholamban sequence into pcDNA3.1 (+) at restriction sites EcoR I and Xba I another approach was tried. Two new oligonucleotides were designed to enable amplification of any completely annealed phospholamban sequence (PLB UPPER 41 MER 5'-AAA ATA GAA TTC CAT CGA TAA AGT CCA ATA CCT CAC TAG-3') and (PLB LOWER 33 MER 5'-GGA GGA TCT AGA TTA GAG AAG CAT CAC AAT GAT-3'). Following the successful amplification of the full-length coding sequence for phospholamban the amplified product was digested with restriction endonucleases EcoR I and Xba I. The digested fragment was then isolated by the freeze-squeeze method (section 2.2.5) and ligated into the pcDNA3.1 (+) vector.

3.2.1 COS 7 cells

COS 7 cells were used for the expression and visualisation of EGFP tagged constructs. These are a widely used cell line derived by transformation of another established cell line (simian CV 1 cells). The CV 1 cells are transformed with an origin defective SV 40 virus, and contain large T-antigen for DNA replication (Gluzman, 1981).

3.2.2 Transfection

COS 7 cells were grown to 60-80% confluence in either 24 well tissue culture plates or large 145 cm^2 culture dishes. These cells were transfected using Fugene-6 (a lipid based transfection reagent). Fugene-6 was diluted in DMEM without antibiotics and foetal

bovine serum at a ratio of 3 µl Fugene-6 to 1 µg construct DNA as described in the manufacturers handbook. The Fugene-6 / DNA mixture was left for 15mins at room temperature before being added to the COS 7 cells drop wise. The cells could then be harvested 2 days after transfection to produce microsomes or viewed by fluorescence microscopy. In later experiments it was necessary to transfect cells with two constructs at the same time that would express two different proteins. Transfecting cells with SERCA1a and phospholamban as well as SERCA1a and sarcolipin expressing constructs was achieved by mixing the expression vector DNA of both SERCA1a and either phospholamban or sarcolipin and transfecting in a similar way as for a single expressing construct.

3.2.3 Visualisation of EGFP tagged constructs in living COS 7 cells

COS 7 cells were grown routinely in 145 cm^2 flasks washed with Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (HBSS) with 10% Trypsin EDTA. The Trypsin / HBSS solution lifts the cells from the bottom of the flask. The flasks are also tapped to split apart large clumps of cells. The cells were then collected and diluted with DMEM containing foetal bovine serum (10%), fungizone (1%) and gentamycin (0.4%) (25 mls) in a 50 ml collecting tube. These cells were then seeded on to 13 mm glass coverslips at 60-80% confluence in 24 well plates. The cells were left to adhere to the coverslips for 3 hrs before being transfected (section 3.2.2) with the appropriate construct. After 48 hrs the coverslips were removed from the culture dishes, washed in PBS and mounted onto microscope slides using mowiol mountant with 0.1% citifluor (Mancini *et al.*, 2003). The

cells were then viewed under a Leica digital epifluorescence microscope (DM IRBE) or a Zeiss confocal microscope (LSM 510 META). The Leica digital epiflourescent was fitted with standard FITC and rhodamine filter sets for the excitation of both EGFP and Texas Red fluorophores. The Zeiss confocal microscope collected images sequentially. The EGFP fluorophore was excited with a laser at 488 nm with a band pass filter of 505 – 530 nm. The Texas Red fluorophore was excited with a laser at 543 nm with a long pass filter of 560 nm.

3.2.4 Immunofluorescence microscopy

The COS 7 cells were treated as outlined for the visualisation of EGFP-tagged proteins except that following the removal and washing of the coverslips in PBS the following protocol was followed. The coverslips were washed twice in PBS for 2 minutes. The coverslips were fixed in 100% methanol for 10 minutes. The methanol was removed and the coverslips were air-dried (these dried coverslips could be kept at –20 ^{0}C for 2-5 weeks before processing). Before processing the coverslips for immunofluorescence examination the coverslips were then soaked in PBS containing 0.1% Triton X-100 (PBST) for 15minutes to permeabilise the membranes. The PBST was removed and 100 µl of diluted primary antibody in PBST was added. The primary antibody was left to incubate for 1 hr at 37 ^{0}C. Each sample was then rinsed in PBST with gentle agitation for 5 minutes at room temperature (this step was repeated 3 times). The secondary antibody was then diluted and 100 µl added to the coverslips and incubated for a further 1 hr at 37 ^{0}C. This secondary antibody was then removed using the same wash procedure. The

coverslips were then rinsed in PBST for 5 minutes, blotted dry and mounted onto glass slides using mowiol mountant with 0.1% citifluor. The cells were then viewed under a Leica digital epifluorescence microscope (DM IRBE) or a Zeiss confocal microscope (LSM 510 META) as outlined in (section 3.2.3).

In the phospholamban and sarcolipin colocalisation with SERCA1 experiments the primary antibody used was Y/1F4 (a mouse monoclonal SERCA specific antibody) at a dilution of 1 : 10 with PBST (Colyer *et al.*, 1989). The secondary antibody used was an anti-mouse Texas Red linked conjugate antibody (Amersham Pharmacia) used at a dilution of 1 : 50 with PBST.

3.2.5 Step-wise permeabilisation of the plasma and endoplasmic reticulum membranes

Transfected cells expressing the appropriate constructs were washed in PBS and then fixed in the 24 well plate using 4% formaldehyde solution in PBS for 15 minutes. The cells were then washed once in PBS, containing either Triton X-100 (0.1%) or saponin (0.2-0.4 mg/ml) and then incubated in buffer P (PBS supplemented with 2% milk powder and either 0.2-0.4 mg/ml saponin or 0.1% Triton X-100 as appropriate) for 30 minutes. Low levels of saponin (0.1-0.4 mg/ml) selectively permeabilise the plasma membrane without any major effect on the endoplasmic reticulum membrane. Triton X-100 (0.1%) on the other hand permeabilises all the cell membranes. The cells were then incubated with primary antibody (mouse anti EGFP 1 : 100) in buffer P for 1 hr at 37 ^{0}C. The cells

were then washed 3 times in buffer P to remove unbound primary antibody. The secondary antibody (anti-mouse Texas Red linked conjugate 1 : 50) was then added to the cells in buffer P and incubated for 1 hr at 37 ^{0}C. The cells were then washed 3 times in buffer P to remove unbound secondary antibody and then washed once in PBS before being viewed under the Leica digital epifluorescence microscope (DM IRBE).

3.2.6 Expression of lumenal endoplasmic reticulum EGFP

COS 7 cells were transfected as outlined in (section 3.2.2) with a construct made by (I.D.A. Van Goethem PhD 2002) designated GAPK. This construct codes for a fusion protein of EGFP attached to an N-terminal signal sequence of human α_1-anti-trypsin containing an additional C-terminal KDEL retrieval sequence. The DNA coding this fusion protein was cloned into pcDNA3.1 (+) for efficient expression in COS 7 cells. This protein was used as an endoplasmic reticulum located marker.

3.2.7 Determination of transmembrane topology

The orientation of phospholamban EGFP and sarcolipin EGFP in the endoplasmic reticulum membrane was determined using the method of (Moss *et al.*, 1998). The method uses the protease susceptibility of phospholamban EGFP and sarcolipin EGFP in microsomes to determine whether the EGFP tag is localised in the cytoplasm or lumenal side of the membrane.

COS 7 cells were grown on a large 145 cm^2 culture plate and transfected with the construct of choice. The cells were washed twice with 5 mls of homogenisation buffer (0.25 M sucrose, 0.05 M potassium acetate, 0.005 M magnesium acetate, 0.001 M DTT, 0.05 M Tris HCl, pH 7.6) this was removed and 450 µl of homogenisation buffer was vigorously added to the centre of the plate. The cells were removed from the surface of the plate using a 5 ml syringe plunger and transferred to a 1.9 ml tube. All the cells were homogenised using 10 strokes of a ground glass homogeniser. The homogenised cells were then used to set up the following reactions or snap frozen for storage at –20 ^{0}C.

Reaction #	1	2	3
Homogenised cells	40 µl	40 µl	40 µl
Protease K	---	4 µl	4 µl
Triton X-100 (12%)	---	---	4 µl
Homogenisation buffer	8 µl	4 µl	---

The digestions were incubated on ice for 2 minutes before the reactions were stopped using 20 µl of preheated (95 ^{0}C) 3x sample buffer containing 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (0.0024 g/ml). The samples were then boiled for 5 minutes and loaded and separated on a 15% polyacrylamide gel. The separated proteins were then transferred to nitrocellulose as outlined in (section 2.3.3) and probed with anti EGFP antibodies (1 : 1000) and anti calregulin antibodies (1 : 2000) using the protocol outlined in (section 2.3.4).

3.2.8 Results

3.2.9 Phospholamban EGFP expression in COS 7 cells

Figure 18 shows the typical EGFP fluorescence of two cells expressing phospholamban EGFP. The nuclei of the cells can be clearly seen in the centre of each cell. The cells show slight perinuclear fluorescence that becomes less the further from the nuclei of the two cells. The network of membranes that runs throughout the cell is the most obvious structure visible. Although this reticular structure appears to take up most of the cell there does not seem to be a defined outline to the cell. There is no evidence of fluorescence at the plasma membrane as no distinct boundary at the edge of the cell can be seen. What does seem to be apparent is a small amount of fluorescence in the cytoplasm of the cell between the bright fluorescence of the highly branched spider web like network. The nuclei of the 2 cells also appear to be surrounded by a brighter region. This fluorescence is often associated with the localisation of the tagged proteins to the ERGIC compartment.

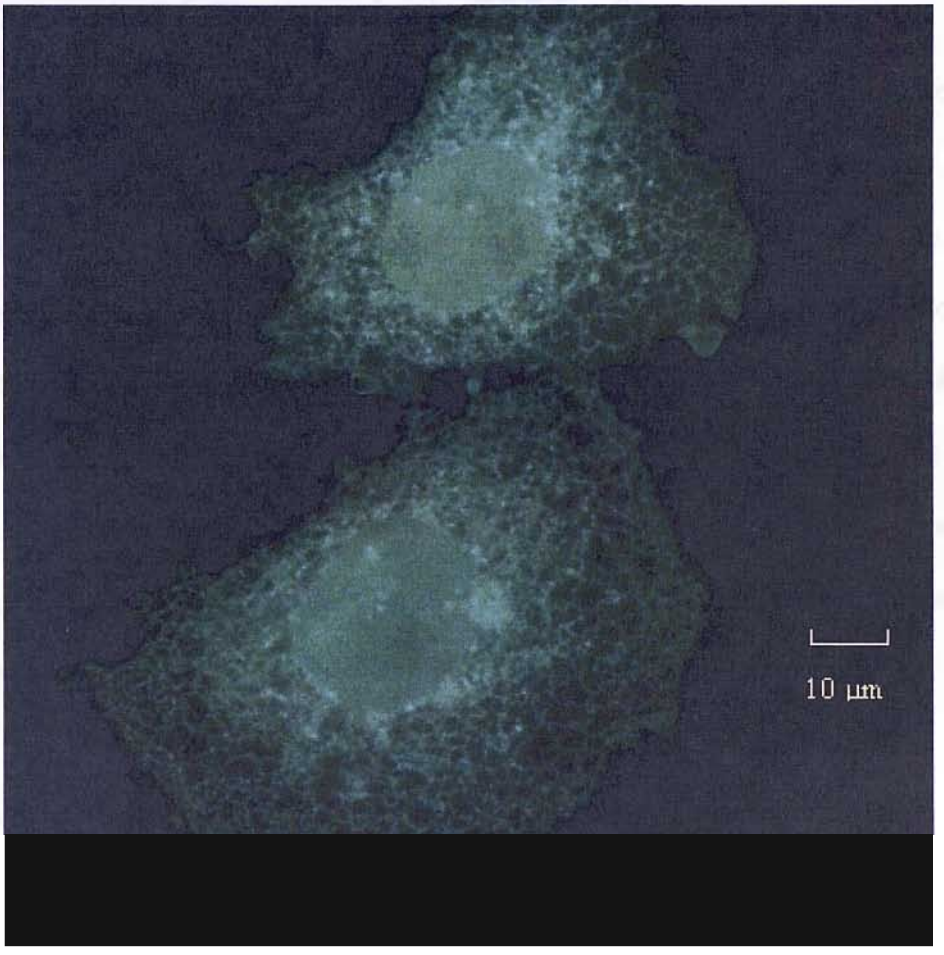

Figure 18: EGFP fluorescence in COS 7 cells transfected with pcDNA3.1 (+) containing an insert coding for phospholamban EGFP

COS 7 cells were seeded onto 13 mm coverslips at a confluency of 40-60%. They were then transfected with the mammalian expression vector pcDNA3.1 (+) containing sequence coding for phospholamban EGFP using Fugene-6 reagent. The cells were then incubated for 2 days and the coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were then viewed under a Leica digital epiflourescent microscope (DM IRBE).

3.3.0 Sarcolipin EGFP expression in COS 7 cells

Figure 19 shows the typical EGFP fluorescence of a single cell expressing sarcolipin EGFP. The pattern of expression is similar to that seen for phospholamban EGFP expression as seen in (figure 18). The nuclei of the cell can be clearly seen in the centre of the cell. The cell shows a high level of perinuclear fluorescence that is often associated with ERGIC localisation. The network that runs throughout the cell is the most obvious structure visible; again although this reticular structure appears to take up most of the cell, there does not seem to be a defined outline to the cell as no plasma membrane fluorescence can be seen. There seems also to be apparent a small amount of fluorescence in the cytoplasm of the cell between the highly fluorescent network structures.

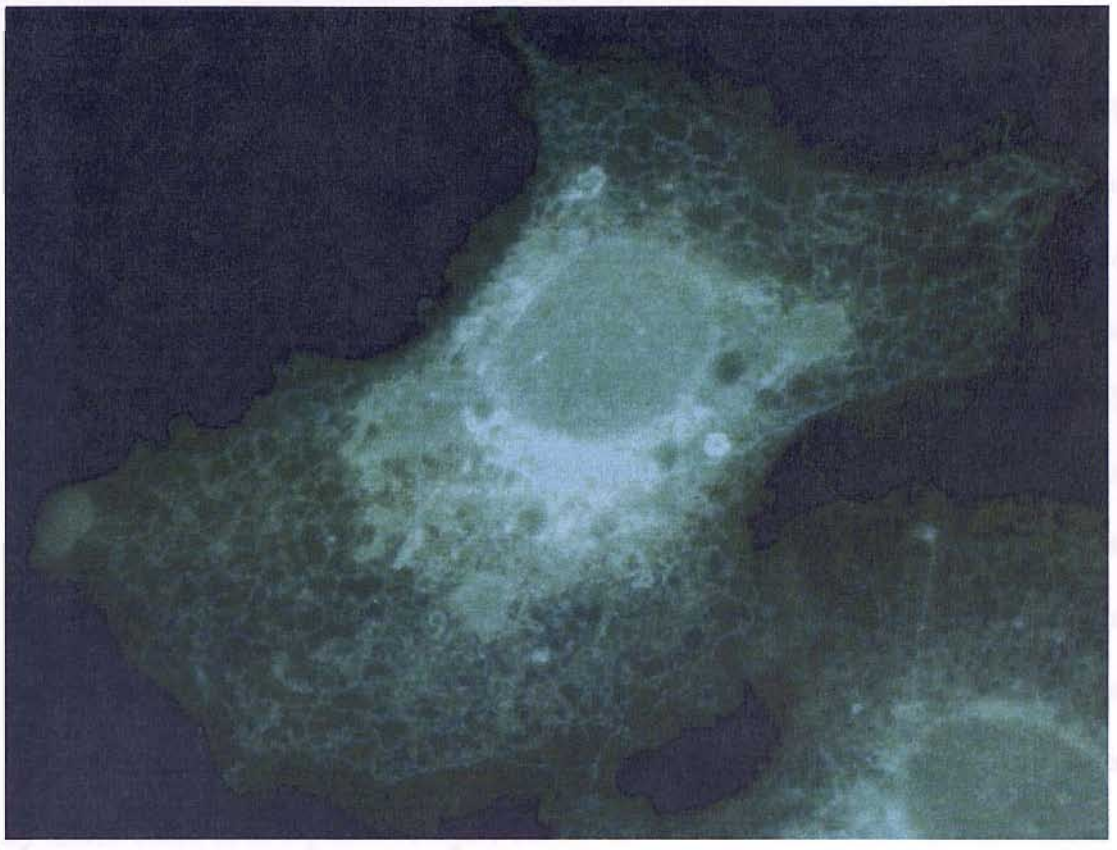

Figure 19: EGFP fluorescence in COS 7 cells transfected with pcDNA3.1 (+) containing an insert coding for sarcolipin EGFP

COS 7 cells were seeded out on to 13 mm coverslips and transfected with pcDNA3.1 (+) containing DNA sequence coding for sarcolipin EGFP fusion proteins. The cells were incubated for 2 days and mounted on microscope slides using mowiol mountant containing 0.1% citifluor. The cells were then viewed under a Leica digital eppifluorescent microscope (DM IRBE).

3.3.1 Colocalisation of SERCA1 with phospholamban EGFP

Figure 20 shows a COS 7 cell that was transfected with both phospholamban EGFP indicated in (panel A) by fluorescence of the EGFP tag, and SERCA1 indicated in (panel B) by immunolocalisation. SERCA1 was detected using a mouse monoclonal SERCA specific antibody (Y/1F4) this in turn was detected using an secondary anti-mouse Texas Red linked conjugate antibody. Panel C shows an overlay of phospholamban EGFP fluorescence and SERCA1 fluorescence. The yellow colour shown in panel C of figure 20 indicates clear colocalisation of both these two proteins to the endoplasmic reticulum. In this cell there appears to be no defined edge as no plasma membrane fluorescence can be seen and there also appears to be a relatively high level of perinuclear fluorescence. The endoplasmic reticulum network is however, the most prominent structure indicated by fluorescence in all 3 panels of figure 20.

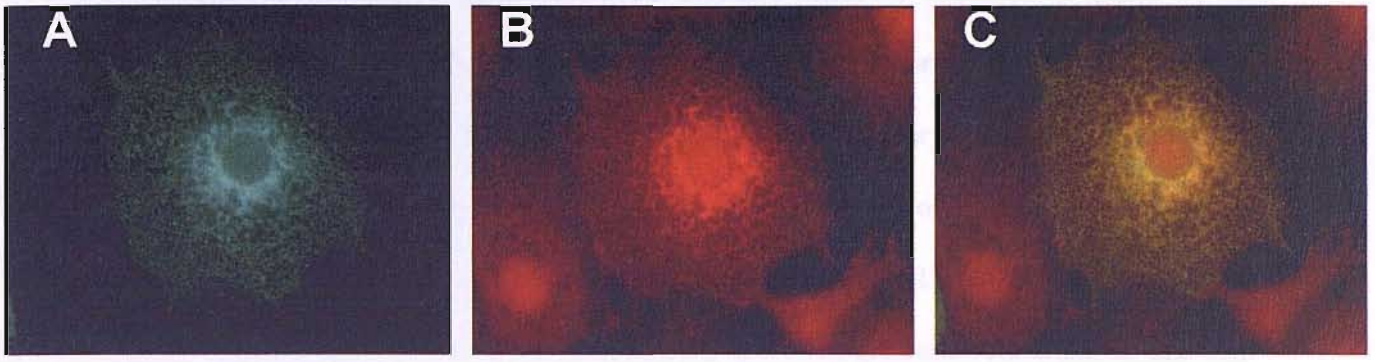

Figure 20

SERCA1 colocalisation with phospholamban EGFP

COS 7 cells were co-transfected with 2 constructs one coding for phospholamban EGFP and another coding for SERCA1. The cells were incubated for 2 days and then SERCA1 was identified by use of an antibody. Y/1F4 antibodies were used to probe the localisation of SERCA1 within the COS 7 cells (see Immunofluorescence microscopy section 3.2.4). The COS 7 cells were mounted and viewed under the Leica digital epiflourescent microscope (DM IRBE). Panel A shows the localisation of phospholamban EGFP, while Panel B shows SERCA1 localisation and SERCA1 and phospholamban EGFP fluorescence are overlaid in panel C.

3.3.2 Colocalisation of SERCA1 with sarcolipin EGFP

Figure 21 shows a COS 7 cell that was transfected with both sarcolipin EGFP indicated in (panel A) by fluorescence of the EGFP tag, and SERCA1 indicated in (panel B) by immunolocalisation. SERCA1 was detected using a mouse monoclonal SERCA specific antibody (Y/1F4) this in turn was detected using an secondary anti-mouse Texas Red linked conjugate antibody. Panel C shows an overlay of sarcolipin EGFP fluorescence and SERCA1 fluorescence. The yellow colour shown in panel C of figure 21 indicates clear colocalisation of both these two proteins to the endoplasmic reticulum. In this cell there appears to be no defined edge as no plasma membrane fluorescence can be seen and there also appears to be a relatively high level of perinuclear fluorescence especially above the nucleus of the cell. The endoplasmic reticulum network is however, the most prominent structure indicated by fluorescence in all 3 panels of figure 21.

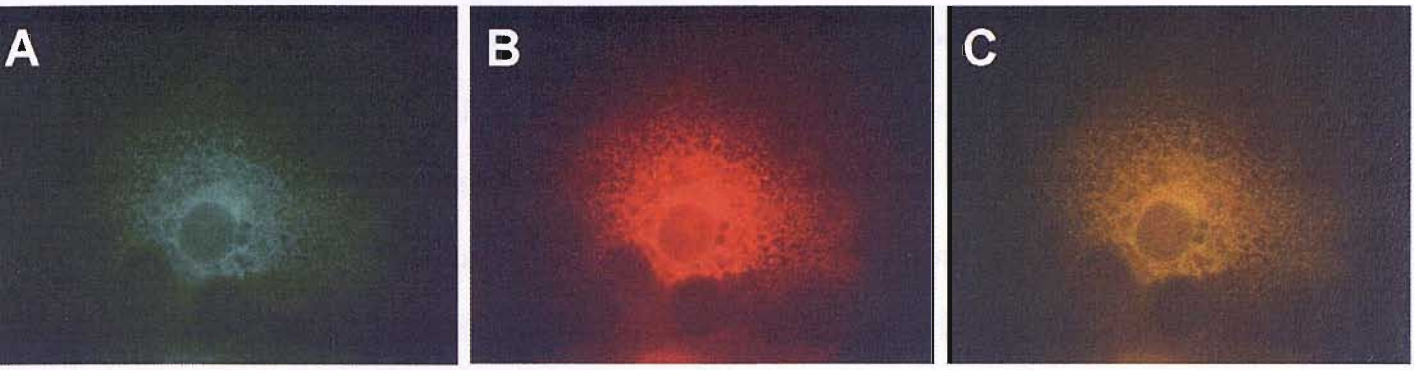

Figure 21

SERCA1 colocalisation with sarcolipin EGFP

COS 7 cells were co-transfected with 2 constructs one coding for sarcolipin EGFP and another coding for SERCA1. The cells were incubated for 2 days and then SERCA1 was identified by use of an antibody. Y/1F4 antibodies were used to probe the localisation of SERCA1 within the COS 7 cells (see Immunofluorescence microscopy section 3.2.4). The COS 7 cells were mounted and viewed under the Leica digital epiflourescent microscope (DM IRBE). Panel A shows the localisation of sarcolipin EGFP, while Panel B shows SERCA1 localisation and SERCA1 and sarcolipin EGFP fluorescence are overlaid in panel C.

3.3.3 Orientation studies of phospholamban and sarcolipin

Figure 22 shows COS 7 cells transfected with constructs coding for phospholamban EGFP (panels A, B, E, F, I and J). As well as cells transfected with a construct coding for an N-terminal leader sequence attached to EGFP tagged at its C-terminus with the endoplasmic reticulum retrieval sequence KDEL that acts as a marker of the endoplasmic reticulum lumen as well as an internal control (panels C, D, G, H, K and L). Cells shown in (panels A - D) have been treated with saline to act as a control. Panel A shows phospholamban EGFP fluorescence where as panel B shows the same cells treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody. Panel B shows a small patch of red fluorescence but no whole cells can be seen. Panel C shows fluorescence of the lumenal EGFP protein and it can be clearly seen in the fine network of the cells. Panel D shows the same cells as panel C treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody this time no red fluorescence can be seen from the Texas Red antibodies.

Cells shown in (panels E - H) have been treated with saponin which selectively permeabilises the plasma membrane. Cells shown in panel E have been transfected with phospholamban EGFP and appear similar in appearance to those in panel A. Cells shown in panel F are the same cells as shown in panel E but have been treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS containing saponin. Panel F shows the same cells, which are visible by phospholamban EGFP fluorescence, fluorescing red with Texas Red fluorescence from the antibodies. It can be seen that it is the network in the cells shown in both panels E and F that becomes visible with fluorescence. Panel G shows the fluorescence from the lumenal EGFP protein.

When these cells are treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS saponin (panel H) no Texas Red fluorescence can be seen.

Cells shown in (panels I - L) have been treated with Triton X-100 which permeabilises all cellular membranes. This time the EGFP fluorescence from both phospholamban EGFP and the lumenal EGFP protein can be seen (panels I and K). When these cells are treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS Triton X-100 the Texas Red fluorescence in these cells can be seen clearly.

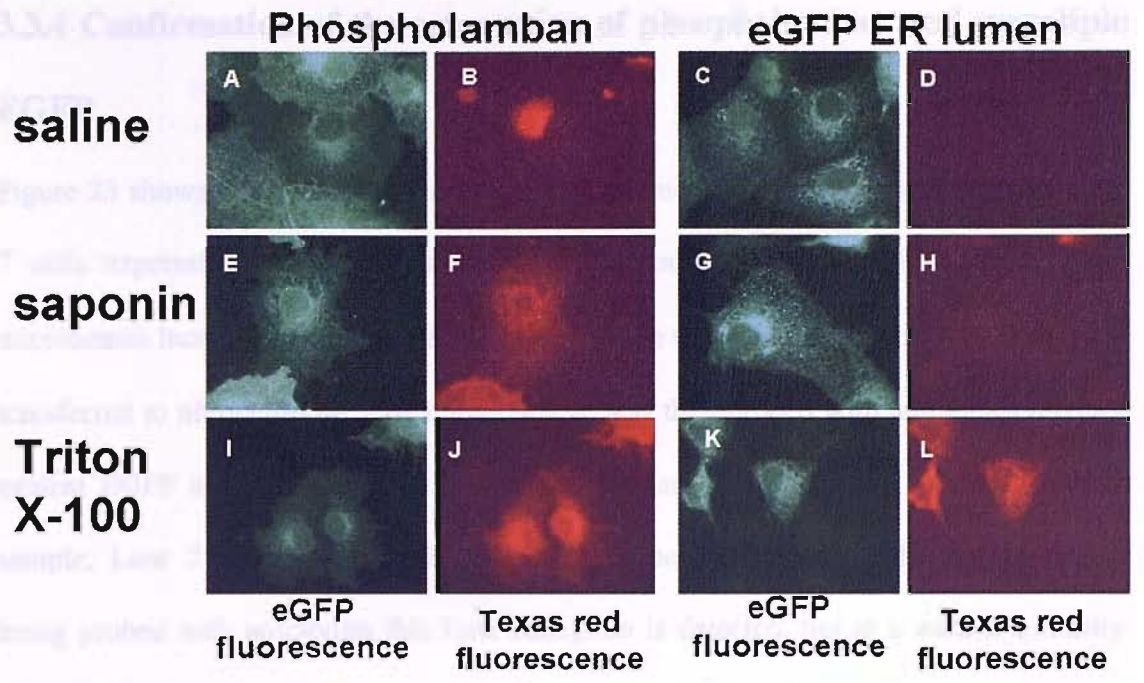

Figure 22

Selective permeabilisation studies to determine protein orientation

Cells were seeded on to 13 mm coverslips at 60-80% confluence and transfected with either constructs coding for phospholamban EGFP or EGFP targeted to the endoplasmic reticulum lumen and containing an endoplasmic reticulum retention signal (KDEL). These cells, after 2 days post transfection, were treated with either PBS / PBS containing saponin or PBS containing Triton X-100 and probed with antibodies directed against EGFP.

3.3.4 Confirmation of the orientation of phospholamban and sarcolipin EGFP

Figure 23 shows the results obtained from the treatment of microsomes made from COS 7 cells expressing either phospholamban EGFP or sarcolipin EGFP. Lane 1 shows microsomes incubated with 8 μl of buffer these were then separated with SDS PAGE and transferred to nitrocellulose. The nitrocellulose was then probed with antibodies directed against EGFP and calregulin. As can be seen in lane 1 a protein is detected for each sample. Lane 2 shows microsomes, which have been incubated with protease before being probed with antibodies this time calregulin is detected, but at a weaker intensity. The antibodies directed against EGFP have not detected any protein in lane 2. Microsomes treated with both protease and Triton X-100 detects no protein when probed with antibodies directed against EGFP or calregulin (lane 3).

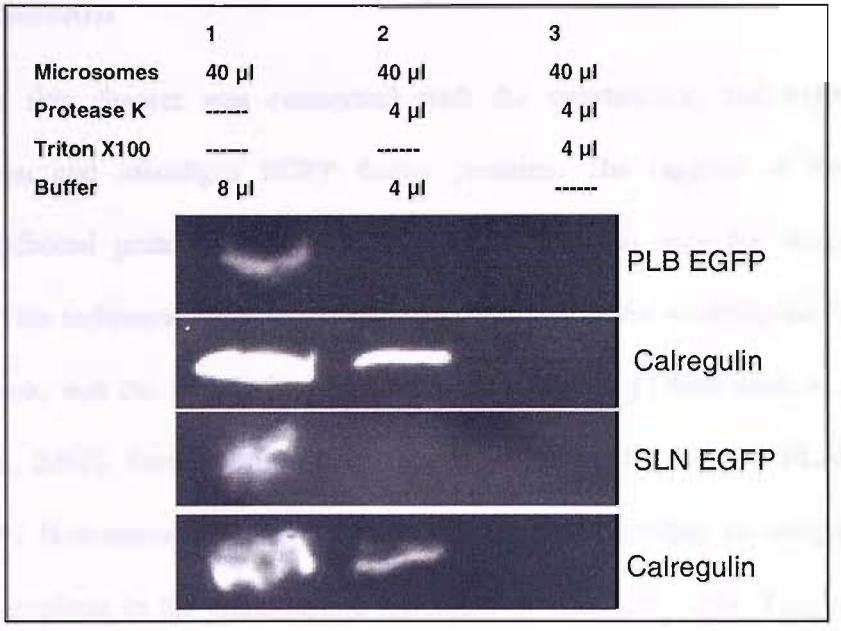

Figure 23

Proteolysis by protease K to examine the orientation of sarcolipin EGFP and phospholamban EGFP in microsomes

Microsomes made from COS 7 cells expressing phospholamban EGFP or sarcolipin EGFP were treated with either buffer (lane 1), protease K (lane 2) or Triton X-100 with protease K (lane 3). The microsomes were incubated on ice and then electrophoresed on SDS PAGE gels. They were then transferred to nitrocellulose and probed with antibodies directed against EGFP and calregulin. The gels were analysed by the use of a Supersignal Western Dura ECL detection kit (Pierce) as instructed by the manufacturer. The signals were then detected using a Biorad Versa Doc detection system.

3.3.5 Discussion

The work in this chapter was concerned with the construction and expression of phospholamban and sarcolipin EGFP fusion proteins. The tagging of the two C-terminally anchored proteins with EGFP provided an ideal way for studying their localisation. This technique has been used many times before for studying the location of various proteins, and the signals involved with their targeting (Beckmann *et al.*, 2002; Newton *et al.*, 2002). Sarcolipin has been tagged before by the use of a FLAG epitope attached to its N-terminus (Odermatt *et al.*, 1998). This provided an insight into the location of sarcolipin in the endoplasmic reticulum of HEK 293 cells. Tagging of both phospholamban and sarcolipin with EGFP however provided not only a convenient means to study their localisation but also their orientation. When a tag such as EGFP is added to a protein it may affect the targeting and the orientation of that protein in its natural environment. The experiments carried out in this chapter were to see whether the tagging of the two proteins had affected the localisation and orientation of the proteins in the endoplasmic reticulum membrane.

Firstly both sarcolipin EGFP and phospholamban EGFP fusion proteins were expressed in COS 7 cells. This was to see whether they could be expressed in COS 7 cells and to determine the types of localisation patterns within the cells. It was clear from the fluorescence micrographs (figures 18 and 19) that both phospholamban and sarcolipin although tagged still appeared to be targeted to the endoplasmic reticulum. The reticular network revealed is similar to that seen by (Odermatt *et al.*, 1998). It was also visible from these micrographs that perinuclear fluorescence from both sarcolipin and

phospholamban EGFP was visible. This is typical ERGIC localisation (Greenfield and High, 1999).

Phospholamban EGFP and sarcolipin EGFP colocalisation studies with SERCA1 support the assumption that they are in the endoplasmic reticulum. This is because SERCA1 is a known resident protein of the endoplasmic reticulum (Odermatt *et al.*, 1998; Asahi *et al.*, 1999) and so by using it as a marker for the endoplasmic reticulum one can determine if another protein has entered the same compartment. When (figures 20 and 21) are viewed it is clear that both phospholamban and sarcolipin show high levels of colocalisation with SERCA1 in the endoplasmic reticulum. This type of study was carried out by Odermatt *et al.*, (1998) with sarcolipin tagged with a FLAG epitope on its N-terminus (NF-SLN). SERCA1 and (NF-SLN) were co-expressed in HEK 293 cells and shown to colocalise to the endoplasmic reticulum by the use of immunolocalisation. These experiments here confirm and substantially back up these earlier results.

As discussed above it may be that the tagged phospholamban and sarcolipin are inserted into the endoplasmic reticulum, but the tag may interfere with the orientation of phospholamban and sarcolipin. Figure 22 shows cells that have been selectively permeabilised after they have been expressing either EGFP located in the endoplasmic reticulum lumen or phospholamban EGFP. These cells were then probed with antibodies directed to EGFP. Panels A - D show cells incubated with PBS this does not breach the plasma membrane of the cell and so antibodies cannot gain access to EGFP epitopes. Thus only EGFP fluorescence can be seen in both panels A and C with no Texas Red

fluorescence in panel D however, when looking at panel B it is possible to see a small amount of Texas Red fluorescence this is because the plasma membrane of the cell in question may be damaged slightly allowing the antibodies to gain access and cause fluorescence. No whole cells can be viewed in panel B and when looking at the red fluorescence under the microscope it was obvious that the breach in the plasma membrane of the cell was relatively small and only allowed a small leak of antibodies into the cell. The cells shown in panels E - H are incubated with saponin which selectively permeabilises the plasma membrane but not the endoplasmic reticulum membrane. These cells were then probed with antibodies directed to EGFP. Panels E and G show EGFP fluorescence but only panel F shows Texas Red fluorescence not panel H. This is due to the antibodies directed to EGFP being able to gain access to phospholamban's EGFP tag as it is protruding into the cytoplasm. The EGFP in the lumen of the endoplasmic reticulum is still protected by the endoplasmic reticulum membrane and this means that the antibodies directed to EGFP can not gain access thus cannot bind and produce a signal. This experiment indicates that phospholamban is inserted in its correct orientation C-terminal first leaving the EGFP tag protruding in to the cytoplasm and as such can be recognised by anti EGFP antibodies. If phospholamban orientation had been reversed then the endoplasmic reticulum membrane would have protected the EGFP tag and no Texas Red fluorescence would have been seen. Panels I - L show cells incubated with Triton X-100. This permeabilises all cellular membranes, hence panels I and K show EGFP fluorescence and panels J and L show Texas Red fluorescence as now the EGFP directed antibodies can gain access not only to the phospholamban EGFP tag but also to the previously protected lumenal EGFP. This

experiment gave a good insight in to the orientation of phospholamban EGFP but due on the basis of this evidence alone it cannot be ruled out that the phospholamban EGFP has been inserted randomly in different orientations. For example it was still possible that this protein was adopting a 50 : 50 orientation where half the phospholamban EGFP was inserted via its C-terminus and half inserted via its N-terminus this would have still yielded the same results using this experimental design. This question has been addressed in the studies employing protease K to proteolyse EGFP tagged phospholamban and sarcolipin in microsomal preparations. COS 7 cells that had been expressing the fusion proteins were homogenised to form microsomes. These microsomes were treated with protease K that would digest any proteins on the extra-lumenal face of the microsome. The EGFP tag on the N-terminus of phospholamban and sarcolipin would therefore be digested if it were on the extra-lumenal face of the microsome. If however, the N-terminus of the protein was inserted into the microsome then the EGFP tag would be protected from digestion. The EGFP tag could again be identified with the use of an anti EGFP antibody. Another antibody was used against calregulin a resident lumenal protein of the endoplasmic reticulum. This could be used to judge the vesicle integrity and act as a control. When intact microsomes were treated with protease K it could be seen that EGFP is susceptible to hydrolysis (figure 23 lane 2) whereas calregulin is protected. The reduction in calregulin detected following protease K treatment maybe explained by partially leaky microsomes. Alternatively some calregulin maybe lost to the external medium during microsome formation and thus would be susceptible to proteolysis. As expected when Triton X-100 is used to permeabilise the microsomes both EGFP and calregulin were susceptible to proteolysis (figure 23 lane 3). This experiment supports the

selective permeabilisation study. It also shows that it is unlikely that the phospholamban and sarcolipin tagged proteins adopt a 50 : 50 orientation. If this was the case one would expect to see some protection from proteolysis from protease K in the absence of Triton X-100, but this did not occur and hence supports the presumption that both phospholamban EGFP and sarcolipin EGFP are inserted into the endoplasmic reticulum membrane via their C-termini.

Together these results show categorically that tagging phospholamban and sarcolipin has no major effects on the targeting or insertion of phospholamban and sarcolipin within the endoplasmic reticulum membrane. These tagged proteins can therefore justifiably be used to examine the processes of targeting and maintenance in the endoplasmic reticulum in more detail.

Chapter four:

The cellular trafficking of sarcolipin EGFP and phospholamban EGFP fusion proteins expressed in COS 7 cells.

4.1.0 Introduction

As discussed in the protein sorting section (1.1.8) proteins start their life being manufactured by ribosomes located in the cytoplasm. If these proteins have a signal sequence at their N-terminus this will cause elongational arrest of the ribosome, as the signal sequence will interact with an SRP. The SRP then directs the partially constructed protein along with the ribosome to the endoplasmic reticulum membrane. Once the ribosome docks on to the translocon within the endoplasmic reticulum membrane translation resumes. The protein can then become a lumenal protein of the endoplasmic reticulum or become a membrane protein of the endoplasmic reticulum. Phospholamban and sarcolipin however, do not reach the endoplasmic reticulum in this manner. This is because their synthesis is completed before they emerge from the ribosome (Whitley *et al.*, 1996). C-terminal anchored proteins like phospholamban and sarcolipin are thought to interact with chaperones in the cytoplasm after their synthesis and release from the ribosome. These chaperones direct these types of proteins to the endoplasmic reticulum membrane for insertion (Wattenberg and Lithgow, 2001). Cross-linking studies by Abell

et al., (2004) show that SRP has a role in the insertion of some C-terminal anchored membrane proteins such as Sec 61β and Syb 2. Cross-linking studies showed strong evidence of interaction of Sec 61β and Syb 2 with SRP after translation by the ribosome. A cell free system in which cytoplasmic components were added to a membrane insertion assay revealed that Sec 61β and Syb 2 require SRP, GTP and an intact SRPR for efficient insertion into the endoplasmic reticulum membrane (Abell *et al.*, 2004). The translocon is still thought to be the main protein responsible for the actual insertion of the C-terminally anchored proteins in to the membrane.

The work in chapter 3 shows that both phospholamban EGFP and sarcolipin EGFP fusion proteins are localised to the endoplasmic reticulum. The data in chapter 3 also show that phospholamban and sarcolipin adopt the correct orientation in the endoplasmic reticulum membrane. However the mechanism by which sarcolipin and phospholamban are maintained in the endoplasmic reticulum has not been addressed. Two possible mechanisms for the location of phospholamban and sarcolipin can be envisaged. They may be maintained there by a process of retention in which the rate at which they exit the endoplasmic reticulum is very slow. Alternatively they could be maintained there by a process of retrieval in which escaping phospholamban and sarcolipin are returned to the endoplasmic reticulum from post endoplasmic reticulum compartments.

Work carried out on cytochrome b5, a typical C-terminal anchored transmembrane protein of the endoplasmic reticulum (Honsho *et al.*, 1998), showed that extension of the transmembrane region caused leakage of this protein from the endoplasmic reticulum to the plasma membrane. Honsho *et al.*, (1998) believed the process responsible for the

residency of cytochrome b5 was a static retention process with no retrieval from later compartments. Although, when this was studied in more depth by (Pedrazzini *et al.*, 2000), it was shown that a small amount of cytochrome b5 does escape the endoplasmic reticulum. This was examined by adding O-linked glycosylation sites to cytochrome b5. The O-linked glycosylation seen with this modified cytochrome b5 using [H^3]-N-acetylgalactosamine proves that the modified cytochrome b5 has visited the Golgi compartment. This is because the enzymes necessary for O-linked glycosylation are located in the Golgi apparatus. A small amount of cytochrome b5 must leak from the endoplasmic reticulum and reach the Golgi in order for the glycosylation to take place. Cell fractionation and lack of colocalisation with giantin, a marker of the later Golgi compartments, indicates retrieval of cytochrome b5 from the ERGIC / cis-Golgi compartments for more details see (Pedrazzini *et al.*, 2000).

Another protein, which is C-terminally anchored in the endoplasmic reticulum, is Sec12p. This protein was shown to be retained by the endoplasmic reticulum by a process of retention by a cytoplasmic domain and retrieval by its transmembrane domain (Sato *et al.*, 1996b). Rer1p is believed to be a receptor responsible for the retrieval of Sec12p. Rer1p has been demonstrated to recognise the transmembrane domains of many membrane proteins resident in the endoplasmic reticulum. Rer1p seems to act as a receptor in post endoplasmic reticulum compartments scanning for escaped proteins and returning them to the endoplasmic reticulum. Besides Sec12p, Rer1p is required for the correct localisation of Sed4p, Mns1p, Sec71p and Sec61p (Sato *et al.*, 1996b; Sato *et al.*, 1997). The main techniques used to study Rer1p interaction with various proteins were confocal microscopy, halo assays and cross-linking studies (Sato *et al.*, 2003). EGFP

tagged Sec12p and Sec71p were shown to localise to the endoplasmic reticulum of wild type *S.cerevisiae*. However, when yeast mutants lacking Rer1p were seen to express EGFP Sec12p and Sec71p these proteins were mistargeted to plasma membrane vacuoles. The halo assay was used to measure the amount of secreted hence non-retrieved protein escaped from *S.cerevisiae* cells. Chimeric proteins were made where Sec12p and Sec71p were fused to α-mating factor precursor (MFα1p). The MFα1p when processed by the Golgi becomes mature and active. When secreted by the α-expressing cell it inhibits the growth of *S.cerevisiae* cells on a selection plate. This leads to a so-called halo around the α-expressing cell. The size of the halo can indicate the amount of protein being secreted and hence give an approximation of the lack of retrieval to the endoplasmic reticulum. The experiment using Sec71p and Sec12p MFα1p chimeras showed that the wild type *S.cerevisiae* cells had high levels of retrieval and so very small halos were seen. But in strains that were Rer1p deficient large halos were seen indicating that Rer1p has a major role in the retrieval of these proteins. This type of experiment enabled further examination of the transmembrane domains of Sec12p and Sec71p and their role in retrieval (Minamisawa *et al.*, 2003a). Further cross-linking studies using the thiol cleavable linker (DSP) showed a direct interaction of Sec12p and Sec71p with Rer1p.

This chapter addresses the question as to whether a process of retention or retrieval is responsible for the maintenance of phospholamban EGFP and sarcolipin EGFP fusion proteins in the endoplasmic reticulum. This chapter also seeks to identify the signals responsible for maintaining these two proteins in the endoplasmic reticulum membrane. In order to follow a protein through the cells secretory pathway compartment-specific markers are needed. Antibodies directed to proteins known to be resident in

particular compartments were used. These antibodies can then be used to see whether sarcolipin EGFP or phospholamban EGFP enter a specific compartment in the cells secretory pathway. If colocalisation of a marker with sarcolipin EGFP or phospholamban EGFP occurs then it is likely that the fusion protein has entered the compartment in which that marker is located. The work with cytochrome b5 highlighted the importance of the transmembrane region in endoplasmic reticulum targeting (Honsho *et al.*, 1998). In this study the role of the transmembrane domains of sarcolipin and phospholamban in targeting has been examined.

4.2.0 Methods

In order to investigate the roles of the transmembrane regions of both phospholamban and sarcolipin, mutants in which the transmembrane regions were extended were made. These mutants were then used in conjunction with the constructs used in chapter 3 to visualise the trafficking of phospholamban and sarcolipin in COS 7 cells.

4.2.1 Construction of a phospholamban EGFP mutant with the extension of the transmembrane region by 4 leucine residues

DNA coding sequence for phospholamban was taken from the constructs previously made in chapter 3. Two oligonucleotides were designed to perform a polymerase chain reaction amplification of phospholamban. At the same time these oligonucleotides introduced 4 extra leucine residues at position 52 of phospholamban. The two oligonucleotides are shown below in the text, restriction endonuclease sites are shown in blue-(EcoR I) and in red-(Xba I). (Plb upper 41mer 5'-AAA ATA GAA TTC CAT CGA TAA AGT CCA ATA CCT CAC TAG-3') (Plb mut1 lower 49mer 5'-AGG ATA TCT AGA TTA GAG GAG GAG GAG GAG AAG CAT CAC AAT AAT ACA G-3')

The polymerase chain reaction was carried out as specified in (section 2.2.9). The resultant coding DNA for the phospholamban mutant was then isolated using a PCR clean up kit (Promega). The coding sequence was then digested along with the vector pcDNA3.1 (+) using restriction endonucleases EcoR 1 and Xba I. The vector DNA was then isolated by the freeze-squeeze method (see section 2.2.5) followed by the use of a DNA clean up kit (Promega). The phospholamban mutant coding sequence was purified

after the restriction digest by the use of a DNA clean up kit (Promega). The vector DNA pcDNA3.1 (+) and the DNA coding for the phospholamban mutant were ligated together in molar ratios ranging from (1 : 1 – 5 : 1) (insert : vector). The DNA after ligation was dialysed to remove salt. *E.coli* DH5-α cells were then transformed with pcDNA3.1 (+) containing the insert coding for the phospholamban mutant. The transformants were then transferred to ampicillin resistant plates. Colonies were identified from bacterial plates and single colonies picked from these plates were cultured overnight and then the DNA was extracted using a Wizard™ Miniprep Kit (Promega).

4.2.2 Construction of sarcolipin EGFP mutant with the extension of the transmembrane region by 7 leucine residues

To start with sarcolipin was mutated with the addition of 4 leucine residues to position 25. This was achieved by quick change site directed mutagenesis. Quick change site directed mutagenesis provides a fast efficient way to produce mutants from virtually any double stranded plasmid containing the DNA coding sequence for the gene of interest. The technique has four basic steps that are shown in figure 24

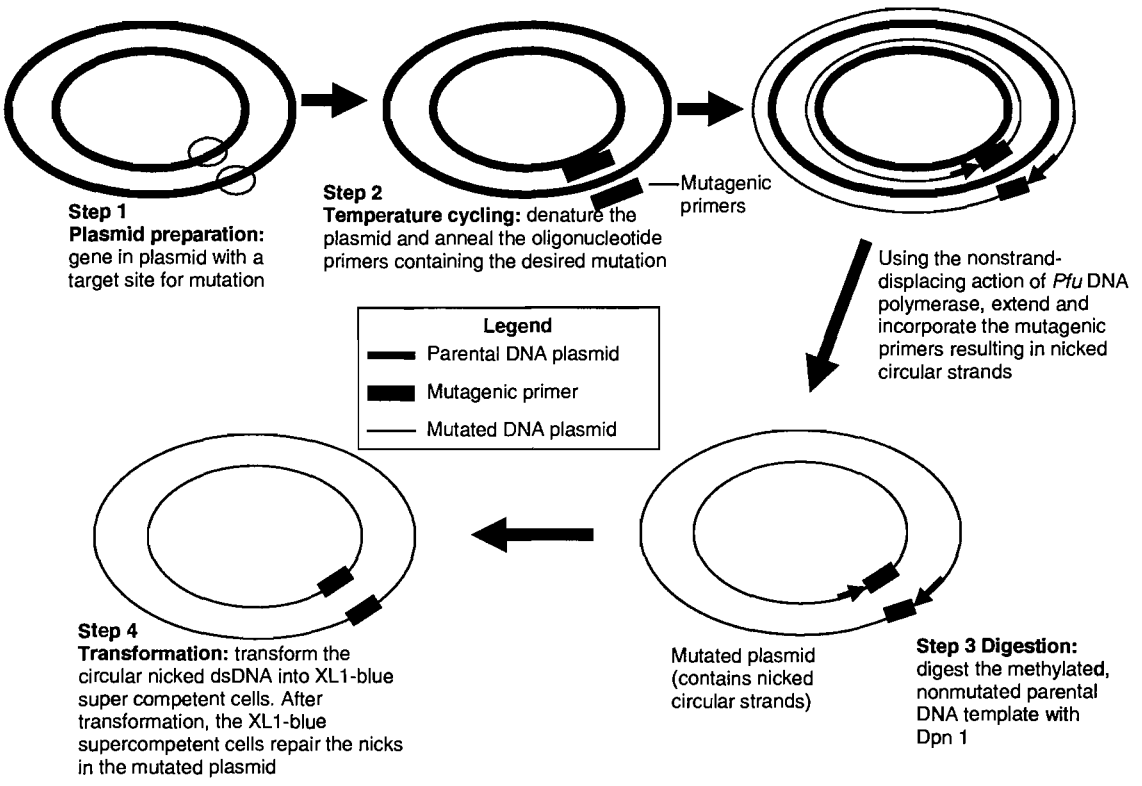

Figure 24

The four steps in quick change site directed mutagenesis

This figure shows the 4 basic steps in producing a mutation by quick change PCR. The first step is to isolate the DNA and design oligonucleotide primers, which will introduce the desired mutations during the PCR cycling. The second step is the temperature cycling and DNA replication in the PCR cycle. The third step involves the digestion of the methylated parental strands of DNA with Dpn I to leave just the newly synthesised DNA containing the mutation. Step four is to transform the plasmid containing the mutant into XL 1-Blue super competent cells. This diagram was modified from the Stratagene protocol (www.stratagene.com/manuals/200518.pdf).

Two oligonucleotides were designed which allowed the introduction of 4 leucine residues at position 25 of sarcolipin. The oligonucleotides annealed to the plasmid DNA and once the polymerised chain reaction was initiated they began to replicate the plasmid DNA incorporating the extra bases coding for the extra leucine residues. The two oligonucleotides used in the quick change reaction are shown below. (SLN MUT 1 U44 5'-CAT CCT TAT TTG GCT ACT ACT CCT CCT ACT AGT GCG GTC TTA CC-3') (SLN MUT 1 L44 5'-GGT AAG ACC GCA CTA GTA GGA GGA GTA GTA GCC AAA TAA GGA TG-3')

The following reaction mixture was set up in the production of the mutant sarcolipin:

The reaction was set up as listed below:

10 X reaction buffer	5 µl
5-50 ng of template DNA	1 µl
125 ng of oligonucleotide primer 1	1.25 µl
125 ng of oligonucleotide primer 2	1.25 µl
20 mM dNTPs	1 µl
Sterile distilled water to a final volume of	50 µl

To the above mixture 1 µl of *Pfu* DNA polymerase was added 2.5 (U/µl)

The following cycling parameters were followed to introduce the mutations into the plasmid:

Segment	Cycles	Temperature	Time
1	1	95 ^{0}C	30 seconds
2	18	95 ^{0}C	30 seconds
		55 ^{0}C	1 minute
		68 ^{0}C	1 minute / Kb

After the polymerase chain reaction had finished the product was separated by electrophoresis on a 1% agarose gel to check to see if the DNA had been amplified (see figure 25).

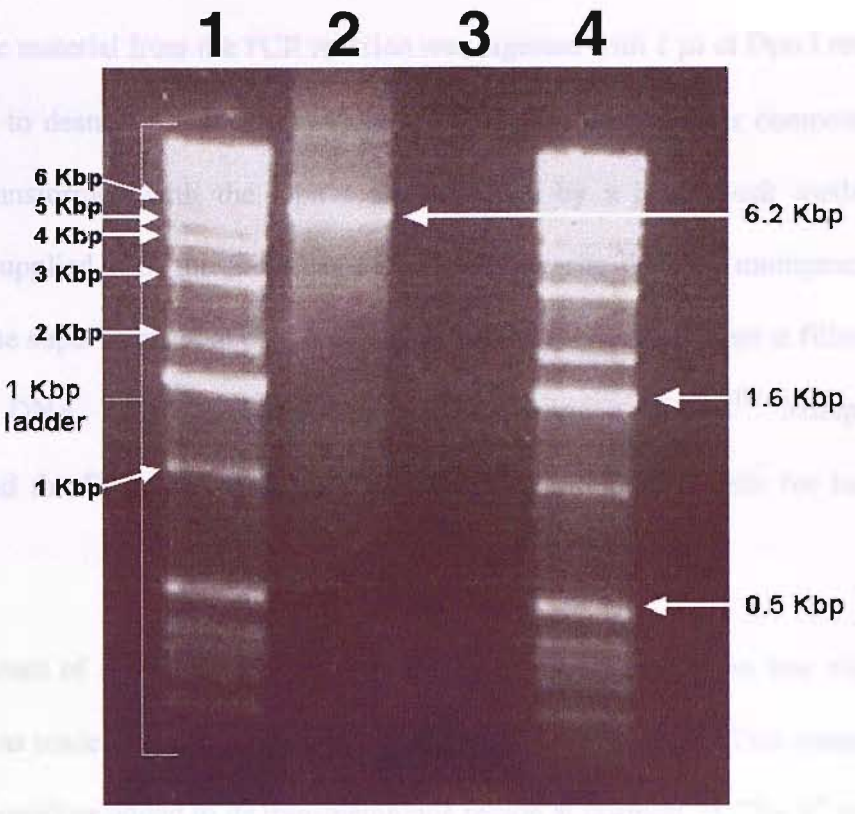

Figure 25

Quick change mutagenesis of sarcolipin to introduce 4 extra leucine residues

The figure shows an agarose gel used to analyse the product of a quick change mutagenesis reaction. Lanes 1 and 4 are 1 Kbp markers (GibcoBRL). The product of the quick change reaction was separated in lane 2 and the products of a control reaction in which the PCR reaction was done without template was separated in lane 3. The gel was electrophoresed at 110 V for 45 minutes and viewed under a UVP GDS 5000 illuminator and camera.

The rest of the material from the PCR reaction was digested with 1 μl of Dpn I restriction endonuclease to destroy the parental strand of DNA. XL 1-Blue super competent cells were then transformed with the Dpn I treated DNA by a heat shock method (see instructions supplied with the Stratagene Quick change site directed mutagenesis kit). The XL 1-Blue super competent cells were used, as they are very efficient at filling in the nicks in the DNA. The DNA was then extracted using a Wizard™ Miniprep Kit (Promega) and the DNA was then transformed into *E.coli* DH5-α cells for long term storage.

A second mutant of sarcolipin with an extended transmembrane region was also made this mutant was made in the same way using the quick change method. This mutant had 7 extra leucine residues added to its transmembrane region at position 25. The 1st sarcolipin mutant served as the template DNA for the mutagenesis reaction. The following oligonucleotides were used to synthesize the 2nd mutant: (SLN MUT 2 QCFWD 5'-GGC TAC TAC TCC TCC TAC TGC TGC TCC TAG TGC GGT CTT ACC AG-3') (SLN MUT 2 QCREV 5'-CTG GTA AGA CCG CAC TAG GAG CAG CAG TAG GAG GAG TAG TAG CC-3'). This second mutant was again produced and transferred to DH5-α cells for long term storage.

4.2.3 TGN / ERGIC immunolocalisation

Transfected COS 7 cell were fixed by incubating them in formaldehyde solution 4% in PBS for 15 minutes. The cells were washed with PBS Triton X-100 (0.01%) and then incubated for 30 minutes in PBS Triton X-100 0.01% with 2% milk powder. This was

then removed by washing twice with PBS Triton X-100 (0.01%). The primary antibodies were then diluted in PBS Triton X-100 (0.01%) sheep anti-human TGN 46 was used at a dilution of (1 : 50) (Serotech) and mouse anti-human ERGIC 53 was used at a dilution of (1 : 100) (a gift from H.P. Hauri). 100 µl of the diluted antibodies were then added to the appropriate cells and incubated for 1hr at 37 ^{0}C. The cells were then washed twice in PBS Triton X-100 (0.01%) to remove unbound primary antibody. The secondary antibodies were then diluted in PBS Triton X-100 (0.01%). The secondary antibody for TGN 46 was donkey anti-sheep IgG conjugated to Texas Red (Amersham life sciences) used at a dilution of (1 : 100). The secondary antibody for ERGIC 53 was sheep anti-mouse Texas Red antibody (Amersham life sciences) used at a dilution of (1 : 50).

The cells were incubated with the appropriate secondary antibody for 1hr. This was washed off using PBS Triton X-100 (0.01%). The cells were then viewed under a Leica digital epiflourescent microscope (DM IRBE) or a Zeiss confocal microscope (LSM 510 META) as outlined in (section 3.2.3).

When preparing cells for the immunolocalisation of the ERGIC compartment. The cells were incubated for 2 hours at 15 ^{0}C before fixation and immunolocalisation took place. This procedure was necessary to cause an increase in vesicularisation of the ERGIC compartment and hence make it a more distinguishable structure when immunolocalised (Saraste and Kuismanen, 1984).

COS 7 cells which were to undergo TGN immunolocalisation were treated with brefeldin A (5 μg/ml) (Sigma) and incubated for 1 hour prior to fixation. The brefeldin A treatment had the effect of separating the endoplasmic reticulum and the TGN and increasing the size of the TGN. Brefeldin A blocks retrograde transport from the Golgi compartment thus increasing its size and making this compartment more visible (Lippincottschwartz *et al.*, 1989).

4.2.4 Labelling of the plasma membrane with concanavlin A conjugate

Concanavalin A conjugate Alexa Fluor 594 (Molecular probes) was used to label the plasma membrane of COS 7 cells to look for colocalisation with phospholamban and sarcolipin mutants with extended transmembrane regions. COS 7 cells were grown on coverslips in a 24 well plate at a confluence of approximately 60-80%. The cells were then washed twice with PBS. They were then incubated with 100 μl (per well) of concanavalin A conjugate Alexa Fluor 594 (250 μg/ml) diluted in PBS containing 1% BSA. The cells were incubated with this mixture for 10 minutes before being washed twice with PBS with 1% BSA and then mounted using mowiol mountant + 0.1% citifuor onto microscope slides. The cells were then viewed under a Leica digital epiflourescent microscope (DM IRBE) or a Zeiss confocal microscope (LSM 510 META) as outlined in (section 3.2.3).

4.2.5 Cellular fractionation (Nycodenz gradient) to separate the cellular compartments of the secretory pathway

This approach is based on the protocol devised by (Hammond and Helenius, 1994) and was also used by (Greenfield and High, 1999).

Preperation of Nycodenz gradients

A solution of 27% Nycodenz 10 mM Tris (pH 7.4), 3 mM KCl, 1 mM EDTA, was made and from this stock solution 24%, 19.33%, 14.66% and 10% nycodenz solutions were made using dilution buffer (0.75% NaCl, 10 mM Tris (pH 7.4), 3 mM KCl and 1mM EDTA). These solutions were then layered in a 13.5 ml centrifuge tube to form a discontinuous gradient. In order to form a continuous gradient these gradients were left upright in the 13.5 ml tube overnight at 4 ^{0}C.

Sub-cellular fractionation

COS 7 cells were seeded onto 145 cm^2 cell culture dishes and transfected with either phospholamban EGFP or sarcolipin EGFP (see section 3.2.2). 2 days post transfection the cells were washed twice with 5 mls of homogenisation buffer (250 mM sucrose, 10 mM triethanalamine, 10 mM acetic acid, 1 mM EDTA and 1 mM DTT). The cells were then dislodged and harvested in 500 µl of homogenisation buffer. The cells were then homogenised by passing them sequentially through syringe needles of decreasing diameter (i.e., 19 gauge, 21 gauge and 25 gauge) 5 times each, the resultant suspension was then centrifuged at 1500 g for 5 minutes to remove undisrupted cells and nuclei. The

supernatants were then loaded onto the pre-made Nycodenz gradients and centrifuged at 37000 rpm for 1.5 hours using a Kontron TST 41.14 swing out rotor. After centrifugation 1.5 ml fractions were collected by piercing the bottom of the 13.5 ml tube with a 19 gauge needle and collecting the drops in a 1.9 ml polypropylene tube. Proteins in each fraction were then precipitated by adding 200 µl of perchloric acid (11.6 M) and incubating on ice for 1 hour with occasional mixing. The precipitated proteins were pelleted by centrifugation at 14000 g for 10 minutes. Some fractions (1 - 4) had to be diluted with water (1ml : 7 ml) (precipitated protein : sterile distilled water) in order to ensure the formation of a pellet on centrifugation. After washing the pellets with 1 ml H_2O the fractions were analysed by SDS PAGE and western blot analysis.

SDS PAGE

The protein pellets were resuspended in 40 µl of 3-fold concentration sample buffer containing 8 M urea. In order to solublise the proteins the pellets had to be sonicated 3 times in a sonicating water bath for 10 minutes and heated to 95 ^{0}C for 5 minutes before being loaded onto a 15% polyacrylamide gel and electrophoresed at 45 mA for 45 minutes.

Western blotting

After SDS PAGE, proteins were transferred to nitrocellulose for western blot analysis. The nitrocellulose membrane was blocked in PBS (containing 0.05% Tween and 5% milk powder) over night. The membrane was then washed 3 times in PBS containing 0.05% Tween (PBST) for 5 minutes per wash. The membrane was then incubated with 5 mls of

diluted primary antibody for 1 hour (see below for dilution). This was then washed off using PBST with 3 washes of 5 minutes each. The secondary antibody (conjugated to HRP) was then added diluted in PBST and incubated for 1 hour (see below for dilution). This was then washed off with 3 washes of 5 minutes with PBST. The blots were then analysed using ECL super signal substrate (Pierce) and a Biorad Versa Doc system. Rabbit calnexin primary antibody (Stressgene) was used at a dilution of (1 : 1000) and probed with anti-rabbit secondary antibody (HRP conjugate) (Amersham life science) (1 : 20000). Mouse β COP primary antibody (Abcam) was used at a dilution of (1 : 500) and probed with anti-mouse secondary antibody (HRP conjugate) (Pierce) (1 : 2000). Sheep TGN 46 primary antibody (Serotech) was used at a dilution of (1 : 1000) and was probed with anti-sheep secondary antibody (HRP conjugate) (Serotech) (1 : 20000). Mouse EGFP primary antibody (Roche) was used at a dilution of (1 : 1000) and was probed with anti-mouse secondary antibody (HRP conjugate) (Pierce) (1 : 2000).

4.3.0 Results

4.3.1 Phospholamban EGFP localisation with the ERGIC compartment

Figure 26 shows a cell expressing phospholamban EGFP (Panel A). The cell membrane has been permeabilised and the ERGIC compartment has been made visible by the use of an anti ERGIC 53 antibody (Panel B). The ERGIC antibody has labelled a region near the nucleus that is presumably the ERGIC compartment. When the EGFP fluorescence is overlaid with the Texas Red fluorescence from the secondary antibody used to identify anti ERGIC 53 antibody it can be clearly seen that there is a high level of colocalisation (Panel C). Panel C shows a distinct yellow fluorescence indicating that both phospholamban EGFP and ERGIC 53 share the same compartment. This type of colocalisation is seen in both cells that have been transfected in the field of view. The other cell shown in the bottom right has not been transfected with phospholamban EGFP and as a result only the ERGIC compartment is visible.

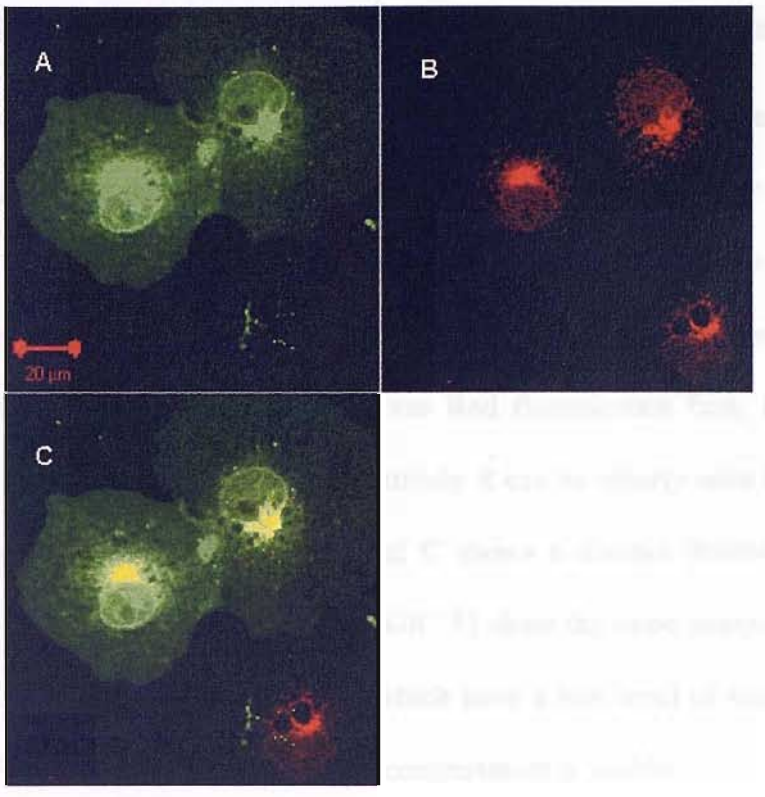

Figure 26

Phospholamban EGFP expression in COS 7 cells colocalised with ERGIC 53

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing an insert coding for phospholamban EGFP using Fugene-6. The cells were then incubated for 2 days before using an anti ERGIC 53 antibody to reveal the ERGIC compartment (see section 4.2.3). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows two cells expressing phospholamban EGFP, panel B shows the same cells treated with an antibody against the ERGIC marker p53 followed by a Texas Red conjugated secondary antibody. Panel C shows an overlay of panels A and B.

4.3.2 Sarcolipin EGFP localisation with the ERGIC compartment

Figure 27 shows 2 cells expressing sarcolipin EGFP (Panel A). The cell membranes have been permeabilised and the ERGIC compartments have been made visible by the use of an anti ERGIC 53 antibody (Panel B). The ERGIC antibody has labelled a region near the nucleus visible, again indicating the location of the ERGIC compartment. When the EGFP fluorescence is overlaid with the Texas Red fluorescence from the secondary antibody used to identify anti ERGIC 53 antibody it can be clearly seen that there is a high level of colocalisation (Panel C). Panel C shows a distinct yellow fluorescence indicating that both sarcolipin EGFP and ERGIC 53 share the same compartment. There are also 3 other cells in the field of view, which have a low level of transfection with sarcolipin EGFP, and hence only the ERGIC compartment is visible.

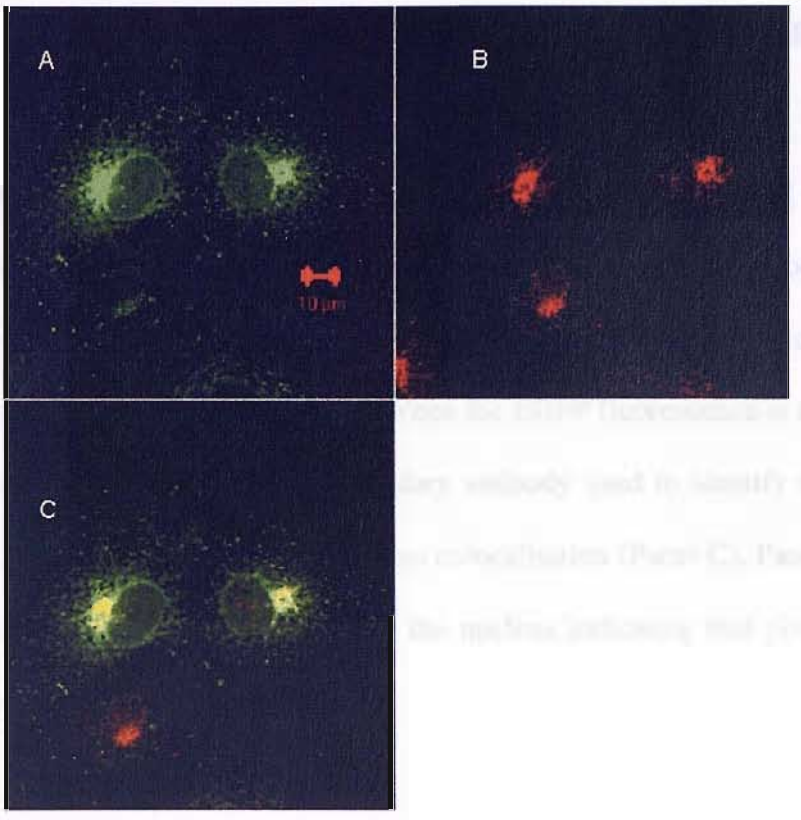

Figure 27

Sarcolipin EGFP expression in COS 7 cells colocalised with ERGIC 53

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for sarcolipin EGFP using Fugene-6. The cells were then incubated for 2 days before being treated to reveal the ERGIC compartment (see section 4.2.3). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were then viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows several cells expressing sarcolipin EGFP, panel B shows the same cells treated with an antibody against the ERGIC marker p53 followed by a Texas Red conjugated secondary antibody. Panel C shows an overlay of panels A and B.

4.3.3 Phospholamban EGFP localisation with the trans-Golgi network (TGN 46)

Figure 28 shows phospholamban EGFP expression in COS 7 cells (Panel A). The cell membrane has been permeabilised and the trans-Golgi network has been labelled with an anti TGN 46 antibody (Panel B). The TGN 46 antibody has revealed a region just above the nucleus of the cell visible as the TGN. When the EGFP fluorescence is overlaid with the Texas Red fluorescence from the secondary antibody used to identify anti TGN 46 antibody, it can be clearly seen that there is no colocalisation (Panel C). Panel C shows a distinct Texas Red fluorescence just above the nucleus indicating that phospholamban EGFP has not colocalised with the TGN.

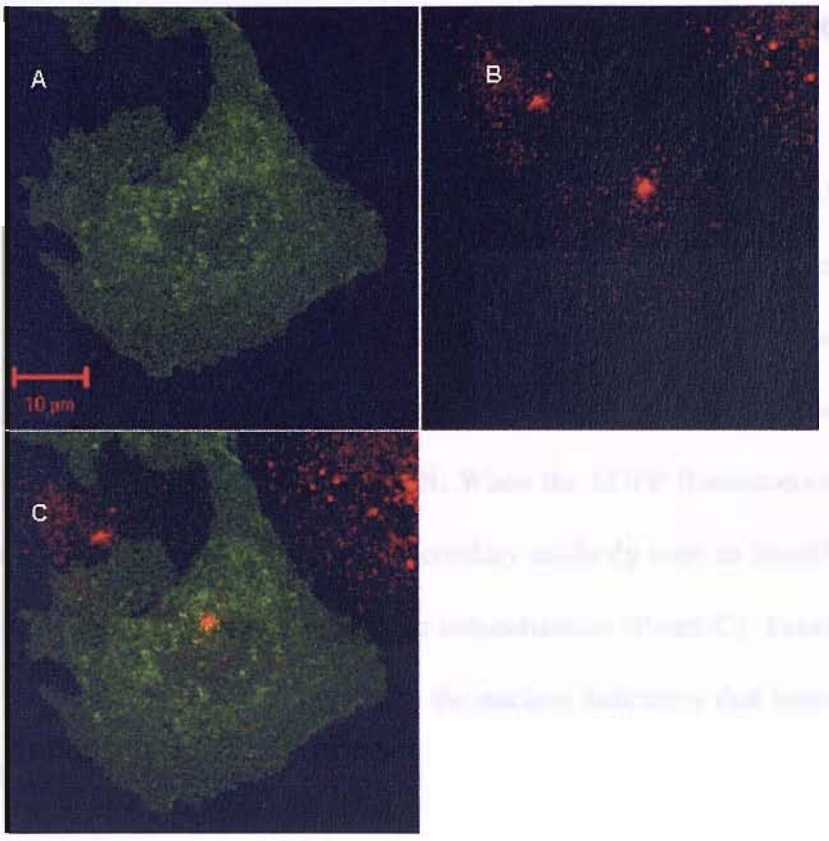

Figure 28

Phospholamban EGFP expressed in COS 7 cells not colocalised with the TGN

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for phospholamban EGFP using Fugene-6. The cells were then incubated for 2 days before fixation and visualisation of the TGN using an anti TGN 46 antibody for immunofluorescence localisation (see section 4.2.3). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows phospholamban EGFP expression. Panel B shows the same cells treated with an antibody against the TGN marker TGN 46 followed by a Texas Red conjugated secondary antibody. Panel C shows an overlay of panels A and B.

4.3.4 Sarcolipin EGFP localisation with the trans-Golgi network (TGN 46)

Figure 29 shows sarcolipin EGFP expression in 2 COS 7 cells (Panel A). The cells membranes have been permeabilised and the trans-Golgi network has been made visible by the use of an anti TGN 46 antibody (Panel B). The TGN 46 antibody has labelled a compartment just below the nucleus of the cell on the right and a region below and to the right of the cell on the left visible as the TGN. When the EGFP fluorescence is overlaid with the Texas Red fluorescence from the secondary antibody used to identify anti TGN 46 antibody, it can be seen that there is little colocalisation (Panel C). Panel C shows a distinct Texas Red fluorescence just out from the nucleus indicating that sarcolipin EGFP has not colocalised with the TGN.

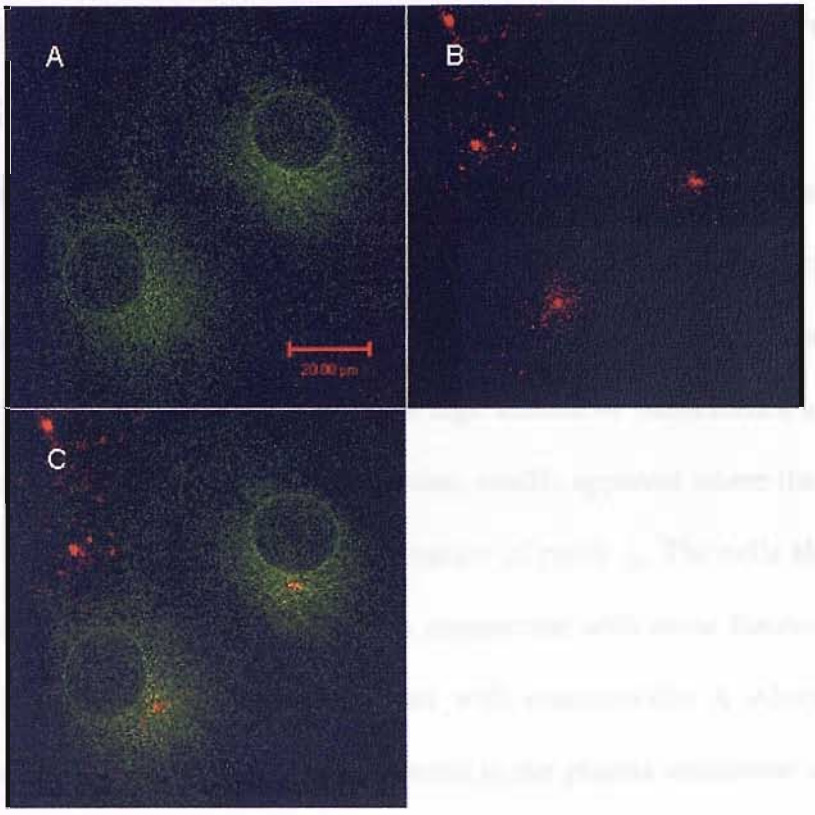

Figure 29

Sarcolipin EGFP expressed in COS 7 cells not colocalised with the TGN

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for sarcolipin EGFP using Fugene-6. The cells were then incubated for 2 days before fixation and visualisation of the TGN using an anti TGN 46 antibody for immunofluorescence localisation (see section 4.2.3). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows sarcolipin EGFP expression. Panel B shows the same cells treated with an antibody against the TGN marker TGN 46 followed by a Texas Red conjugated secondary antibody. Panel C shows an overlay of panels A and B.

4.3.5 Mutant phospholamban EGFP with 4 extra leucine residues at position 52 expressed in COS 7 cells

Figure 30 shows 2 COS 7 cells, which have been transfected with phospholamban EGFP that has had the transmembrane part of this protein extended by 4 extra leucine residues at position 52. Panel A shows the EGFP fluorescence of the phospholamban mutant, it can clearly be seen that there appears to be a high amount of fluorescence at the plasma membrane of the two cells shown. This becomes readily apparent where the two plasma membranes of the cell are in contact in the centre of panel A. The cells also appear to have endoplasmic reticulum fluorescence in conjunction with some fluorescence in the cytoplasm. Panel B shows the cells treated with concanavalin A Alexa Fluor 594 conjugate antibody which binds to glycoproteins in the plasma membrane and acts as a marker of the plasma membrane. In panel B the plasma membrane can clearly be seen as a red fluorescent signal around the perimeter of the cell. When the fluorescence of the EGFP tag and the fluorescence from the plasma membrane marker are overlaid in panel C, it is clear by the yellow fluorescence that they are colocalised at the plasma membrane. Panel D shows a close up of the junction between the two plasma membranes of the transfected cells, here it can clearly be seen that the red and green signals overlay highlighting the plasma membrane in yellow.

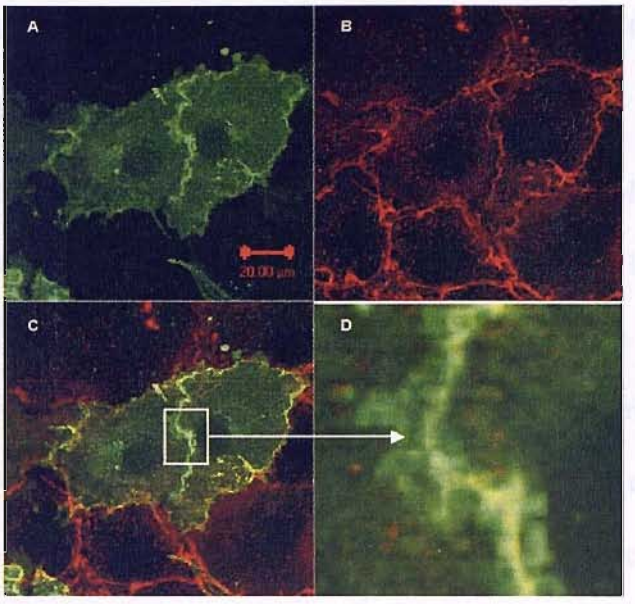

Figure 30

Cellular localisation of mutant phospholamban EGFP containing 4 extra leucine residues at position 52 in the transmembrane domain

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant phospholamban EGFP containing 4 extra leucine residues at position 52 using Fugene-6. The cells were then incubated for 2 days before being treated to reveal the plasma membrane using a concanavalin A Alexa Fluor 594 conjugate antibody (see section 4.2.4). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows two COS 7 cells expressing phospholamban mutant with 4 extra leucine residues. Panel B shows the plasma membrane of the two cells tagged with concanavalin A Alexa Fluor 594 conjugate antibody thus making it fluoresce red. Panel C shows an overlay of panels A and B. Panel D shows an expanded version of the two plasma membranes in contact highlighting the colocalisation.

4.3.6 Mutant sarcolipin EGFP with 4 extra leucine residues at position 25 expressed in COS 7 cells

Figure 31 shows COS 7 cells, which have been transfected with sarcolipin EGFP that has had the transmembrane part of this protein extended by 4 extra leucine residues at position 25. The EGFP fluorescence of the sarcolipin mutant can be seen clearly in the endoplasmic reticulum. This type of fluorescence distribution within the cell is very much like that of wild type sarcolipin (see figure 19). The nuclei of the cells can be clearly seen in the centre of each cell. The cell shows a high level of perinuclear fluorescence that is often associated with ERGIC localisation. Although most of the fluorescent signal is seen in the endoplasmic reticulum there does appear to be a low level of fluorescence in the cytoplasm, but there is no indication that sarcolipin EGFP has migrated to the plasma membrane.

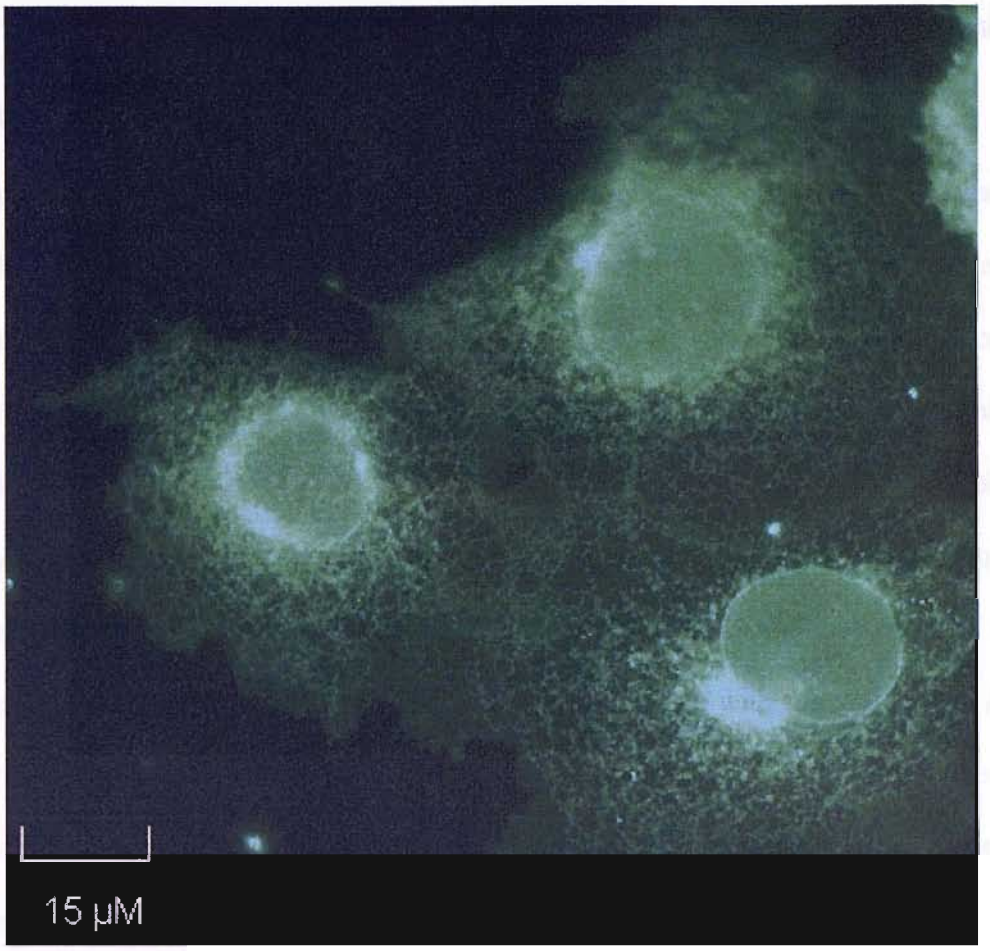

Figure 31

COS 7 cells expressing mutant sarcolipin EGFP containing 4 extra leucine residues at position 25 in the transmembrane domain

COS 7 cells were seeded on to 13 mm coverslips and transfected with pcDNA3.1 (+) containing DNA sequence coding for sarcolipin EGFP mutant protein. The cells were incubated for 2 days and mounted on microscope slides using mowiol mountant containing 0.1% citifluor. The cells were then viewed under a Leica digital eppifluorescent microscope (DM IRBE).

4.3.7 Mutant sarcolipin EGFP with 7 extra leucine residues at position 25 expressed in COS 7 cells

Figure 32 shows COS 7 cells, which have been transfected with sarcolipin EGFP that has had the transmembrane part of this protein extended by 7 extra leucine residues at position 25. Panel A shows the EGFP fluorescence of the sarcolipin mutant. It can be seen clearly that there is a significant amount of fluorescence localised at the plasma membranes of the cells shown. This becomes really apparent where the plasma membranes of the two cells are in contact with each other. The cells also appear to have fluorescence associated with the endoplasmic reticulum in conjunction with some fluorescence in the cytoplasm. Panel B shows the cells treated with concanavalin A Alexa Fluor 594 conjugate antibody which binds to glycoproteins in the plasma membrane and acts as a plasma membrane marker. In panel B the plasma membrane can clearly be seen as a red fluorescent perimeter around the cells. When the fluorescence of the EGFP tag and the fluorescence from the plasma membrane marker are overlaid in panel C it is clear by the yellow signal that they are colocalised at the plasma membrane. Panel D shows a close up of the plasma membrane of the transfected cell at the bottom right. Here it can clearly be seen that the red and green signals are colocalised at the plasma membrane.

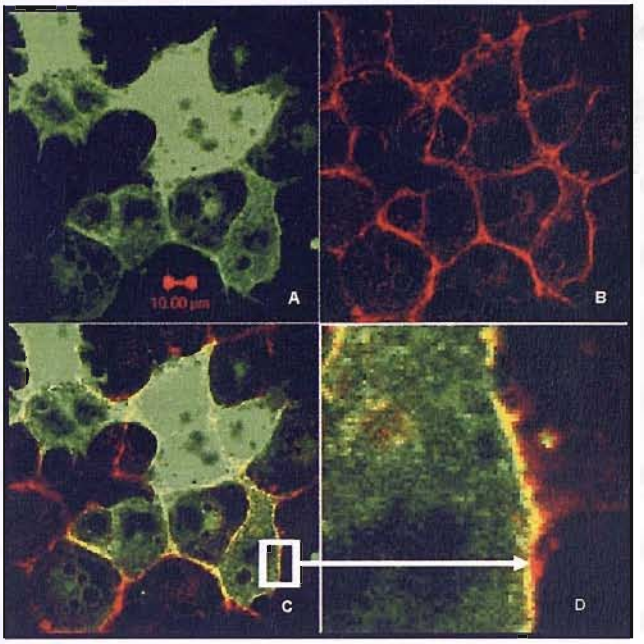

Figure 32

Cellular localisation of mutant sarcolipin EGFP containing 7 extra leucine residues at position 25 in the transmembrane domain

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant sarcolipin EGFP containing 7 extra leucine residues at position 25 using Fugene-6. The cells were then incubated for 2 days before being treated to reveal the plasma membrane using a concanavalin A Alexa Fluor 594 conjugate antibody (see section 4.2.4). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows cells expressing sarcolipin mutant with 7 extra leucine residues. Panel B shows the plasma membrane of the cells tagged with concanavalin A Alexa Fluor 594 conjugate antibody. Panel C shows an overlay of panels A and B. Panel D shows an expanded version of the plasma membrane of one cell highlighting the colocalisation.

4.3.8 Localisation of phospholamban EGFP by cell fractionation

Figure 33 shows western blot analysis of material generated from COS 7 cells transfected with phospholamban EGFP and then subjected to cell fractionation (section 4.2.5). Fractions were taken from the bottom of the Nycodenz gradient after centrifugation, so fraction 1 contains the heaviest material and fraction 9 contains the lighter organelles. The western blot was performed to determine the compartments in which phospholamban EGFP was located. Antibodies against calnexin were used to identify the fraction containing the endoplasmic reticulum, as this protein is resident in this compartment (Ou *et al.*, 1993). Antibodies against β COP were used to identify the ERGIC compartment (Griffiths *et al.*, 1995) and antibodies against TGN 46 were used to identify the TGN (Prescott *et al.*, 1997). Calnexin can be seen by immunoblotting to be restricted to fraction 1, whereas β COP tends to reside in fractions 6 – 9 with smaller amounts in fractions 1 and 5. TGN 46 is mainly found in fractions 6 and 7. Antibodies against EGFP were used to identify the localisation of phospholamban EGFP. Figure 32 shows that phospholamban EGFP can be seen in the same fraction as calnexin, β COP and TGN 46. However the strongest signal for phospholamban EGFP is found in fraction 1 and to a lesser extent fraction 7.

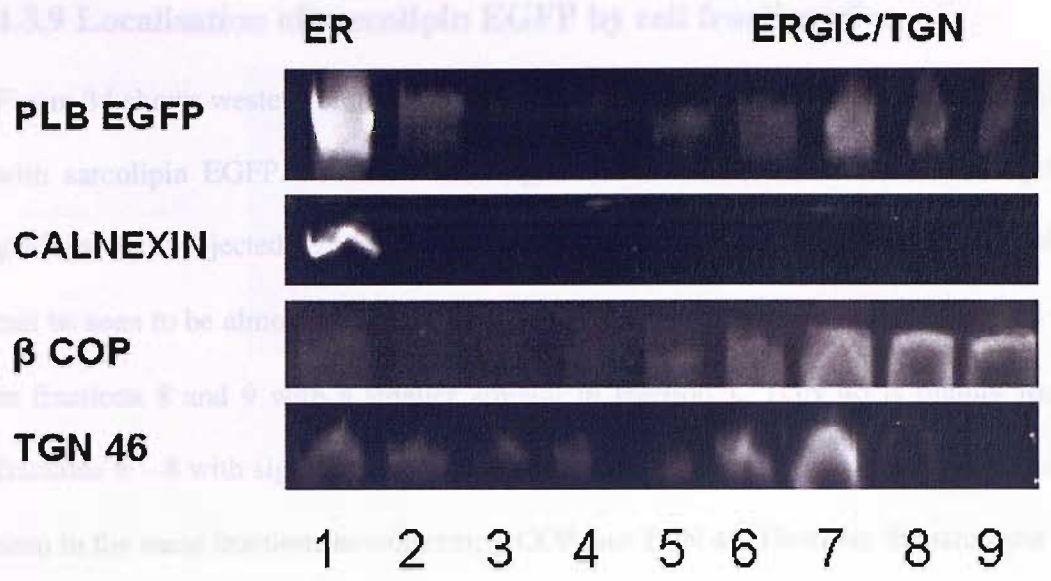

Figure 33

Localisation of phospholamban EGFP by cell fractionation

COS 7 cells transfected with phospholamban EGFP were disrupted as described in (section 4.2.5) and after removing the nuclei and undisrupted cells with a preliminary centrifugation step, material was applied to a Nycodenz gradient and centrifuged. 9 fractions were collected fraction 1 being the densest fraction. Proteins from each fraction were precipitated using perchloric acid and the pellets obtained by centrifugation were solubilised in sample buffer containing 8 M urea. The proteins from the 9 different fractions were separated by SDS-PAGE (15 % gel). The proteins were then subjected to western blot analysis using antibodies directed to EGFP, calnexin, β COP and TGN 46. These primary antibodies were then detected using secondary antibodies conjugated to horseradish peroxidase. Peroxidase activity was visualised by enhanced chemiluminescence and recorded digitally using a Biorad Versa Doc system.

4.3.9 Localisation of sarcolipin EGFP by cell fractionation

Figure 34 shows western blot analysis of material generated from COS 7 cells transfected with sarcolipin EGFP. Fractions were again taken from the bottom of the Nycodenz gradient and subjected to western blot analysis as discussed above. As before calnexin can be seen to be almost exclusively located in fraction 1 whereas β COP tends to reside in fractions 8 and 9 with a smaller amount in fraction 1. TGN 46 is mainly found in fractions 6 – 8 with significantly less material in fractions 1 – 5. Sarcolipin EGFP can be seen in the same fractions as calnexin, β COP and TGN 46. However the strongest signal for sarcolipin EGFP is found in fractions 1 – 3 and also fraction 8.

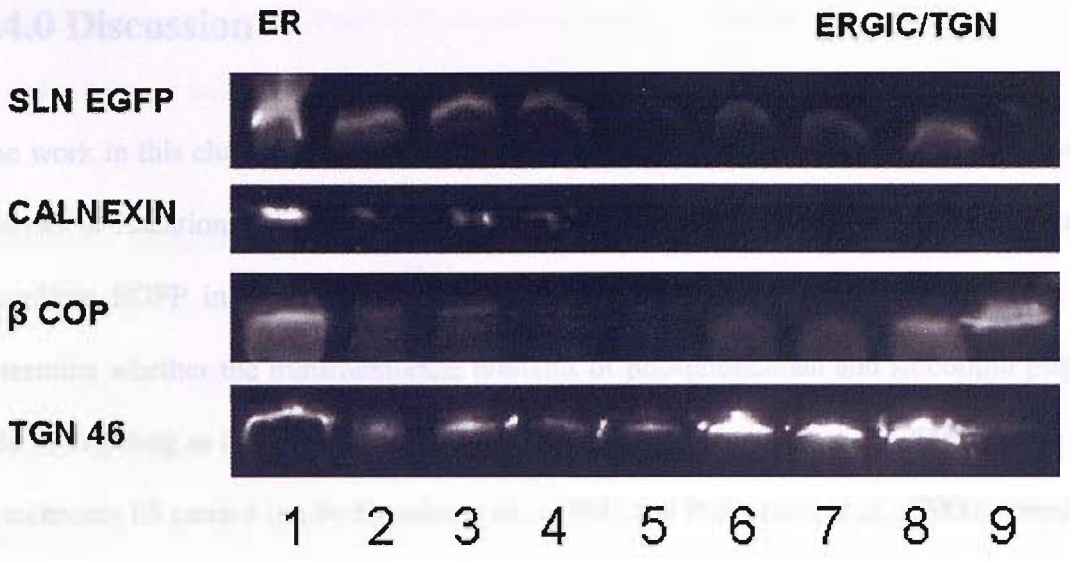

Figure 34

Localisation of sarcolipin EGFP by cell fractionation

COS 7 cells transfected with sarcolipin EGFP were disrupted as described in (section 4.2.5) and after removing the nuclei and undisrupted cells with a preliminary centrifugation step, material was applied to a Nycodenz gradient and centrifuged. 9 fractions were collected fraction 1 being the densest fraction. Proteins from each fraction were precipitated using perchloric acid and the pellets obtained by centrifugation were solubilised in sample buffer containing 8 M urea. The proteins from the 9 different fractions were separated by SDS-PAGE (15 % gel). The proteins were then subjected to western blot analysis using antibodies directed to EGFP, calnexin, β COP and TGN 46. These primary antibodies were then detected using secondary antibodies conjugated to horseradish peroxidase. Peroxidase activity was visualised by enhanced chemiluminescence and recorded digitally using a Biorad Versa Doc system.

4.4.0 Discussion

The work in this chapter was concerned with answering the question of whether it was a process of retention or retrieval responsible for maintaining phospholamban EGFP and sarcolipin EGFP in the endoplasmic reticulum membrane. The chapter also seeks to determine whether the transmembrane domains of phospholamban and sarcolipin play a role in targeting as has been found for cytochrome b5 (Pedrazzini *et al.*, 2000). Work on cytochrome b5 carried out by Honsho *et al.*, (1998) and Pedrazzini *et al.*, (2000) revealed that the transmembrane domain is a key area important in maintaining this C-terminal anchored protein in the endoplasmic reticulum. It was therefore logical to extend the transmembrane regions of both phospholamban and sarcolipin and see if this had a similar effect causing leakage from the endoplasmic reticulum.

Figure 26 indicates that a significant fraction of the expressed phospholamban EGFP is colocalised to the ERGIC compartment in COS 7 cells (panel C) as shown by the overlapping fluorescence signals from phospholamban EGFP and the ERGIC marker P53. Sarcolipin EGFP produced a similar result shown in figure 27 colocalising with the ERGIC compartment marker. These results are interesting because in order for both these fusion proteins to enter the ERGIC compartment they must have exited the endoplasmic reticulum. This means that these two proteins have entered secretory vesicles, which have trafficked and formed the ERGIC compartment. This indicates that at least a proportion of both phospholamban EGFP and sarcolipin EGFP have left the endoplasmic reticulum. If this is the case then it appears that if these proteins have a retention signal then it is

weak or perhaps the mechanism for retention is being overloaded with the huge amounts of heterologous protein being expressed.

The results in figures 28 and 29 both show that phospholamban EGFP and sarcolipin EGFP do not colocalise with the trans-Golgi network as this compartment can be seen fluorescing red where no EGFP signal is seen. This indicates that even though both phospholamban EGFP and sarcolipin EGFP enter the ERGIC compartment they do not move on to the later parts of the Golgi apparatus. Sec 61 has been shown to have a similar distribution to phospholamban and sarcolipin. Sec 61 was studied using GFP tagging, immunolocalisation and sub cellular fractionation (Greenfield and High, 1999). It was found that Sec 61 localised to the endoplasmic reticulum and the ERGIC but did not localise to the TGN. The authors concluded that this protein must be maintained in the endoplasmic reticulum by a process of retrieval and perhaps to a lesser extent retention. A similar study using SERCA 1 / plasma membrane calcium ATPase (PMCA) chimeras showed that SERCA 1 had a similar distribution as phospholamban and sarcolipin with the SERCA 1 localising to the endoplasmic reticulum and the ERGIC compartment but not the TGN (Newton *et al.*, 2002). When residues 1 – 211 were replaced for PMCA residues it was seen that mistargeting of SERCA 1 to the plasma membrane resulted. This indicated a region containing a targeting motif perhaps a retrieval sequence for this protein see (Newton *et al.*, 2002) for more details. Immunofluorescence and cell fractionation studies on cytochrome b5 have shown that retention is the major process responsible for its maintenance in the endoplasmic reticulum even though a small amount of the protein can be seen to traffic to the ERGIC

compartment (Pedrazzini *et al.*, 2000). The endoplasmic reticulum localisation of sarcolipin EGFP and phospholamban EGFP contrasts with that of cytochrome b5 as a large proportion of sarcolipin EGFP and phospholamban EGFP localise to the ERGIC compartment (see figures 26 and 27) but do not localise to the TGN. This indicates retrieval is the main process at work in phospholamban and sarcolipin maintenance in the endoplasmic reticulum. Proteins such as calnexin are seen to have a very slow leak rate from the endoplasmic reticulum and are found to localise exclusively to the endoplasmic reticulum by a process of retention (Bannykh *et al.*, 1995). Although there seems to be a number of mechanisms for the retrieval of escaped endoplasmic reticulum resident proteins phospholamban and sarcolipin do not have any of the typical retrieval sequences such as KDEL / HDEL, di lysine motifs or di arganine motifs. This leaves one receptor that may be responsible for the retrieval of phospholamban and sarcolipin from the ERGIC compartment back to the endoplasmic reticulum. Sec 12p and Sec 71p are C-terminally anchored membrane proteins without identifiable retrieval sequences. Sec 12p has been shown to interact with Rer1p a retrieval receptor responsible for helping to maintain this protein in the endoplasmic reticulum (Sato *et al.*, 1996a). If Rer1p is responsible for retrieving phospholamban EGFP and sarcolipin EGFP it must play a major role in maintaining these proteins in the endoplasmic reticulum as it is clear from figures 26 and 27 that a large fraction of phospholamban EGFP and sarcolipin EGFP leaks from the endoplasmic reticulum to the ERGIC compartment. If this is the case then it appears that retrieval is the major mechanism responsible for the maintenance of phospholamban EGFP and sarcolipin EGFP to the endoplasmic reticulum rather than retention.

Figure 30 shows a mutant containing 4 extra leucine residues at position 52 of phospholamban EGFP being expressed in COS 7 cells. These extra residues seem to cause the protein to leak freely from the endoplasmic reticulum, and traffic through the cells secretory pathway, where it ends up following the default pathway in to the plasma membrane of the cell. This can be seen in figure 30 (panels C and D) where colocalisation of the mutant phospholamban can be seen colocalised with the plasma membrane marker Concanavlin A Alexa Fluor 594 conjugate. The addition of the 4 extra leucine residues probably affects the retrieval mechanisms. Perhaps receptors responsible for the retrieval of phospholamban EGFP and sarcolipin EGFP are unable to recognise the extended transmembranous region. This type of extension in a α helix spanning the membrane is likely to cause tilting of the transmembrane region of the protein (Biggin and Sansom, 1999). If this occurs then perhaps the receptor responsible for retrieval cannot align itself appropriately to bind the protein for retrieval. As a result the proteins are not recycled to the endoplasmic reticulum and enter the default pathway ending up in the plasma membrane.

The extension of the transmembrane region of sarcolipin EGFP by 4 leucine residues appeared to have little effect on the localisation from wild type sarcolipin EGFP (figure 31). When analysing the length of the sarcolipin mutant with 4 extra leucine residues at position 25 it was noted that this extension made the transmembrane region of sarcolipin approximately the same length as wild type phospholamban. It was logical to assume from this that the extension of the transmembrane region of sarcolipin by 4 leucine

residues was not enough to stop its interaction with the retrieval process. Extending sarcolipin EGFP's transmembrane region by 7 leucine residues resulted in the same localisation pattern as the mutant phospholamban EGFP. These results indicate that the transmembrane region of both phospholamban and sarcolipin plays a key role in the maintenance of these proteins in the endoplasmic reticulum. It appears that the length of the transmembrane region plays a critical role in determining whether phospholamban and sarcolipin are to be endoplasmic reticulum resident proteins or not. When cytochrome b5 was mutated to extend the transmembrane region of the protein it was shown to reduce retention by the endoplasmic reticulum (Honsho *et al.*, 1998). However, when comparing the extension of the transmembrane regions of sarcolipin and phospholamban to that of cytochrome b5 it can be shown that it is more likely that the extension of sarcolipin and phospholamban caused the retrieval mechanisms to fail, as the proteins have already been shown to exit the endoplasmic reticulum more readily than cytochrome b5. It is also possible that the extension in the transmembrane region of both phospholamban and sarcolipin could increase the rate of leak of these proteins from the endoplasmic reticulum thus overwhelming the retrieval mechanisms resulting in relocation of the proteins to the plasma membrane. However this seems unlikely since the very high levels of expression of wild type sarcolipin EGFP and phospholamban EGFP appear unable to overwhelm the retrieval apparatus.

Cell fractionation experiments followed by western blot analysis were used as another technique to show that both phospholamban EGFP and sarcolipin EGFP trafficked from the endoplasmic reticulum to the ERGIC compartment. Unfortunately this technique is

not capable of distinguishing between the ERGIC compartment and the TGN. Figure 33 shows clearly that phospholamban EGFP is in the endoplasmic reticulum as it is in the same fraction as calnexin a resident protein of the endoplasmic reticulum. Calnexin can only be seen in fraction 1 indicating that it is strongly retained in the endoplasmic reticulum as reported by (Ou *et al.*, 1993). This distribution is typically seen in cellular fractionation experiments (Greenfield and High, 1999). Significant amounts of phospholamban EGFP are also found in fraction 7 showing phospholamban EGFP to have a bimodal pattern of distribution. This coincides with β COP a marker used to identify the ERGIC compartment, which also recycles back to the endoplasmic reticulum as a component of COP I vesicles performing retrograde transport. This indicates that a proportion of phospholamban EGFP appears to be in both the endoplasmic reticulum as well as the ERGIC compartment. TGN 46 is present in fraction 7 but the immunolocalisation studies shown in figure 28 show clearly that phospholamban EGFP does not make it to this compartment. The reason for this discrepancy is that it is hard to separate the ERGIC compartment from the TGN and as such you end up with both compartments in the same fraction. This does not mean that phospholamban EGFP has entered the TGN. Figure 34 shows the same type of cellular fractionation and western blot analysis but this time the cells have been transfected with sarcolipin EGFP. Again as for phospholamban EGFP, sarcolipin EGFP can be seen to have a bimodal pattern of distribution being in the same fraction as calnexin indicating it is in the endoplasmic reticulum and it can also be seen in the same fractions as β COP indicating its trafficked to the ERGIC compartment. This experiment is another way to show clear evidence that both phospholamban EGFP and sarcolipin EGFP do leave the endoplasmic reticulum and

are most likely returned back to the endoplasmic reticulum by some type of retrieval mechanism.

The purpose of this chapter was to determine whether retrieval plays a larger role than retention in maintaining phospholamban EGFP and sarcolipin EGFP to the endoplasmic reticulum. The evidence shows that sarcolipin EGFP and phospholamban EGFP leave the endoplasmic reticulum and traffic as far as the ERGIC compartment as judged by immunolocalisation and cellular fractionation experiments. Neither protein enters the TGN and as a result they must be recycled back to the endoplasmic reticulum by some sort of retrieval receptor mediated system. This chapter has also demonstrated the importance of the transmembranous region in phospholamban EGFP and sarcolipin EGFP in maintaining these proteins in the endoplasmic reticulum. It appears that extension of this region by too many residues causes leakage from the endoplasmic reticulum, possibly due to the loss of interaction between the transmembrane regions of phospholamban and sarcolipin with receptor proteins responsible for the retrieval of these proteins from downstream compartments. Theoretically it is possible that extending the transmembrane region might interfere with a retention signal, but since significant amounts of sarcolipin and phospholamban exit the endoplasmic reticulum anyway it is difficult to envisage why they would not be retrieved if that system were still able to recognise the retrieval sequences in sarcolipin and phospholamban.

Chapter 5:

Factors affecting the orientation of sarcolipin EGFP and phospholamban EGFP

5.1.0 Introduction

As shown in chapter 3 the fusion proteins phospholamban EGFP and sarcolipin EGFP adopt the correct orientation in the endoplasmic reticulum membrane. The orientation of these proteins was judged using selective permeabilisation of the cellular membranes followed by immunolocalisation to assess the accessibility of the fusion proteins. This was then followed by a protease K assay (see sections 3.3.2 and 3.3.3 for more detail). Both these methods showed phospholamban EGFP and sarcolipin EGFP to adopt C-terminal anchoring in the endoplasmic reticulum membrane. Chapter 4 indicated that the transmembrane region of these C-terminal anchored proteins was very important in their localisation to the endoplasmic reticulum. However, no studies on the orientation of these extended transmembrane mutants of either phospholamban EGFP or sarcolipin EGFP were carried out. This chapter investigates the role of the length of the transmembrane region and how this affects the orientation that the protein adopts.

Three main factors determine the topology of a membrane protein, firstly the folding state of the portion of the protein to be translocated across the membrane. Secondly the length of the hydrophobic transmembrane domain and finally and most

importantly charged residues flanking the membrane spanning segment (Spiess, 1995). Statistical analysis of membrane proteins from both prokaryotes and eukaryotes revealed an enrichment of positive charges in the cytoplasmic sequences and a depletion of charges in the lumen. This was initially termed the 'positive inside rule' (with respect to the plasma membrane). Post-translational protein insertion and co-translational protein insertion also differ in terms of the potential obstacles that may affect protein orientation. For example a co-translational protein is translocated across the membrane as it is being synthesised by the ribosome whereas a post-translational protein has already emerged from a ribosome and may already be folded. This may mean that the portion of the protein to be translocated and the size of this portion are very important in determining the proteins orientation. It is even likely that post-translational proteins are aided by cytoplasmic chaperones to stop unfavourable folding interactions before insertion (Wattenberg and Lithgow, 2001) or if they have folded then perhaps they are unfolded before translocation takes place (Spiess, 1995).

Orientation of post-translational membrane proteins is probably governed by at least two other proteins these being cytoplasmic chaperones that direct proteins to the endoplasmic reticulum membrane for insertion and the translocating machinery itself. It is relatively easy to understand how charges on a protein can dictate the orientation of that protein (see figure 35). It is less easy however, to understand how the length of the hydrophobic domain can influence the orientation of a protein. It is known that the longer the transmembrane region the more likely that the N-terminus will be the anchoring termini all other things being equal (Higy *et al.*, 2004).

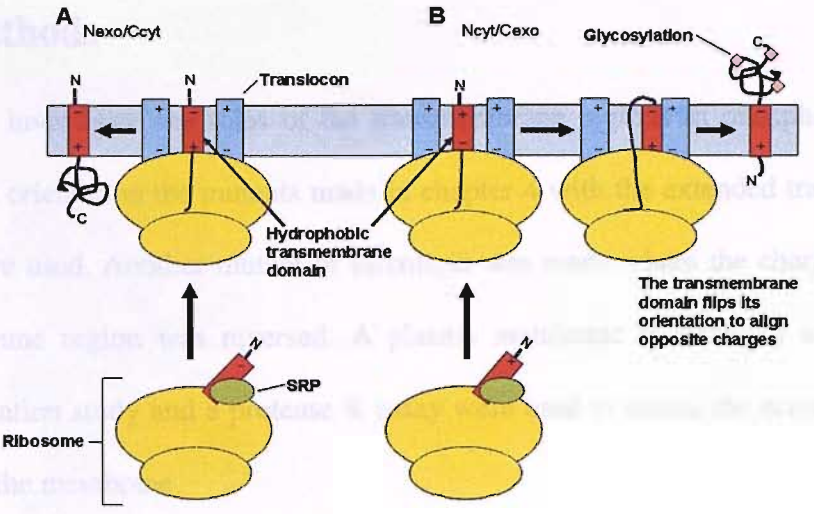

Figure 35

Orientation of membrane proteins

When a protein is delivered to the translocon the charges flanking the transmembranous region dictate the orientation of the protein. Panel A shows the protein in the orientation of N-terminus in the lumen of the endoplasmic reticulum. This is stabilised by the charges flanking the hydrophobic stretch. The positive charges on the flanking sequence align next to negative charges on the translocon and vice versa. This is a favourable interaction as opposite charges attract each other. Panel B shows how a transmembranous protein may re orientate to achieve a more favourable interaction in the translocon. This time the protein arrives in the translocon in an orientation where like charges are opposite each other, the protein thus flips to realign a positive charge next to a negative one and a negative charge next to a positive one. This diagram shows that proteins generally align to give the most positive side of the protein cytoplasmic location. This is known as the positive inside rule. The diagram depicts co-translational insertion of membrane proteins but a similar mechanism can be envisaged for post-translational membrane protein insertion. Modified from Higy *et al.*, (2004).

5.2.0 Methods

In order to investigate the roles of the transmembrane regions in phospholamban and sarcolipin's orientation the mutants made in chapter 4 with the extended transmembrane regions were used. Another mutant of sarcolipin was made where the charge across the transmembrane region was reversed. A plasma membrane localisation study, a semi permeabilisation study and a protease K assay were used to assess the orientation of the proteins in the membrane.

5.2.1 Construction of a sarcolipin EGFP mutant with an exchange of arginine at position 27 for glutamate

A mutant of sarcolipin with an exchange of residue arginine 27 for glutamate was made using the quick change PCR method (see section 4.2.2). The construct sarcolipin EGFP made in chapter 3 served as a template for the PCR reaction. The following oligonucleotides were designed to make the point mutation in sarcolipin: (SLN FLIP 1L 5'-GTA CTG GTA AGA CTC GAC TAG TAG CCA AAT AAG G-3') (SLN FLIP 1U 5'-GGC TAC TAG TCG AGT CTT ACC AGT ACT GAT CTA G-3'). This mutant was again produced and propogated in DH5-α cells for long-term storage.

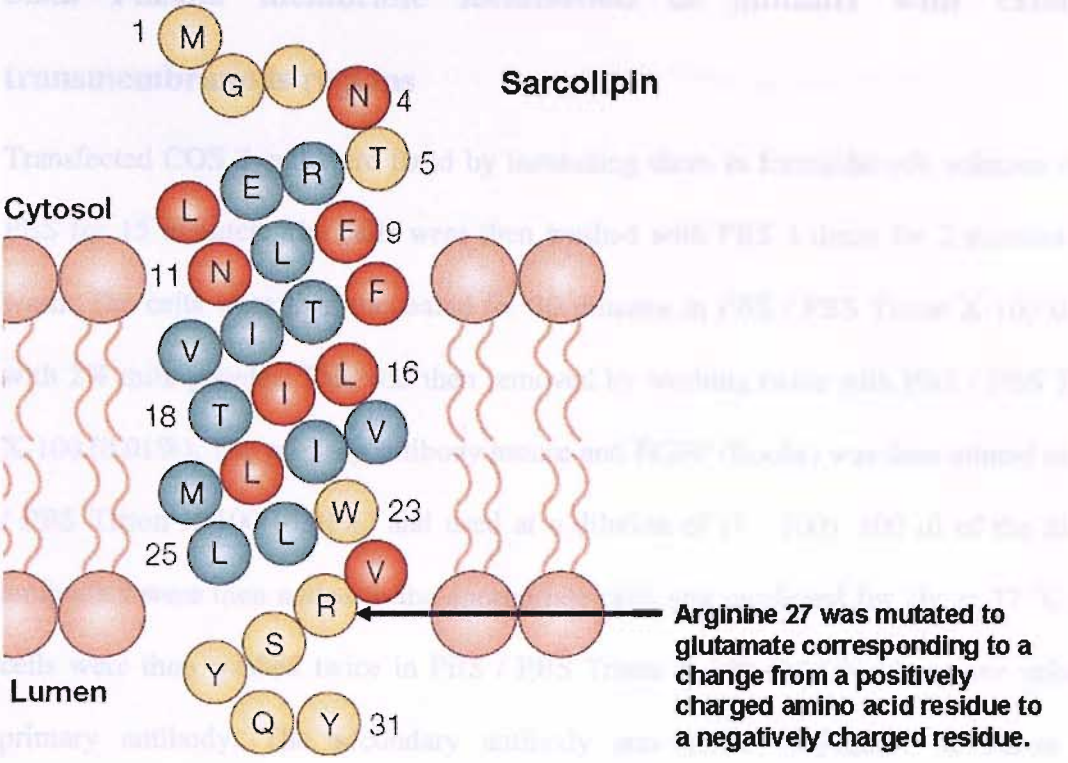

Figure 36

Quick change mutagenesis of sarcolipin to exchange arginine 27 for glutamate

The above diagram shows a schematic representation of sarcolipin in the endoplasmic reticulum membrane. The arrow indicates the position of the amino acid residue arginine 27 that was replaced by quick change mutagenesis. This exchange in amino acid residues from arginine to glutamate meant a change in charge across the transmembrane region as this single amino acid alteration changes a positive charge to a negative one. Modified from MacLennan and Kranias (2003).

5.2.2 Plasma membrane localisation of mutants with extended transmembranous regions

Transfected COS 7 cell were fixed by incubating them in formaldehyde solution 4% in PBS for 15 minutes. The cells were then washed with PBS 3 times for 2 minutes each wash. The cells were then incubated for 30 minutes in PBS / PBS Triton X-100 0.01% with 2% milk powder. This was then removed by washing twice with PBS / PBS Triton X-100 (0.01%). The primary antibody mouse anti EGFP (Roche) was then diluted in PBS / PBS Triton X-100 (0.01%) and used at a dilution of (1 : 100). 100 µl of the diluted antibodies were then added to the appropriate cells and incubated for 1hr at 37 ^{0}C. The cells were then washed twice in PBS / PBS Triton X-100 (0.01%) to remove unbound primary antibody. The secondary antibody anti-mouse conjugated to Texas Red (Amersham life sciences) was used at a dilution of (1 : 50) in PBS / PBS Triton X-100 (0.01%) and incubated for 1hr at 37 ^{0}C. The secondary antibody was then washed off using PBS / PBS Triton X-100 (0.01%). The cells were then viewed under a Leica digital epiflourescent microscope (DM IRBE) or a Zeiss confocal microscope (LSM 510 META) as outlined in (section 3.2.3).

5.2.3 Step-wise permeabilisation of the plasma and endoplasmic reticulum membranes

Transfected cells expressing the sarcolipin EGFP, lumenal EGFP and sarcolipin EGFP mutant with residue arginine 27 replaced with glutamate underwent the same treatment as outlined in (section 3.2.5) in order to judge the topology of these proteins.

5.2.4 Determination of transmembrane topology

The orientation of the sarcolipin mutant in the endoplasmic reticulum membrane was determined using the method of (Moss *et al.*, 1998). The method uses the protease susceptibility of the EGFP tag in microsomes to determine whether the EGFP tag is localised in the cytoplasm or lumenal side of the membrane. The microsomes were treated as outlined in (section 3.2.7).

5.3.0 Results

5.3.1 Extra cellular immunolocalisation of mutant phospholamban EGFP with 4 extra leucine residues at position 52 expressed in COS 7 cells

Figure 37 shows 2 COS 7 cells, which have been transfected with phospholamban EGFP that has had the transmembrane part of this protein extended by 4 extra leucine residues at position 52. Panel A shows the EGFP fluorescence of the phospholamban mutant, it can clearly be seen that there appears to be a high amount of fluorescence at the plasma membrane of the two cells shown. The cells also appear to have endoplasmic reticulum fluorescence in conjunction with some fluorescence in the cytoplasm. Panel B shows the cells treated with PBS and incubated with anti EGFP antibody this was then detected by anti-mouse Texas Red conjugate secondary antibody. This should bind to any EGFP protruding from the plasma membrane and act as a label. In panel B no red fluorescence can be seen around the cell indicating no binding of anti EGFP antibody. Panel C shows the EGFP fluorescence of the mutant phospholamban with no red signal.

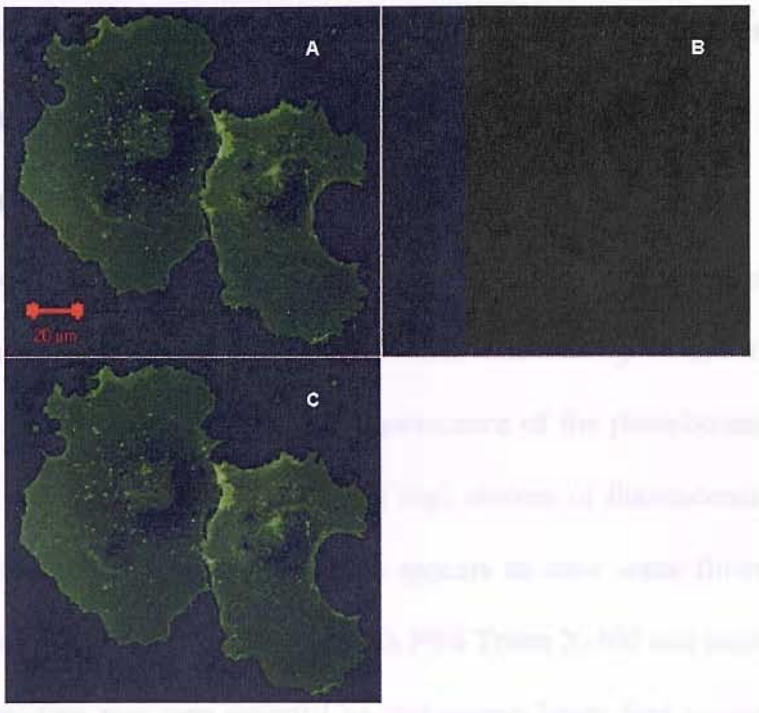

Figure 37

Immunolocalisation of mutant phospholamban EGFP with 4 extra leucine residues at position 52 expressed in intact COS 7 cells

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant phospholamban EGFP containing 4 extra leucine residues at position 52 using Fugene-6. The cells were then incubated for 2 days before being probed with anti EGFP antibodies to reveal any EGFP protruding from the exterior of the plasma membrane (see section 5.2.2). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows two COS 7 cells expressing phospholamban mutant with 4 extra leucine residues. Panel B shows no red signal indicating no binding of anti EGFP antibodies. Panel C shows an overlay of panels A and B, only EGFP fluorescence can be seen, as no red signal is apparent.

5.3.2 Plasma membrane permeabilised immunolocalisation of mutant phospholamban EGFP with 4 extra leucine residues at position 52 expressed in COS 7 cells

Figure 38 shows a COS 7 cell, which has been transfected with phospholamban EGFP that has had the transmembrane part of this protein extended by 4 extra leucine residues at position 52. Panel A shows the EGFP fluorescence of the phospholamban mutant, it can clearly be seen that there appears to be a high amount of fluorescence at the plasma membrane of the cell shown. The cell also appears to have some fluorescence in the cytoplasm. Panel B shows the cell treated with PBS Triton X-100 and incubated with anti EGFP antibody. This was then detected by anti-mouse Texas Red conjugate secondary antibody. In panel B a high fluorescent red signal is seen where the anti EGFP antibody has detected EGFP. The red fluorescence can be seen around the cell perimeter indicating binding to the phospholamban mutants EGFP tag on the cytoplasmic side of the plasma membrane. Panel C shows the EGFP fluorescence of the mutant phospholamban overlaid with the Texas Red fluorescence from the secondary antibody detecting anti EGFP antibody bound to EGFP. A high level of colocalisation is seen all around the perimeter of the cell especially around the lower left hand side of the cell.

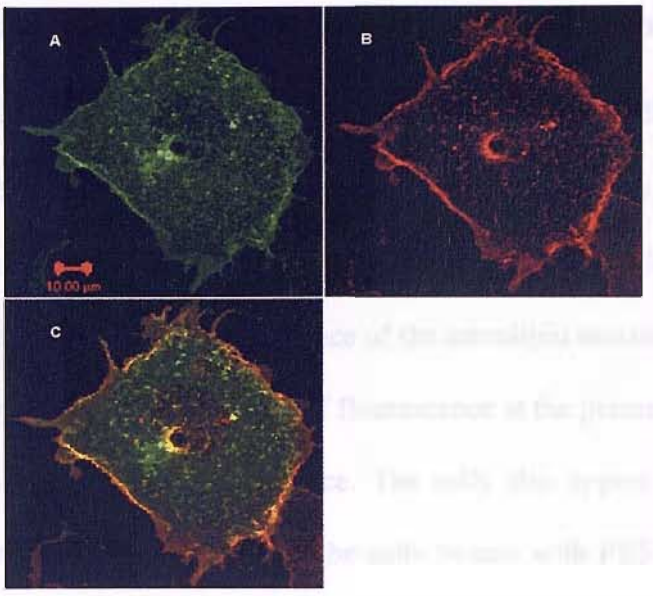

Figure 38

Immunolocalisation of mutant phospholamban EGFP with 4 extra leucine residues at position 52 expressed in permeabilised COS 7 cells

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant phospholamban EGFP containing 4 extra leucine residues at position 52 using Fugene-6. The cells were then incubated for 2 days before being permeabilised and probed with anti EGFP antibodies to reveal any EGFP protruding from the plasma membrane (see section 5.2.2). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows a COS 7 cell expressing phospholamban mutant with 4 extra leucine residues. Panel B shows a high red fluorescent signal around the perimeter of the cell indicating the binding of anti EGFP antibodies to phospholamban mutant EGFP. Panel C shows an overlay of panels A and B, indicating a high level of colocalisation to the plasma membrane.

5.3.3 Extra cellular immunolocalisation of mutant sarcolipin EGFP with 7 extra leucine residues at position 25 expressed in COS 7 cells

Figure 39 shows a COS 7 cell, which has been transfected with sarcolipin EGFP that has had the transmembrane part of this protein extended by 7 extra leucine residues at position 25. Panel A shows the EGFP fluorescence of the sarcolipin mutant, it can clearly be seen that there appears to be a high amount of fluorescence at the plasma membrane of the cell shown by several strips of fluorescence. The cells also appear to have some fluorescence in the cytoplasm. Panel B shows the cells treated with PBS and incubated with anti EGFP antibody this was then detected by anti-mouse Texas Red conjugate secondary antibody. This should bind to any EGFP protruding from the plasma membrane and act as a label. In panel B no red fluorescence can be seen around the cell indicating no binding of anti EGFP antibody. Panel C just shows the EGFP fluorescence of the mutant sarcolipin with no red signal.

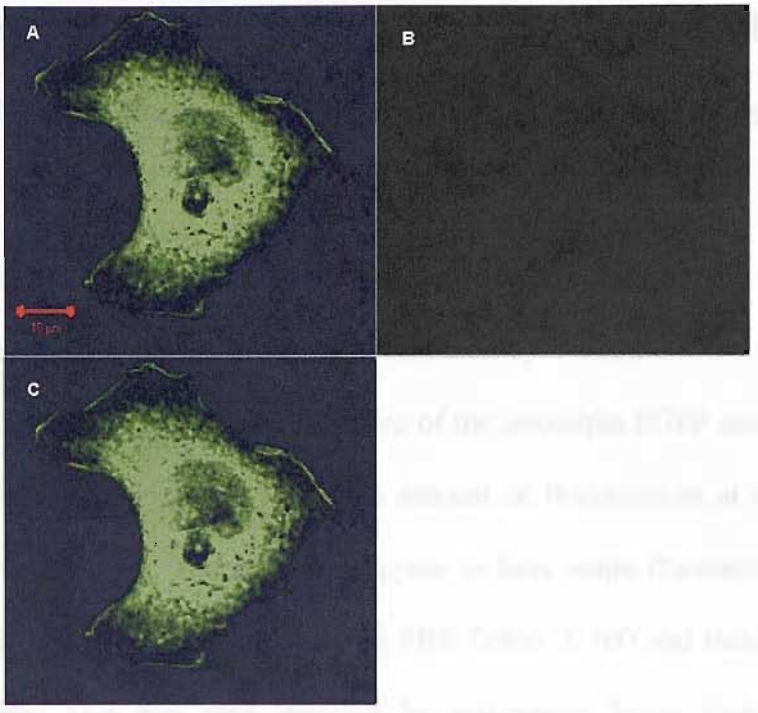

Figure 39

Immunolocalisation of mutant sarcolipin EGFP with 7 extra leucine residues at position 25 expressed in intact COS 7 cells

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant sarcolipin EGFP containing 7 extra leucine residues at position 25 using Fugene-6. The cells were then incubated for 2 days before being probed with anti EGFP antibodies to reveal any EGFP protruding from the exterior of the plasma membrane (see section 5.2.2). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A show a COS 7 cell expressing sarcolipin mutant with 7 extra leucine residues. Panel B shows no red signal indicating no binding of anti EGFP antibodies. Panel C shows an overlay of panels A and B, only EGFP fluorescence can be seen, as no red signal is apparent.

5.3.4 Plasma membrane permeabilised immunolocalisation of mutant sarcolipin EGFP with 7 extra leucine residues at position 25 expressed in COS 7 cells

Figure 40 shows 2 COS 7 cells, which have been transfected with sarcolipin EGFP that has had the transmembrane part of this protein extended by 7 extra leucine residues at position 25. Panel A shows the EGFP fluorescence of the sarcolipin EGFP mutant, it can clearly be seen that there appears to be a high amount of fluorescence at the plasma membrane of the cells shown. The cells also appear to have some fluorescence in the cytoplasm. Panel B shows the cells treated with PBS Triton X-100 and incubated with anti EGFP antibody. This was then detected by anti-mouse Texas Red conjugate secondary antibody. In panel B a high red fluorescent signal is seen where the anti EGFP antibody has detected EGFP. The red fluorescence can be seen around the cell perimeter indicating binding to the sarcolipin EGFP mutants EGFP tag on the cytoplasmic side of the plasma membrane. Panel C shows the EGFP fluorescence of the mutant sarcolipin EGFP overlaid with the Texas Red fluorescence from the secondary antibody detecting anti EGFP antibody bound to EGFP. A high level of colocalisation is seen all around the perimeter of the cell especially on the bottom of the lower cell.

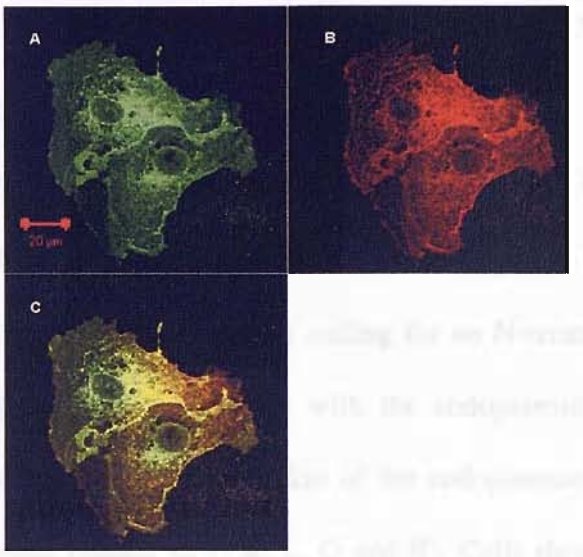

Figure 40

Immunolocalisation of mutant sarcolipin EGFP with 7 extra leucine residues at position 25 expressed in permeabilised COS 7 cells

COS 7 cells were seeded onto coverslips and transfected with pcDNA3.1 (+) containing the sequence coding for mutant sarcolipin EGFP containing 7 extra leucine residues at position 25 using Fugene-6. The cells were then incubated for 2 days before being permeabilised and probed with anti EGFP antibodies to reveal any EGFP protruding from the plasma membrane (see section 5.2.2). The coverslips were mounted onto microscope slides using mowiol mountant + 0.1% citifluor. The cells were viewed with a Zeiss confocal microscope (section 3.2.3). Panel A shows 2 COS 7 cells expressing sarcolipin EGFP mutant with 7 extra leucine residues. Panel B shows a high red fluorescent signal around the perimeter of the cells indicating the binding of anti EGFP antibodies to sarcolipin EGFP mutant. Panel C shows an overlay of panels A and B, indicating a high level of colocalisation to the plasma membrane.

5.3.5 Orientation studies of sarcolipin EGFP mutant with arginine 27 replaced by glutamate

Figure 41 shows COS 7 cells transfected with constructs coding for sarcolipin EGFP mutant (panels A, B, G, H, M and N) and sarcolipin EGFP (panels C, D, I, J, O and P). As well as cells transfected with a construct coding for an N-terminal leader sequence attached to EGFP tagged at its C-terminus with the endoplasmic reticulum retrieval sequence KDEL. This protein acts as a marker of the endoplasmic reticulum lumen as well as an internal control (panels E, F, K, L, Q and R). Cells shown in (panels A - F) have been treated with saline to act as a control. Panel A shows sarcolipin EGFP fluorescence where as panel B shows the same cells treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody. Panel B shows a very small patch of red fluorescence. Panel C shows fluorescence of the sarcolipin EGFP mutant protein. Panel D shows the same cells as panel C treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody this time very little red fluorescence can be seen from the Texas Red antibodies. Panel E shows the EGFP fluorescence from the lumenal marker. Panel F shows the same cells treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody again no red signal can be seen.

Cells shown in (panels G - L) have been treated with saponin which selectively permeabilises the plasma membrane. Cells shown in panel G have been transfected with sarcolipin EGFP and appear similar in appearance to those in panel A. Cells shown in panel H are the same cells as shown in panel G but have been treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS containing saponin. Panel H shows the same cells, which are visible by sarcolipin EGFP

fluorescence, fluorescing red with Texas Red fluorescence from the antibodies. It can be seen that it is the network in the cells shown in both panels G and H that becomes visible with fluorescence. Panel I shows the fluorescence from the sarcolipin EGFP mutant. When these cells are treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS saponin (panel J) very little Texas Red fluorescence can be seen. Panel K shows the lumenal EGFP marker fluorescence. Where as panel L again shows no Texas Red fluorescence from the antibodies.

Cells shown in (panels M-R) have been treated with Triton X-100 which permeabilises all cellular membranes. This time the EGFP fluorescence from sarcolipin EGFP, sarcolipin EGFP mutant and the lumenal EGFP protein can be seen (panels M, O and Q) when these cells are treated with an anti EGFP antibody and a secondary Texas Red conjugate antibody diluted in PBS Triton X-100 the Texas Red fluorescence in these cells can all be seen clearly.

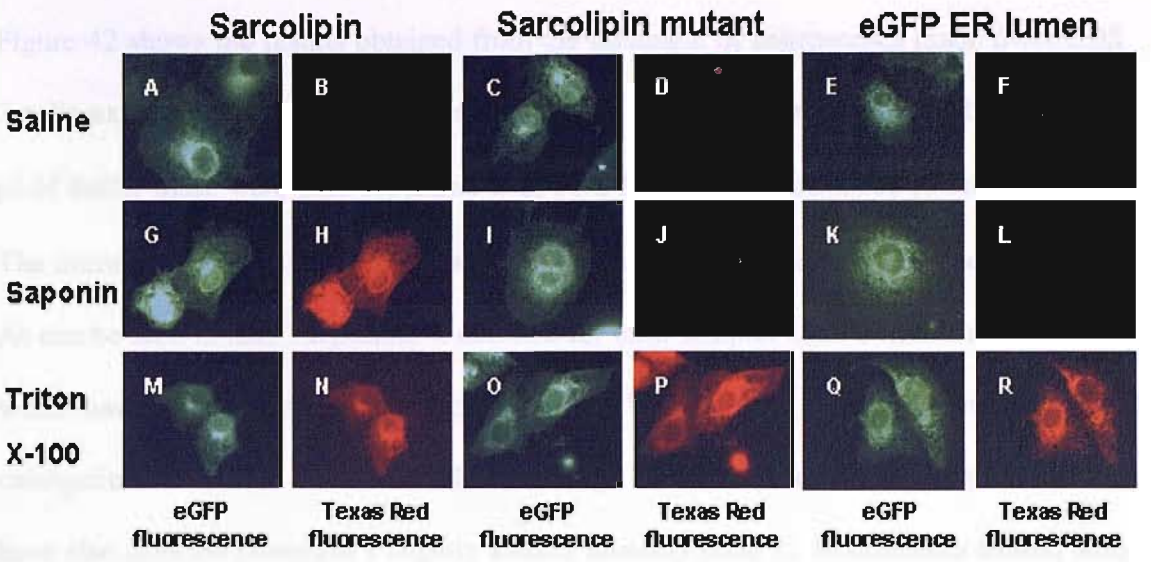

Figure 41

Selective permeabilisation studies to determine the protein orientation of sarcolipin EGFP mutant

Cells were seeded on to 13 mm coverslips at 60-80% confluence and transfected with either constructs coding for sarcolipin EGFP, sarcolipin EGFP mutant or endoplasmic reticulum located lumenal EGFP protein. These cells after 2 days post transfection were treated with either PBS, PBS containing saponin or PBS containing Triton X-100 and probed with antibodies directed against EGFP.

5.3.6 Confirmation of the orientation for sarcolipin EGFP mutant

Figure 42 shows the results obtained from the treatment of microsomes made from COS 7 cells expressing sarcolipin EGFP mutant. Lane 1 shows microsomes incubated with 8 μl of buffer these were then separated with SDS PAGE and transferred to nitrocellulose. The nitrocellulose was then probed with antibodies directed against EGFP and calregulin. As can be seen in lane 1 a protein is detected for each sample. Lane 2 shows microsomes, which have been incubated with protease before being probed with antibodies this time calregulin is detected, but at a weaker intensity. The antibodies directed against EGFP have also detected protein at a slightly weaker intensity (lane 2). Microsomes treated with both protease and Triton X-100 detects no protein when probed with antibodies directed against EGFP and calregulin (lane 3).

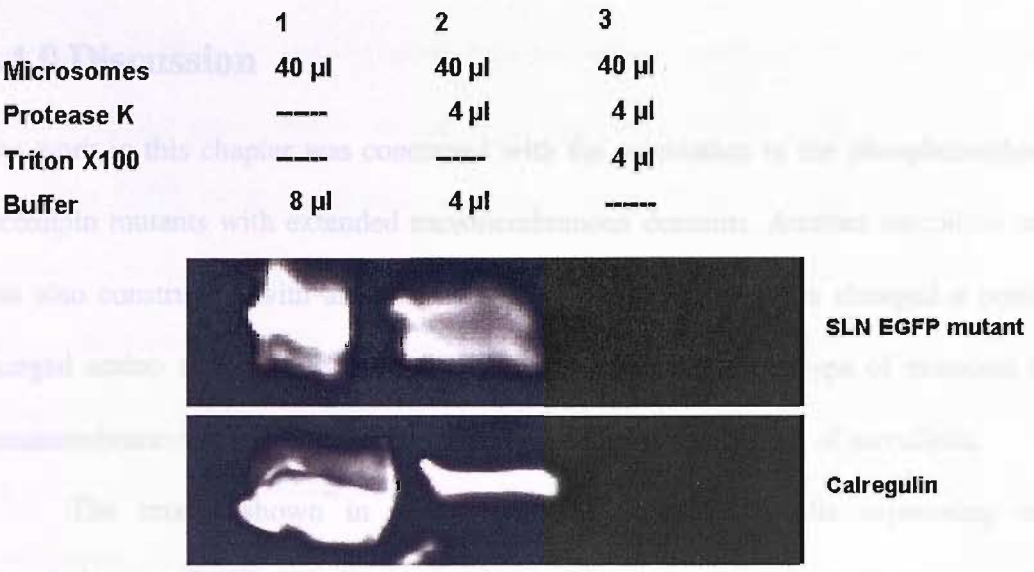

Figure 42

Proteolysis by protease K to examine the orientation of sarcolipin EGFP mutant in microsomes

Microsomes made from COS 7 cell expressing sarcolipin EGFP mutant were treated with either buffer (lane 1), protease K (lane 2) or Triton X-100 with protease K (lane 3). The microsomes were incubated on ice and then electrophoresed on SDS PAGE gels. They were then transferred to nitrocellulose and probed with antibodies directed against EGFP and calregulin. The gels were analysed by the use of a Supersignal Western Dura ECL detection kit (Pierce) as instructed by the manufacturer. The signals were then detected using a Biorad Versa Doc detection system.

5.4.0 Discussion

The work in this chapter was concerned with the orientation of the phospholamban and sarcolipin mutants with extended transmembranous domains. Another sarcolipin mutant was also constructed with arginine 27 replaced by glutamate this changed a positively charged amino acid residue for a negatively charged one. This type of mutation in the transmembrane region of the protein may influence the orientation of sarcolipin.

The results shown in figure 37 show 2 COS 7 cells expressing mutant phospholamban EGFP with 4 extra leucine residues at position 52 in the transmembrane region of the protein. The cells have been incubated with anti EGFP antibodies in PBS so the plasma membrane remains intact. This would thus detect any mutant phospholamban, which had reversed its orientation and hence ended up with its EGFP tag protruding from the extra cellular side of the plasma membrane. Panel B shows no red signal from the secondary antibodies used to detect anti EGFP antibodies. This indicates that no mutant phospholamban EGFP has flipped in orientation the only thing that has resulted from this mutation appears to be mistargeting to the plasma membrane of the cell. When this experiment is repeated with the plasma membrane permeabilised with Triton X-100 (see figure 38) the result changes because the antibodies can now gain access to the EGFP tag protruding from the plasma membrane on the cytoplasmic side. Figure 38 (panel B) shows a high red fluorescent signal where the anti EGFP antibodies have bound. When this signal is overlaid with EGFP fluorescence in panel C convincing plasma membrane localisation is seen. This result shows that the EGFP tag is in the cytoplasm of the cells. The extension of the transmembrane domain of phospholamban has not flipped the

orientation of phospholamban. When these experiments are repeated for the mutant sarcolipin EGFP with the extended transmembrane region by 7 leucine residues at position 25 the same results are obtained. Figure 39 shows a cell expressing mutant sarcolipin EGFP with the extension of the transmembrane region. Again the protein appears to locate to the plasma membrane of the cell. When the cell is incubated with anti EGFP antibodies in PBS to maintain an intact plasma membrane no Texas Red fluorescence is seen from the secondary antibodies (Panel B). This means that the EGFP tag on the sarcolipin mutant as for the phospholamban mutant is again located on the cytoplasmic side of the plasma membrane. When the cells are treated with Triton X-100 the plasma membrane is permeabilised and the anti EGFP antibodies can gain access to the EGFP tag (figure 40, panel B) again indicating the tag is on the cytoplasmic face of the plasma membrane. These results indicate that like phospholamban the extension of the transmembrane region of sarcolipin has no effect on the orientation of this protein in the membrane.

Orientation studies on the sarcolipin EGFP mutant with arginine 27 replaced for glutamate showed some interesting results. Figure 40 shows cells that have been selectively permeabilised after they have been expressing sarcolipin EGFP / sarcolipin EGFP mutant or EGFP located in the lumen of the endoplasmic reticulum. These cells were then probed with antibodies directed against EGFP. Panels A - F show cells incubated with anti EGFP antibodies in PBS this does not breach the plasma membrane of the cells and so antibodies cannot gain access to the EGFP epitopes. Thus only EGFP fluorescence is seen in panels A, C and E. No Texas Red fluorescence from the secondary antibody used to detect the anti EGFP antibody was seen in panels B and F.

Panel D does show a very slight amount of Texas Red fluorescence but no whole cells can be seen. This is probably due to the membranes being damaged slightly, which allows a small amount of antibodies in to the cells, or due to artefacts in the sample. Panels G - L show cells treated with saponin. This treatment selectively permeabilises the plasma membrane, leaving the endopasmic reticulum membrane of the cells intact. These cells were then probed with anti EGFP antibodies as before. Panels G, I and K show EGFP fluorescence but only panel H shows Texas Red fluorescence of 2 whole cells. Panel J also shows a very small amount of Texas Red fluorescence again probably due to damage of the membranes or artefacts in the sample. Panel L shows no Texas Red fluorescence at all. The fluorescence in panel H is due to the antibodies directed to EGFP being able to gain access to sarcolipin EGFP's tag as its protruding into the cytoplasm. The EGFP of the sarcolipin mutant and the EGFP located in the lumen of the endoplasmic reticulum are still protected by the endoplasmic reticulum membrane. This means that the antibodies directed to EGFP cannot gain access to their EGFP epitopes and therefore cannot bind and produce a signal. This experiment therefore indicates that the sarcolipin mutant does not share the orientation of the wild type sarcolipin EGFP but that its EGFP tag is located in the lumen of the endoplasmic reticulum. Therefore it has flipped in orientation to N-terminal anchoring. Panels M - R show cells treated with Triton X-100 which permeabilises all cellular membranes. Hence panels M, O and Q show EGFP fluorescence and panels N, P and R all show high Texas Red fluorescence as the antibodies can now gain access to the EGFP epitope whether its cytoplasmicly located (wild type sarcolipin EGFP) or lumenaly located (sarcolipin mutant EGFP / EGFP lumenal protein).

Figure 42 shows COS 7 cells that have been transfected with sarcolipin EGFP mutant with arginine 27 replaced by glutamate and made into microsmes (see section 3.2.7). These microsomes were then treated with protease K that would digest away any proteins on the extra-lumenal face of the microsome. The EGFP tag on the N-terminus of sarcolipin EGFP mutant would therefore be digested if on the outside of the microsome. If however, the N-terminus of the protein were inserted into the microsome then the EGFP would be protected from digestion. The EGFP tag was again detected with the use of anti EGFP antibodies. Another antibody was also used against calregulin as a resident protein of the endoplasmic reticulum lumen. This protein could be used to judge the vesicle integrity and act as a control. Lane 1 (figure 42) shows two good western blots for both EGFP and calregulin indicating the antibodies are working correctly. Lane 2 shows the microsomes treated with protease K this would digest any protein on the outside of the microsomes away and yet there is still a detection of the EGFP epitope and calregulin epitope. As expected when the microsomes are treated with Triton X-100 they are permeabilised and the epitopes are digested hence the antibodies are unable to detect any protein for either EGFP or calregulin. This experiment is again confirming that the sarcolipin EGFP mutant appears to have inserted in a opposite orientation from wild type sarcolipin EGFP (see figure 23 for wild type sarcolipin EGFP results). The EGFP tag of the mutant appears to be inside the lumen of the microsome and as such is protected from the protease K digestion and hence a signal is still produced in lane 2 for EGFP.

According to Spiess, (1995) this type of mutation with arginine 27 being replaced by glutamate at this position in the transmembrane domain of sarcolipin should have stabilised the sarcolipin anchoring in the C-terminal orientation. However, the results

have shown that this mutant protein appears to have flipped orientation. Higy *et al.*, (2004) suggest that the charges in the translocon type machinery favour a protein to anchor with the most positive side facing into the cytoplasm (the positive inside rule). There are many reasons why perhaps the sarcolipin EGFP mutant did not anchor in this orientation. It may be that the EGFP tag effects the translocating process of the post-translationally inserted protein thus causing sarcolipin EGFP mutant to flip in orientation. It is also worth noting that many post-translationally inserted proteins like sarcolipin interact with chaperones in the cytoplasm which direct them to the endoplasmic reticulum insertion machinery (Wattenberg and Lithgow, 2001). Perhaps the cytoplasmic chaperone binds to the sarcolipin EGFP mutant differently than wild type sarcolipin and hence the protein is in a different orientation when delivered to the translocating machinery. This may result in the protein being inserted in a different orientation. There are many hypotheses as to why this sarcolipin mutant is inserted in the opposite orientation to wild type sarcolipin. They would however all require further investigation to understand fully why this mutant appears to flip in orientation. According to Higy *et al.*, (2004) extension of the hydrophobic transmembrane domain should favour N-terminal insertion of the transmembrane protein. This was not found in the mutants of sarcolipin and phospholamban that had extended transmembranous regions. These mutants were found to mis-target from the endoplasmic reticulum to the plasma membrane but the results shown in (figures 37 - 40) show that the orientation of sarcolipin and phospholamban mutants remains the same as for the wild type proteins.

Chapter 6

General discussion

6.0.0 The nature of phospholamban and sarcolipin targeting

The targeting of phospholamban and sarcolipin has been investigated using several different approaches. The first objective was to tag both phospholamban and sarcolipin with EGFP so that the trafficking of these peptides could be followed. The work in chapter 3 was essential in ensuring that EGFP tagging was a suitable way of following the trafficking of phospholamban and sarcolipin within a mammalian cell line. EGFP as a fluorescent tag for both phospholamban and sarcolipin is not ideal because of its size (26 kDa) compared to phospholamban (6.1 kDa) and sarcolipin (3.7 kDa). This large bulky attachment to the native proteins may have affected their normal trafficking / insertion. For this reason it may have been preferential to use epitope tags which are a lot smaller as a means of following the trafficking of phospholamban and sarcolipin through the cell. The use of antibodies however to follow proteins through a cell are not without drawbacks. There is no guarantee that the addition of an epitope will not influence the targeting off the protein to which it has been attached. In order for the cells to be viewed they also have to be fixed and permeabilised and thus are non living at the time of viewing. The antibodies themselves are costly and take time to bind to their epitopes. The use of antibodies to view the location of a protein in a cell does not give an instant answer as to whether it's being expressed whereas EGFP tagging can be seen in seconds in living

cells with no processing. Although EGFP may have not been ideal this tag provided a convenient way to study the trafficking of these proteins with in COS 7 cells. This tagging also provided a means of studying the targeting of these proteins in living cells after these preliminary experiments, whereas the use of antibodies would not have allowed this to occur. In chapter 3 it was demonstrated that not only could the fusion proteins be expressed in COS 7 cells but that the expressed proteins adopted the correct orientation in the membrane (C-terminal anchoring). Colocalisation studies with SERCA1 revealed that the proteins localised to the endoplasmic reticulum as expected. After discovering that tagging phospholamban and sarcolipin had no major effects on the targeting or insertion of these proteins into the endoplasmic reticulum it was possible to investigate in more detail the trafficking of these proteins within the cell. The cells used for expressing phospholamban EGFP and sarcolipin EGFP were COS 7 cells. These cells were derived from african green monkey kidney fibroblast cells (www.ecacc.org.uk). These cells are very different from the types of muscle cells in which native phospholamban and sarcolipin would normally be expressed in and targeted to the sarcoplasmic reticulum. COS 7 cells were used in this study as a preliminary cell line because although their morphology is very different than that of muscle cells they are relatively easy to grow and are easy to transfect. Muscle cell lines however are more difficult to grow and are relatively resistant to transfection (I.D.A. Van Goethem PhD 2002). COS 7 cell lines and their derivatives have also been used widely before for the preliminary study of the trafficking of numerous membrane bound proteins (Greenfield and High, 1999; Odermatt *et al.*, 1998; Asahi *et al.*, 1999; Newton *et al.*, 2002). Although COS 7 cell structure is very different from that of muscle cells the organelle we are

interested in is the endoplasmic reticulum / sarcoplasmic reticulum. The sarcoplasmic reticulum in muscle cells is a specialized form of endoplasmic reticulum. The sarcoplasmic reticulums principle function in muscle is to act as a storage / release compartment for Ca^{2+} ions. This means that the sarcoplasmic reticulum is arranged differently than the endoplasmic reticulum in COS 7 cells. The sarcoplasmic reticulum is divided into regions which surround the contractile apparatus of the myocyte. These regions are filled with Ca^{2+} release channels which can release Ca^{2+} quickly into the contractile apparatus and cause muscle contraction. These regions are also rich in SERCA so that the Ca^{2+} can be quickly removed from the contractile apparatus and cause relaxation of the muscle. Although these differences exist there are also similarities between muscle cell sarcoplasmic reticulum and COS 7 cell endoplasmic reticulum and this is the region involved with protein insertion into the membrane. The basic components for protein insertion and trafficking in both cell types are likely to be the same. This is the area that this study is interested in and thus it is logical to use COS 7 cells to undertake these preliminary studies of the targeting and insertion of phospholamban and sarcolipin. In chapter 4 the trafficking of phospholamban and sarcolipin through the cell was studied. Using immunoblotting techniques it was seen that both phospholamban and sarcolipin exit the endoplamic reticulum and localise to the ERGIC compartment along with the ERGIC marker p53. Using TGN 46, a marker of the trans-Golgi network, revealed that neither phospholamban nor sarcolipin progress this far in the secretory pathway. These results were further supported by cellular fractionation followed by western blot analysis to reveal which compartments phospholamban and sarcolipin were resident in. This study showed that significant amounts of

phospholamban and sarcolipin are located outside the endoplasmic reticulum, consistent with their localisation in the ERGIC. Together these studies indicate that a process of retrieval from the ERGIC must be the major process by which both phospholamban and sarcolipin are maintained in the endoplasmic reticulum. Mutation of the transmembrane region of phospholamban and sarcolipin by extending it with extra leucine residues revealed that this region is important in maintaining these proteins in the endoplasmic reticulum. The mutants which had the transmembrane region extended were seen to migrate from the endoplasmic reticulum and localise to the plasma membrane. This was judged by colocalisation with the plasma membrane marker concanavalin A conjugated with Alexafluor 594. The data revealed that it is likely that the transmembrane region of both phospholamban and sarcolipin are key areas involved with the retrieval process and hence maintenance of these proteins in the endoplasmic reticulum. Although extension of the transmembrane region in phospholamban and sarcolipin caused mistargeting of the proteins to the plasma membrane it appeared not to alter the orientation of the proteins in the membrane (chapter 5). However, altering arginine 27 to glutamate in sarcolipin appeared to reverse the orientation of sarcolipin from C-terminal anchoring to N-terminal anchoring. The orientation was analysed by several means. Sequential permeabilisation of the plasma and endoplasmic reticulum membranes by saponin and Triton X-100 followed by labelling of the EGFP tag on the sarcolipin EGFP mutant. The other method used involved assessing the susceptibility of the EGFP tag to digestion by protease K after cells transfected with the mutant form of sarcolipin were homogenised to provide microsomes. Both techniques revealed a reverse in orientation from the earlier experiments carried out on the wild type protein tagged only with EGFP at the N-

terminus. This mutation reveals how important charge can be in determining the orientation of a protein in a membrane. What is unexpected is that this change in charge usually favours the original orientation with the more positive side of the transmembrane region facing into the cytoplasm (Higy *et al.*, 2004). The reasons for this reversal in orientation are at present unknown. Perhaps the cytoplasmic chaperones that guide post-translationally inserted proteins to the endoplasmic reticulum handle this protein differently than the wild type counterpart. This may limit the orientation of the sarcolipin EGFP mutant to N-terminal anchoring in the endoplasmic reticulum membrane. This mutation could also affect the folding of the protein and hence the translocation of the protein across the membrane. Alternatively introducing this mutation may interfere with the insertion machinery of the endoplasmic reticulum.

There are a number of ways the studies reported here could be developed. Firstly more mutants, introducing charge changes in the region flanking the transmembranous domain could be produced to shed more light on the factors affecting the orientation of these types of proteins. Secondly cross-linking studies of the type undertaken by Abell *et al.*, (2004) could be performed to identify key proteins involved with insertion of phospholamban and sarcolipin as well as identify receptors responsible for the retrieval of these two proteins from the ERGIC compartment. Once cross-linking had revealed interactions it would be possible to identify other protein parterners with antibodies directed against known candidates. Another approach in studying proteins responsible for the insertion / trafficking of phospholamban and sarcolipin would be the use of mass spectroscopy. In this approach the COS 7 cells would be transfected with phospholamban EGFP / sarcolipin EGFP. A cross linking reagent would then be added and the cells

homogenised. Affinity chromatography could then be used to separate proteins which have cross-linked to phospholamban EGFP / sarcolipin EGFP. These separated proteins could then be run on SDS PAGE where you would look for bands that have shifted in molecular mass from phospholamban EGFP / sarcolipin EGFP indicating interaction with another protein hence an increase in mass. These bands would then be excised from the gel and digested with a protease such as trypsin. After the digest the proteins would then undergo mass spectroscopy in order to identify molecular ions produced. These ions could then hopefully be identified via a large online database which would indicate the types of proteins that are interacting with phospholamban and sarcolipin in trafficking through the cell as well as proteins involved with the insertion into the endoplasmic reticulum of these C-terminally anchored membrane proteins.

Reference List

Abell,B.M., Jung,M., Oliver,J.D., Knight,B.C., Tyedmers,J., Zimmermann,R., High,S. (2003). Tail-anchored and signal-anchored proteins utilize overlapping pathways during membrane insertion. Journal Of Biological Chemistry *278*, 5669-5678.

Abell,B.M., Pool,M.R., Schlenker,O., Sinning,I., High,S. (2004). Signal recognition particle mediates post-translational targeting in eukaryotes. Embo Journal *23*, 2755-2764.

Ahmed,Z., Reid,D.G., Watts,A., Middleton,D.A. (2000). A solid-state NMR study of the phospholamban transmembrane domain: local structure and interactions with Ca2+-ATPase. Biochimica Et Biophysica Acta-Biomembranes *1468*, 187-198.

Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K., Watson,J.D. (1994). Molecular Biology of the Cell. Chapter 12 (Intracellular Compartments and Protein Sorting), Chapter 13 (Vesicle Traffic in the Secretory and Endocytic Pathway) and Chapter 16 (The Cytoskeleton) Garland Publishing, Inc. NewYork and London

Allan,V.J., Thompson,H.M., McNiven,M.A. (2002). Motoring around the Golgi. Nature Cell Biology *4*, E236-E242.

Andersson,H., Kappeler,F., Hauri,H.P. (1999). Protein targeting to endoplasmic reticulum by dilysine signals involves direct retention in addition to retrieval. Journal Of Biological Chemistry *274*, 15080-15084.

Antonny,B., Madden,D., Hamamoto,S., Orci,L., Schekman,R. (2001). Dynamics of the COPII coat with GTP and stable analogues. Nature Cell Biology *3*, 531-537.

Arar,C., Carpentier,V., Lecaer,J.P., Monsigny,M., Legrand,A., Roche,A.C. (1995). ERGIC-53, A membrane-protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. Journal Of Biological Chemistry *270*, 3551-3553.

Aridor,M., Bannykh,S.I., Rowe,T., Balch,W.E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic-reticulum to Golgi transport. Journal of Cell Biology *131*, 875-893.

Arkin,I.T., Adams,P.D., Brunger,A.T., Smith,S.O., Engelman,D.M. (1997). Structural perspectives of phospholamban, a helical transmembrane pentamer. Annual Review of Biophysics and Biomolecular Structure *26*, 157-179.

Armstrong,J. (2000). How do Rab proteins function in membrane traffic? International Journal of Biochemistry & Cell Biology *32*, 303-307.

Asahi,M., Kimura,Y., Kurzydlowski,K., Tada,M., MacLennan,D.H. (1999). Transmembrane helix M6 in sarco(endo)plasmic reticulum Ca2+- ATPase (SERCA) forms a functional interaction site with phospholamban. Circulation *100*, 2213.

Asahi,M., Kurzydlowski,K., Tada,M., MacLennan,D.H. (2002). Sarcolipin inhibits polymerization of phospholamban to induce superinhibition of sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs). Journal Of Biological Chemistry *277*, 26725-26728.

Asahi,M., Sugita,Y., Kurzydlowski,K., De Leon,S., Tada,M., Toyoshima,C., MacLennan,D.H. (2003). Sarcolipin regulates sarco(endo)plasmic reticulurn Ca2+-ATPase (SERCA) by binding to transmembrane helices alone or in association with phospholamban. Proceedings Of The National Academy Of Sciences Of The United States Of America *100*, 5040-5045.

Bannykh,S., Aridor,M., Plutner,H., Rowe,T., Balch,W.E. (1995). Regulated export of cargo from the endoplasmic reticulum of mammalian cells. Cold Spring Harbor Symposia on Quantitative Biology *60*, 127-137.

Bannykh,S.I., Rowe,T., Balch,W.E. (1996). The organization of endoplasmic reticulum export complexes. Journal of Cell Biology *135*, 19-35.

Barlowe,C. (1998). COPII and selective export from the endoplasmic reticulum. Biochimica et Biophysica Acta-Molecular Cell Research *1404*, 67-76.

Barlowe,C., Orci,L., Yeung,T., Hosobuchi,M., Hamamoto,S., Salama,N., Rexach,M.F., Ravazzola,M., Amherdt,M., Schekman,R. (1994). COPII – a membrane coat formed by

SEC proteins that drive vesicle budding from the endoplasmic-reticulum. Cell *77*, 895-907.

Beckmann,R., Toye,A.M., Smythe,J.S., Anstee,D.J., Tanner,M.J.A. (2002). An N-terminal GFP tag does not alter the functional expression to the plasma membrane of red cell and kidney anion exchanger (AE1) in mammalian cells. Molecular Membrane Biology *19*, 187-200.

Belden,W.J., Barlowe,C. (2001). Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. Science *294*, 1528-1531.

Biggin,P.C., Sansom,M.S.P. (1999). Interactions of alpha-helices with lipid bilayers: a review of simulation studies. Biophysical Chemistry *76*, 161-183.

Block,B.A. (1994). Thermogenesis in muscle. Annual Review of Physiology *56*, 535-577.

Block,B.A., Obrien,J., Meissner,G. (1994). Characterization of the sarcoplasmic-reticulum proteins in the thermogenic muscles of fish. Journal of Cell Biology *127*, 1275-1287.

Boehm,J., Letourneur,F., Ballensiefen,W., Ossipov,D., Demolliere,C., Schmitt,H.D. (1997). Sec12p requires Rer1p for sorting to coatomer (COPI)-coated vesicles and retrieval to the ER. Journal of Cell Science *110*, 991-1003.

Bos,K., Wraight,C., Stanley,K.K. (1993). TGN38 is maintained in the trans-Golgi network by a tyrosine- containing motif in the cytoplasmic domain. Embo Journal *12*, 2219-2228.

Bremser,M., Nickel,W., Schweikert,M., Ravazzola,M., Amherdt,M., Hughes,C.A., Sollner,T.H., Rothman,J.E., Wieland,F.T. (1999). Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. Cell *96*, 495-506.

Bryant,N.J., Verma,S., James,D.E. (2001). Syntaxin - Sec1p/Munc18 interactions are highly specific and represent an important regulatory node for vesicle transport. Molecular Biology of the Cell *12*, 408.

Buck,B., Zamoon,J., Kirby,T.L., DeSilva,T.M., Karim,C., Thomas,D., Veglia,G. (2003). Overexpression, purification, and characterization of recombinant Ca-ATPase regulators for high-resolution solution and solid-state NMR studies. Protein Expression and Purification *30*, 253-261.

Cantilina,T., Sagara,Y., Inesi,G., Jones,L.R. (1993). Comparative-studies of cardiac and skeletal sarcoplasmic-reticulum ATPases – effect of a phospholamban antibody on enzyme activation by Ca^{2+}. Circulation *88*, 624.

Carafoli,E. (1994). Biogenesis – plasma-membrane calcium-ATPase - 15 years of work on the purified enzyme. Faseb Journal *8*, 993-1002.

Chardin,P., McCormick,F. (1999). Brefeldin A: The advantage of being uncompetitive. Cell *97*, 153-155.

Chavrier,P., Goud,B. (1999). The role of ARF and Rab GTPases in membrane transport. Current Opinion in Cell Biology *11*, 466-475.

Chen,Z.H., Stokes,S.L., Rice,W.J., Jones,L.R. (2003). Spatial and dynamic interactions between phospholamban and the canine cardiac Ca2+ pump revealed with use of heterobifunctional cross-linking agents. Journal Of Biological Chemistry *278*, 48348-48356.

Clary,D.O., Griff,I.C., Rothman,J.E. (1990). SNAPS, a family of NSF attachment proteins involved in intracellular membrane-fusion in animals and yeast. Cell *61*, 709-721.

Cole,N.B., Ellenberg,J., Song,J., DiEuliis,D., Lippincott-Schwartz,J. (1998). Retrograde transport of Golgi-localized proteins to the ER. Journal of Cell Biology *140*, 1-15.

Colyer,J., Mata,A.M., Lee,A.G., East,J.M. (1989). Effects on ATPase activity of monoclonal-antibodies raised against (Ca^{-2+} Mg^{-2+})-ATPase from rabbit skeletal-muscle sarcoplasmic-reticulum and their correlation with epitope location. Biochemical Journal *262*, 439-447.

Colyer,J., Wang,J.H. (1991). Dependence of cardiac sarcoplasmic-reticulum calcium-pump activity on the phosphorylation status of phospholamban. Journal Of Biological Chemistry *266*, 17486-17493.

Cosson,P., Letourneur,F. (1994). Coatomer interaction with di-lysine endopasmic-reticulum retention motifs. Science *263*, 1629-1631.

East,J.M. (2000). Sarco(endo)plasmic reticulum calcium pumps: recent advances in our understanding of structure/function and biology (Review). Molecular Membrane Biology *17*, 189-200.

Ellgaard,L., Helenius,A. (2003). Quality control in the endoplasmic reticulum. Nature Reviews Molecular Cell Biology *4*, 181-191.

Feher,J.J., Lebolt,W.R. (1990). Stabalization of rat cardiac-sarcoplasmic-reticulum Ca^{2+} uptake activity and isolation of vesicles with improved calcium-uptake activity. Molecular And Cellular Biochemistry *99*, 41-52.

Feldheim,D.A., Yoshimura,K., Admon,A., Schekman,R. (1993). Characterization of SEC63P and associated ER membrane-proteins implicated in protein translocation. Journal of Cellular Biochemistry 10.

Fiedler,K., Veit,M., Stamnes,M.A., Rothman,J.E. (1996). Bimodal interaction of coatomer with the p24 family of putative cargo receptors. Science *273*, 1396-1399.

Fons,R.D., Bogert,B.A., Hegde,R.S. (2003). Substrate-specific function of the translocon-associated protein complex during translocation across the ER membrane. Journal of Cell Biology *160*, 529-539.

Fujii,J., Kadoma,M., Tada,M., Toda,H., Sakiyama,F. (1986). Characterization of a structural unit of phospholamban by amino-acid sequencing and electrophoretic analysis. Biochemical And Biophysical Research Communications *138*, 1044-1050.

Fujii,J., Ueno,A., Kitano,K., Tanaka,S., Kadoma,M., Tada,M. (1987). Complete complimentary DNA-derived amino-acid-sequence of canine cardiac phospholamban. Journal of Clinical Investigation *79*, 301-304.

Gayan-Ramirez,G., Vanzeir,L., Wuytack,F., Decramer,M. (2000). Corticosteroids decrease mRNA levels of SERCA pumps, whereas they increase sarcolipin mRNA in the rat diaphragm. Journal Of Physiology-London *524*, 387-397.

Girotti,M., Banting,G. (1996). TGN38-GFP hybrids expressed in stably transfected NRK cells provide a tool for the real-time, in vivo study of membrane traffic pathways and suggest a possible role for rat TGN38. Molecular Biology of the Cell *7*, 1885.

Gluzman,Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell *23*, 175-182.

Gonzalez,L., Scheller,R.H. (1999). Regulation of membrane trafficking: Structural insights from a Rab/effector complex. Cell *96*, 755-758.

Goode,B.L., Drubin,D.G., Barnes,G. (2000). Functional cooperation between the microtubule and actin cytoskeletons. Current Opinion in Cell Biology *12*, 63-71.

Greenfield,J.J.A., High,S. (1999). The Sec61 complex is located in both the ER and the ER-Golgi intermediate compartment. Journal of Cell Science *112*, 1477-1486.

Griffiths,G. (2000). Gut thoughts on the Golgi complex. Traffic *1*, 738-745.

Griffiths,G., Pepperkok,R., Locker,J.K., Kreis,T.E. (1995). Immunocytochemical localisation of beta-COP to the ER-Golgi boundry and the TGN. Journal of Cell Science *108*, 2839-2856.

Hammond,C., Helenius,A. (1994). Folding of VSV G-protein – sequential interaction with BIP and calnexin. Science *266*, 456-458.

Hammond,C., Helenius,A. (1995). Quality-control in the secretory pathway. Current Opinion in Cell Biology *7*, 523-529.

Higy,M., Junne,T., Spiess,M. (2004). Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry *43*, 12716-12722.

Hirschberg,K., Presley,J., Cole,N., Lippincott,J. (1997). Golgi to plasma membrane trafficking in living cells: Characterization of post-Golgi intermediates. Molecular Biology of the Cell *8*, 1121.

Honsho,M., Mitoma,J.Y., Ito,A. (1998). Retention of cytochrome b(5) in the endoplasmic reticulum is transmembrane and luminal domain-dependent. Journal Of Biological Chemistry *273*, 20860-20866.

Hoogenraad,C.C., Akhmanova,A., Howell,S.A., Dortland,B.R., De Zeeuw,C.I., Willemsen,R., Visser,P., Grosveld,F., Galjart,N. (2001). Mammalian Golgi-associated Bicaudal-D2 functions in the dynein- dynactin pathway by interacting with these complexes. Embo Journal *20*, 4041-4054.

Horng,J.T., Tan,C.Y. (2004). Biochemical characterization of the coating mechanism of the endosomal donor compartment of synaptic vesicles. Neurochemical Research *29*, 1411-1416.

Horsnell,W.G.C., Steel,G.J., Morgan,A. (2002). Analysis of NSF mutants reveals residues involved in SNAP binding and ATPase stimulation. Biochemistry *41*, 5230-5235.

Hughes,G., East,J.M., Lee,A.G. (1994). The hydrophobic domain of phospholamban inhibits the Ca^{2+} transport step of the Ca^{2+}-ATPase. Biochemical Journal *303*, 511-516.

Hughes,G., Starling,A.P., Sharma,R.P., East,J.M., Lee,A.G. (1996). An investigation of the mechanism of inhibition of the Ca2+- ATPase by phospholamban. Biochemical Journal *318*, 973-979.

Hunziker,W., Geuze,H.J. (1996). Intracellular trafficking of lysosomal membrane proteins. Bioessays *18*, 379-389.

Hutter,M.C., Krebs,J., Meiler,J., Griesinger,C., Carafoli,E., Helms,V. (2002). A structural model of the complex formed by phospholamban and the calcium pump of sarcoplasmic reticulum obtained by molecular mechanics. Chembiochem *3*, 1200-1208.

Jackson,M.R., Nilsson,T., Peterson,P.A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic-reticulum. Embo Journal *9*, 3153-3162.

Jackson,M.R., Nilsson,T., Peterson,P.A. (1993). Retrieval of transmembrane proteins to th endoplasmic-reticulum. Journal of Cell Biology *121*, 317-333.

Jahn,R., Sudhof,T.C. (1999). Membrane fusion and exocytosis. Annual Review of Biochemistry *68*, 863-911.

James,P., Inui,M., Tada,M., Chiesi,M., Carafoli,E. (1989). Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic-reticulum. Nature *342*, 90-92.

Jones,B.G., Thomas,L., Molloy,S.S., Thulin,C.D., Fry,M.D., Walsh,K.A., Thomas,G. (1995). Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase-II site in its cytoplasmic tail. Embo Journal *14*, 5869-5883.

Kaiser,C. (2000). Thinking about p24 proteins and how transport vesicles select their cargo. Proceedings Of The National Academy Of Sciences Of The United States Of America *97*, 3783-3785.

Kaiser,C.A., Schekman,R. (1990). Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell *61*, 723-733.

Kamal,A., Goldstein,L.S.B. (2000). Connecting vesicle transport to the cytoskeleton. Current Opinion in Cell Biology *12*, 503-508.

Katz,A.M. (1998). Regression of left ventricular hypertrophy - New hope for dying hearts. Circulation *98*, 623-624.

Keenan,R.J., Freymann,D.M., Stroud,R.M., Walter,P. (2001). The signal recognition particle. Annual Review of Biochemistry *70* , 755-775.

Keller,P., Simons,K. (1997). Post-Golgi biosynthetic trafficking. Journal of Cell Science *110*, 3001-3009.

Kelly,R.B., Burgess,T., Matsuuchi,L. (1987). Sorting it out in secretory-cells. Journal of Cellular Biochemistry 240.

Kimura,Y., Asahi,M., Kurzydlowski,K., Tada,M., MacLennan,D.H. (1998). Phospholamban domain I cytochrome b(5) transmembrane sequence chimeras do not inhibit SERCA2a. Febs Letters *425*, 509-512.

Kirchberger,M.A., Tada,M., Repke,D.I., Katz,A.M. (1972). cyclic adenosine 3', 5'-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. J.Mol.Cell Cardiol *4*, 673-680.

Klumperman,J. (2000). Transport between ER and Golgi. Current Opinion in Cell Biology *12*, 445-449.

Kornfeld,R., Kornfeld,S. (1985). Assembly of asparagine-linked oligosacharides. Annual Review of Biochemistry *54*, 631-664.

Kozak,M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell *44*, 283-292.

Kremer,W., Steiner,G., Beraud-Dufour,S., Kalbitzer,H.R. (2004). Conformational states of the small G protein Arf-1 in complex with the guanine nucleotide exchange factor ARNO-Sec7. Journal Of Biological Chemistry *279*, 17004-17012.

Kuehn,M.J., Schekman,R. (1997). COPII and secretory cargo capture into transport vesicles. Current Opinion in Cell Biology *9* , 477-483.

Lalli,J., Harrer,J.M., Luo,W.S., Kranias,E.G., Paul,R.J. (1997). Targeted ablation of the phospholamban gene is associated with a marked decrease in sensitivity in aortic smooth muscle. Circulation Research *80*, 506-513.

Lamb,G.D. (2000). Excitation-contraction coupling in skeletal muscle: Comparisons with cardiac muscle. Clinical and Experimental Pharmacology and Physiology *27*, 216-224.

Lamberth,S., Schmid,H., Muenchbach,M., Vorherr,T., Krebs,J., Carafoli,E., Griesinger,C. (2000). NMR solution structure of phospholamban. Helvetica Chimica Acta *83*, 2141-2152.

Lee,A.G., East,J.M. (2001). What the structure of a calcium pump tells us about its mechanism. Biochemical Journal *356*, 665-683.

Letourneur,F., Cosson,P. (1998). Targeting to the endoplasmic reticulum in yeast cells by determinants present in transmembrane domains. Journal Of Biological Chemistry *273*, 33273-33278.

Lewis,M.J., Pelham,H.R.B. (1990). A human homolog of the yeast HDEL receptor. Nature *348*, 162-163.

Li,C.F., Wang,J.H., Colyer,J. (1990). Immunological detection of phospholamban phosphorylation states facilitates the description of the mechanism of phosphorylation and dephosphorylation. Biochemistry *29* , 4535-4540.

Lippincott-Schwartz,J. (1998). Cytoskeletal proteins and Golgi dynamics. Current Opinion in Cell Biology *10*, 52-59.

Lippincott-Schwartz,J., Presley,J., Phair,R., Hirschberg,K. (2000). Secretory protein trafficking in single live cells. Biophysical Journal *78*, 812Symp.

LippincottSchwartz,J., Cole,N.B. (1995). Roles for microtubules and kinesin in membrane traffic between the endoplasmic-reticulum and the Golgi-complex. Biochemical Society Transactions *23*, 544-548.

LippincottSchwartz,J., Yuan,L.C., Bonifacino,J.S., Klausner,R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin-A- evidence for membrane cycling from Golgi to ER. Cell *56*, 801-813.

MacLennan,D.H. (2000). Ca2+ signalling and muscle disease. European Journal Of Biochemistry *267*, 5291-5297.

MacLennan,D.H., Kranias,E.G. (2003). Phospholamban: A crucial regulator of cardiac contractility. Nature Reviews Molecular Cell Biology *4*, 566-577.

Mancini,R., Piccolo,E., Mariggio,S., Filippi,B.M., Iurisci,C., Pertile,P., Berrie,C.P., Corda,D. (2003). Reorganization of actin cytoskeleton by the phosphoinositide metabolite glycerophosphoinositol 4-phosphate. Molecular Biology of the Cell *14*, 503-515.

Marsh,M., McMahon,H.T. (1999). Cell biology - The structural era of endocytosis. Science *285*, 215-220.

Martire,G., Mottola,G., Pascale,M.C., Malagolini,N., Turrini,I., SerafiniCessi,F., Jackson,M.R., Bonatti,S. (1996). Different fate of a single reporter protein containing KDEL or KKXX targeting signals stably expressed in mammalian cells. Journal Of Biological Chemistry *271*, 3541-3547.

Martoglio,B., Dobberstein,B. (1998). Signal sequences: more than just greasy peptides. Trends in Cell Biology *8*, 410-415.

Marx,S.O., Dilly,K., Cruz,J.D., Gaburjakova,M., Soble,E.A., Ondrias,K., Jafri,M.S., Henrikson,C., Lederer,W.J., Marks,A.R. (2000). Coupled ryanodine receptor gating in the heart: Role in controlling contractility. Circulation *102*, 434.

Mascioni,A., Karim,C., Barany,G., Thomas,D.D., Veglia,G. (2002). Structure and orientation of sarcolipin in lipid environments. Biochemistry *41*, 475-482.

Mcintosh,D.B., Woolley,D.G., Berman,M.C. (1992). 2',3'-O-(2,4,6-Trinitrophenyl)-8-Azido-AMP and ATP photolabel lys-492 at the active-site of sarcoplasmic-reticulum Ca^{2+}-ATPase. Journal Of Biological Chemistry *267*, 5301-5309.

Miller,E., Antonny,B., Hamamoto,S., Schekman,R. (2002). Cargo selection into COPII vesicles is driven by the Sec24p subunit. Embo Journal *21*, 6105-6113.

Minamisawa,S., Sato,Y., Tatsuguchi,Y., Fujino,T., Imamura,S., Uetsuka,Y., Nakazawa,M., Matsuoka,R. (2003a). Mutation of the phospholamban promoter associated with hypertrophic cardiomyopathy. Biochemical And Biophysical Research Communications *304*, 1-4.

Minamisawa,S., Wang,Y.B., Chen,J., Ishikawa,Y., Chien,K.R., Matsuoka,R. (2003b). Atrial chamber-specific expression of sarcolipin is regulated during development and hypertrophic remodeling. Journal Of Biological Chemistry *278*, 9570-9575.

Mizuno,M., Singer,S.J. (1994). A possible role for stable microtubules in intracellular transport from the endoplasmic-reticulumto the Golgi-apparatus. Journal of Cell Science *107*, 1321-1331.

Moller,J.V., Juul,B., leMaire,M. (1996). Structural organization, ion transport, and energy transduction of P-type ATPases. Biochimica et Biophysica Acta-Reviews on Biomembranes *1286*, 1-51.

Mortishire-Smith,R.J., Broughton,H., Garsky,V.M., Mayer,E.J., Johnson,R.G. (1998). Structural studies on phospholamban and implications for regulation of the Ca2+-ATPase. Cardiac Sarcoplasmic Reticulum Function and Regulation of Contractility *853*, 63-78.

Moss,K., Helm,A., Lu,Y., Bragin,A., Skach,W.R. (1998). Coupled translocation events generate topological heterogeneity at the endoplasmic reticulum membrane. Molecular Biology of the Cell *9*, 2681-2697.

Mostov,K.E., Verges,M., Altschuler,Y. (2000). Membrane traffic in polarized epithelial cells. Current Opinion in Cell Biology *12*, 483-490.

Munro,S. (1995). An investigation of the role of transmembrane domains in Golgi protein retention. Embo Journal *14*, 4695-4704.

Munro,S., Pelham,H.R.B. (1987). A C-terminal signal prevents secretion of lumenal ER proteins. Cell *48*, 899-907.

Muth,T.R., Caplan,M.J. (2003). Transport protein trafficking in polarized cells. Annual Review of Cell and Developmental Biology *19*, 333-366.

Nakano,A., Brada,D., Schekman,R. (1988). A membrane glycoprotein, SEC12P, required fror protein-transport from the endoplasmic-reticulum to the Golgi-apparatus in yeast. Journal of Cell Biology *107*, 851-863.

Narasimhan,S., Freed,J.C., Schachter,H. (1985). Control of glycoprotein - synthesis .10. Control of glycoprotein - synthesis - bovine-milk UDP-galactose - N-acetylglucosamine beta-4-galactosyltransferase catalyzes the preferential transfer of galactose to the GLCNAC-beta-1,2MAN-alpha-1,3 branch of both bisected and non bisected complex biantennary asparagine-linked oligosacharidess. Biochemistry *24*, 1694-1700.

Newton,T., Chad,J.E., Lee,A.G., East,J.M. (2002). Calcium pump targeting. Faseb Journal *15*, A1176.

Nichols,B.J., Pelham,H.R.B. (1998). SNAREs and membrane fusion in the Golgi apparatus. Biochimica et Biophysica Acta-Molecular Cell Research *1404*, 9-31.

Nishikawa,S., Nakano,A. (1993). Identification of a gene required for membrane-protein retention in the early secretory pathway. Proceedings Of The National Academy Of Sciences Of The United States Of America *90*, 8179-8183.

Nishimura,N., Balch,W.E. (1997). A di-acidic signal required for selective export from the endoplasmic reticulum. Science *277*, 556-558.

Nishimura,N., Bannykh,S., Slabough,S., Matteson,J., Altschuler,Y., Hahn,K., Balch,W.E. (1999). A di-acidic (DXE) code directs concentration of cargo during export from the endoplasmic reticulum. Journal Of Biological Chemistry *274*, 15937-15946.

Nobe,K., Sutliff,R.L., Kranias,E.G., Paul,R.J. (2000). Phospholamban (PLB) regulation of bladder contractility: Evidence from gene targeted (PLB-KO) and smooth muscle specific expression (PLB-SMOE) of PLB in mice. Biophysical Journal *78*, 656Pos.

Novick,P., Ferro,S., Schekman,R. (1981). Order of events in the yeast secretory pathway. Cell *25*, 461-469.

Odermatt,A., Becker,S., Khanna,V.K., Kurzydlowski,K., Leisner,E., Pette,D., MacLennan,D.H. (1998). Sarcolipin regulates the activity of SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca2+-ATPase. Journal Of Biological Chemistry *273*, 12360-12369.

Odermatt,A., Taschner,P.E.M., Scherer,S.W., Beatty,B., Khanna,V.K., Cornblath,D.R., Chaudhry,V., Yee,W.C., Schrank,B., Karpati,G., Breuning,M.H., Knoers,N., MacLennan,D.H. (1997). Characterization of the gene encoding human sarcolipin (SLN), a proteolipid associated with SERCA1: Absence of structural mutations in five patients with Brody disease. Genomics *45*, 541-553.

Orci,L., Palmer,D.J., Amherdt,M., Rothman,J.E. (1993). Coated vesicle assembly in the Golgi requires only coatamer and ARF proteins from the cytosol. Nature *364*, 732-734.

Orci,L., Perrelet,A., Rothman,J.E. (1998). Vesicles on strings: Morphological evidence for processive transport within the Golgi stack. Proceedings Of The National Academy Of Sciences Of The United States Of America *95*, 2279-2283.

Orci,L., Ravazzola,M., Volchuk,A., Engel,T., Gmachl,M., Amherdt,M., Perrelet,A., Sollner,T.H., Rothman,J.E. (2000). Anterograde flow of cargo across the Golgi stack potentially mediated via bidirectional "percolating" COPI vesicles. Proceedings Of The National Academy Of Sciences Of The United States Of America *97*, 10400-10405.

Orci,L., Stamnes,M., Ravazzola,M., Amherdt,M., Perrelet,A., Sollner,T.H., Rothman,J.E. (1997). Bidirectional transport by distinct populations of COPI-coated vesicles. Cell *90*, 335-349.

Ou,W.J., Cameron,P.H., Thomas,D.Y., Bergeron,J.J.M. (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature *364*, 771-776.

Pedrazzini,E., Villa,A., Longhi,R., Bulbarelli,A., Borgese,N. (2000). Mechanism of residence of cytochrome b(5), a tail-anchored protein, in the endoplasmic reticulum. Journal of Cell Biology *148*, 899-913.

Pelham,H.R.B. (1994). About turn for the COPS. Cell *79*, 1125-1127.

Pelham,H.R.B. (1997). Traffic control in the secretory pathway. Faseb Journal *11*, 903.

Pelham,H.R.B. (1998). Getting through the Golgi complex. Trends in Cell Biology *8*, 45-49.

Pelham,H.R.B., Rothman,J.E. (2000). The debate about transport in the Golgi - Two sides of the same coin? Cell *102*, 713-719.

Pevsner,J., Hsu,S.C., Braun,J.E.A., Calakos,N., Ting,A.E., Bennett,M.K., Scheller,R.H. (1994). Specificity and regulation of a synaptic vesicle docking complex. Neuron *13*, 353-361.

Pfeffer,S.R. (1999). Transport-vesicle targeting: tethers before SNAREs. Nature Cell Biology *1*, E17-E22.

Pfeffer,S.R., Rothman,J.E. (1987). Biosynthetic protein – transport and sorting by the endoplasmic- reticulum and Golgi. Annual Review of Biochemistry *56*, 829-852.

Pollesello,P., Annila,A., Ovaska,M. (1999). Structure of the 1-36 amino-terminal fragment of human phospholamban by nuclear magnetic resonance and modeling of the phospholamban pentamer. Biophysical Journal *76*, 1784-1795.

Ponnambalam,S., Rabouille,C., Luzio,J.P., Nilsson,T., Warren,G. (1994). The TGN38 glycoprotein contains 2 nonoverlapping signals that mediate localisation to the trans-Golgi network. Journal of Cell Biology *125*, 253-268.

Prescott,A.R., Lucocq,J.M., James,J., Lister,J.M., Ponnambalam,S. (1997). Distinct compartmentalization of TGN46 and beta 1,4- galactosyltransferase in HeLa cells. European Journal of Cell Biology *72*, 238-246.

Presley,J.F., Cole,N.B., Schroer,T.A., Hirschberg,K., Zaal,K.J.M., Lippincottschwartz,J. (1997). ER-to-Golgi transport visualized in living cells. Nature *389*, 81-85.

Robinson,M.S., Bonifacino,J.S. (2001). Adaptor-related proteins. Current Opinion in Cell Biology *13*, 444-453.

Roquemore,E.P., Banting,G. (1998). Efficient trafficking of TGN38 from the endosome to the trans-Golgi network requires a free hydroxyl group at position 331 in the cytosolic domain. Molecular Biology of the Cell *9*, 2125-2144.

Rothman,J.E., Orci,L. (1992). Molecular dissection of the secretory pathway. Nature *355*, 409-415.

Rothman,J.E., Wieland,F.T. (1996). Protein sorting by transport vesicles. Science *272*, 227-234.

Rouille,Y., Rohn,W., Hoflack,B. (2000). Targeting of lysosomal proteins. Seminars in Cell & Developmental Biology *11*, 165-171.

Sadler,I., Chiang,A., Kurihara,T., Rothblatt,J., Way,J., Silver,P. (1989). A yeast gene important for protein assembly into the endoplasmic-reticulum and the nucleus has homology to DNAJ, an escherichia-coli heat shock protein. Journal of Cell Biology *109*, 2665-2675.

Saraste,J., Kuismanen,E. (1984). Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. Cell *38*, 535-549.

Sato,K., Sato,M., Nakano,A. (1997). Rer1p as common machinery for the endoplasmic reticulum localisation of membrane proteins. Proceedings Of The National Academy Of Sciences Of The United States Of America *94*, 9693-9698.

Sato,K., Sato,M., Nakano,A. (2001). Rer1p-dependent localisation signals of endoplasmic reticulum membrane proteins. Molecular Biology of the Cell *12*, 1355.

Sato,K., Sato,M., Nakano,A. (2003). Rer1p, a retrieval receptor for ER membrane proteins, recognizes transmembrane domains in multiple modes. Molecular Biology of the Cell *14*, 3605-3616.

Sato,M., Sato,K., Nakano,A. (1996a). Endoplasmic reticulum localisation of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. Journal of Cell Biology *134*, 279-293.

Sato,M., Sato,K., Nakano,A. (1996b). Identification of two ER localisation signals of Sec12p. Molecular Biology of the Cell *7*, 2943.

Scales,S.J., Pepperkok,R., Kreis,T.E. (1997). Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. Cell *90*, 1137-1148.

Schekman,R., Orci,L. (1996). Coat proteins and vesicle budding. Science *271*, 1526-1533.

Schmid,S.L. (1997). Clathrin-coated vesicle formation and protein sorting: An integrated process. Annual Review of Biochemistry *66*, 511-548.

Schutze,M.P., Peterson,P.A., Jackson,M.R. (1994). An N-terminal double-arginine motif maintains type-II membrane- proteins in the endoplasmic-reticulum. Embo Journal *13*, 1696-1705.

Shweizer,A., Matter,K., Ketcham,C.M., Hauri,H.P. (1991). The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and Cis-Golgi. Journal of Cell Biology *113*, 45-54.

Semenza,J.C., Hardwick,K.G., Dean,N., Pelham,H.R.B. (1990). ERD2, a yeast gene required for the receptor-mediated retrieval of lumenal ER proteins from the secretory pathway. Cell *61*, 1349-1357.

Sheu,S.S., Sharma,V.K., Uglesity,A. (1986). Na^{+}-Ca-$^{2+}$ exchange contributes to increase of cytosolic Ca^{2+} concentration during depolarization in heart-muscle. American Journal of Physiology *250*, C651-C656.

Simmerman,H.K.B., Collins,J.H., Theibert,J.L., Wegener,A.D., Jones,L.R. (1986). Sequence-Analysis of phospholamban - identification of phosphorylation sites and 2 major structural domains. Journal Of Biological Chemistry *261*, 3333-3341.

Simmerman,H.K.B., Lovelace,D.E., Jones,L.R. (1989). Secondary structure of detergent-solubilized phospholamban, a phosphorylatable, oligomeric protein of cardiac sarcoplasmic - reticulum. Biochimica Et Biophysica Acta *997*, 322-329.

Simons,K., Ikonen,E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.

Slack,J.P., Grupp,I.L., Luo,W.S., Kranias,E.G. (1997). Phospholamban ablation enhances relaxation in the murine soleus. American Journal of Physiology-Cell Physiology *42*, C1-C6.

Smith,W.S., Broadbridge,R., East,J.M., Lee,A.G. (2002). Sarcolipin uncouples hydrolysis of ATP from accumulation of Ca2+ by the Ca2+-ATPase of skeletal-muscle sarcoplasmic reticulum. Biochemical Journal *361*, 277-286.

Sollner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R.H., Rothman,J.E. (1993). A protein assembly- disassembly pathway in-vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. Cell *75*, 409-418.

Sonnichsen,B., Fullekrug,J., Van,P.N., Diekmann,W., Robinson,D.G., Mieskes,G. (1994). Retention and retrieval - both mechanisms cooperate to maintain calreticulin in the endoplasmic-reticulum. Journal of Cell Science *107*, 2705-2717.

Spiess,M. (1995). Heads or tails - what determines the orientation of proteins in the membrane . Febs Letters *369*, 76-79.

Springer,S., Chen,E., Duden,R., Marzioch,M., Rowley,A., Hamamoto,S., Merchant,S., Schekman,R. (2000). The p24 proteins are not essential for vesicular transport in Saccharomyces cerevisiae. Proceedings Of The National Academy Of Sciences Of The United States Of America *97*, 4034-4039.

Springer,S., Schekman,R. (1998). Sorting of membrane proteins into COPII ER to Golgi vesicles. Molecular Biology of the Cell *9*, 1968.

Stephens,D.J., Pepperkok,R. (2001). The many ways to cross the plasma membrane. Proceedings Of The National Academy Of Sciences Of The United States Of America *98*, 4295-4298.

Stokes,D.L. (1997). Keeping calcium in its place: Ca2+-ATPase and phospholamban. Current Opinion in Structural Biology *7*, 550-556.

Stokes,D.L., Wagenknecht,T. (2000). Calcium transport across the sarcoplasmic reticulum - Structure and function of Ca2+-ATPase and the ryanodine receptor. European Journal Of Biochemistry *267*, 5274-5279.

Sutliff,R.L., Bowman,P.S., Kranias,E.G., Paul,R.J. (2000). Phospholamban regulates resistance vessel contractility. Faseb Journal *14*, A662.

Suzuki,T., Wang,J.H. (1986). Stimulation of bovine cardiac sarcoplasmic-reticulum Ca^{2+} pump and blocking of phospholamban phosphorylation and dephosphorylation by a phospholamban monoclonal-antibody. Journal Of Biological Chemistry *261*, 7018-7023.

Tai,A.W., Chuan,J.Z., Sung,C.H. (1999). Characterization of the two cytoplasmic dynein light chains Tetex-1 and RP3. Molecular Biology of the Cell *10*, 2125.

Tatulian,S.A., Jones,L.R., Reddy,L.G., Stokes,D.L., Tamm,L.K. (1995). Secondary structure and orientation of phospholamban reconstituted in supported bilayers from polarized attenuated total-reflection FTIR spectroscopy. Biochemistry *34*, 4448-4456.

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

Toole,G., Toole,s. (1995). Understanding Biology, for advanced level. Chapter 4 (cellular organization) and Chapter 21 (blood and circulation) Stanley Thornes (Publishers) LTD Cheltenham

Toomre,D., Keller,P., White,J., Olivo,J.C., Simons,K. (1999). Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. Journal of Cell Science *112*, 21-33.

Toyofuku,T., Kurzydlowski,K., Tada,M., MacLennan,D.H. (1993). Identification of regions in the Ca^{2+}-ATPase of sarcoplasmic-reticulum that affect functional association with phospholamban. Journal Of Biological Chemistry *268*, 2809-2815.

Toyoshima,C., Asahi,M., Sugita,Y., Khanna,R., Tsuda,T., MacLennan,D.H. (2003). Modeling of the inhibitory interaction of phospholamban with the Ca2+ ATPase. Proceedings Of The National Academy Of Sciences Of The United States Of America *100*, 467-472.

Toyoshima,C., Mizutani,T. (2004). Crystal structure of the calcium pump with a bound ATP analogue. Nature *430*, 529-535.

Toyoshima,C., Nakasako,M., Nomura,H., Ogawa,H. (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 angstrom resolution. Nature *405*, 647-655.

Toyoshima,C., Nomura,H. (2002). Structural changes in the calcium pump accompanying the dissociation of calcium. Nature *418*, 605-611.

Tsien,R.Y. (1998). The green fluorescent protein. Annual Review of Biochemistry *67*, 509-544.

Tupling,A.R., Asahi,M., MacLennan,D.H. (2002). Sarcolipin overexpression in rat slow twitch muscle inhibits sarcoplasmic reticulum Ca2+ uptake and impairs contractile function. Journal Of Biological Chemistry *277*, 44740-44746.

Vanderbliek,A.M., Redelmeier,T.E., Damke,H., Tisdale,E.J., Meyerowitz,E.M., Schmid,S.L. (1993). Mutations in human dynamin block an intermediates stage in coated vesicle formation. Journal of Cell Biology *122*, 553-563.

Voss,J., Jones,L.R., Thomas,D.D. (1994). The physical-mechanism of calcium-pump regulation in the heart. Biophysical Journal *67*, 190-196.

Walker,C.A., Spinale,F.G. (1999). The structure and function of the cardiac myocyte: A review of fundamental concepts. Journal of Thoracic and Cardiovascular Surgery *118*, 375-382.

Walter,P., Johnson,A.E. (1994). Signal Sequence recognition and protein targeting to the endoplasmic-reticulum mrmbrane. Annual Review of Cell Biology *10*, 87-119.

Wang,S.R., Trumble,W.R., Liao,H., Wesson,C.R., Dunker,A.K., Kang,C.H. (1998). Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. Nature Structural Biology *5*, 476-483.

Warren,G., Mellman,I. (1999). Bulk flow redux? Cell *98*, 125-127.

Waters,M.G., Hughson,F.M. (2000). Membrane tethering and fusion in the secretory and endocytic pathways. Traffic 588-597.

Waters,M.G., Pfeffer,S.R. (1999). Membrane tethering in intracellular transport. Current Opinion in Cell Biology *11*, 453-459.

Waters,M.G., Serafini,T., Rothman,J.E. (1991). Coatamer - a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. Nature *349*, 248-251.

Wattenberg,B., Lithgow,T. (2001). Targeting of C-terminal (tail)-anchored proteins: Understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic *2*, 66-71.

Weber,T., Zemelman,B.V., McNew,J.A., Westermann,B., Gmachl,M., Parlati,F., Sollner,T.H., Rothman,J.E. (1998a). SNARE dependent membrane fusion. Molecular Biology of the Cell *9*, 1925.

Weber,T., Zemelman,B.V., McNew,J.A., Westermann,B., Gmachl,M., Parlati,F., Sollner,T.H., Rothman,J.E. (1998b). SNARE pins: Minimal machinery for membrane fusion. Cell *92*, 759-772.

Wegener,A.D., Simmerman,H.K.B., Liepnieks,J., Jones,L.R. (1986). Proteolytic cleavage of phospholamban purified from canine cardiac sarcoplasmic-reticulum vesicles – generation of a low resolution model of phospholamban structure. Journal Of Biological Chemistry *261*, 5154-5159.

Wegener,A.D., Simmerman,H.K.B., Lindemann,J.P., Jones,L.R. (1989). Phospholamban phosphorylation in intact ventricles – phosphorylation of serine-16 and threonine-17 in response to beta-adrenergic stimulation. Journal Of Biological Chemistry *264*, 11468-11474.

Weiland,E., Weiland,F., Grossmann,A. (1987). Lactate dehydrogenase-elevating virus induces anti-Golgi apparatus antibodies. Journal of General Virology *68*, 1983-1991.

Whitley,P., Nilsson,I., vonHeijne,G. (1996). A nascent secretory protein may traverse the ribosome endoplasmic reticulum translocase complex as an extended chain. Journal Of Biological Chemistry *271*, 6241-6244.

Wong,S.H., Hong,W.J. (1993). The SXYQRL sequence in the cytoplasmic domain of TGN38 plays a major role in trans-Golgi Network localisation. Journal Of Biological Chemistry *268*, 22853-22862.

Woodman,P. (1998). Vesicle transport: More work for the Rabs? Current Biology *8*, R199-R201.

Yeung,T., Yoshihisa,T., Schekman,R. (1995). Purification of SEC23P-SEC24P complex. Small Gtpases and Their Regulators, Pt C *257*, 145-151.

Young,E.F., Mckee,M.J., Ferguson,D.G., Kranias,E.G. (1989). Structural characterization of phospholamban in cardiac sarcoplasmic-reticulum membranes by cross-linking. Membrane Biochemistry *8*, 95-106.

Zaitseva,E., Weston,C.A., Prives,J.M., Goldgaber,D.Y. (1999). BiP and calnexin interact with APP in HEK293 cells. Molecular Biology of the Cell *10*, 2391.

Zhang,J.X., Braakman,I., Matlack,K.E.S., Helenius,A. (1997). Quality control in the secretory pathway: The role calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. Molecular Biology of the Cell *8*, 1943-1954.