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Targeting of the Sarco/Endoplasmic Calcium ATPase

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Abstract

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Nucleated eukaryotic cells have two types of calcium pump belonging to the family of P-type ATPases; sarco/endoplasmic calcium ATPases (SERCAs) are located in the endo/sarcoplasmic reticulum (ER/SR) whereas plasma membrane calcium ATPases (PMCAs) are located at the plasma membrane. The mechanism(s) by which SERCAs are maintained in the ER/SR is unknown and in this study I have identified the segment of SERCA1b containing the retention/retrieval signal by producing SERCA/PMCA chimeras linked to enhanced green fluorescent protein (eGFP). Pairs of chimeras have been produced in which the structure of one chimera is the obverse of its partner. For example, one chimera is comprised of the central domain of SERCA linked to the N- and C-termini of PMCA. This contrasts with a construct containing N- and C-termini of SERCA in combination with the central domain of PMCA. When comparing the chimeras one of a pair should lose the retention/retrieval signal, while the other should still contain it. In addition, to determine whether SERCA is truly retained in the ER, or retrieved from a post-ER compartment, co-localisation studies have been performed using antibodies against ERGIC-53, which is a marker for the ER-Golgi intermediate compartment (ERGIC). Laser confocal microscopy clearly shows that SERCA-eGFP has entered the ERGIC, and this is confirmed by cell fractionation experiments. This strongly suggests that retrieval plays a significant role in maintaining SERCA1b in the ER.

Acknowledgements

Well here it is! And this is the bit where I get to thank everyone for being supportive during my PhD.

Thanks to the supervision and support of Dr. Malcolm East I got through it all with my sanity relatively in tact and knowing one or two things about molecular biology etc. Thank you also to all of the people who came and went in the Lee/East group during my time there, you all helped me enjoy my time at Southampton.

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My family has been very supportive for the years I've been doing all this and especially my Dad, who always was able to inject a little bit of enthusiasm by talking about proteins after Sunday lunch. But last and not least, thank you Helen for always being on hand with loving support and/or a firm kick in the rear at just the right time. I love you Hel, you're great!

For Mum

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
Ca^{2+} ATPase	Calcium and Magnesium activated ATPase
DAG	Diacyl glycerol
DTT	Dithiothrietol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
GFP	Green fluorescent protein
eGFP	Enhanced green fluorescent protein
IP_3	Inositol triphosphate
kb	Kilobase
kDa	Kilodalton
MOPS	3-(N-morpholino)propanesulphonic acid
PCR	Polymerase chain reaction
PMCA	Plasma membrane Ca^{2+} ATPase
PLC	Phospholipase C
PKC	Protein kinase C
SDS	Sodium dodecyl sulphate

SR	Sarcoplasmic reticulum
TAE	Tris-acetate EDTA buffer
AMP	Adenosine monophosphate
Tris	Tris(hydroxymethyl)aminomethane
T-tubule	Transverse tubule
GPLR	G-protein linked receptor
RTK	Receptor tyrosine kinase
DHPR	Dihydropyridine receptor
TC	Terminal cisternae
BiP	Binding Protein
PDI	Protein Disulphide Isomerase
SRP	Signal recognition particle
COP I and COP II	Coat proteins I and II
ARF	ADP ribosylation factor
GEF	Guanodine exchange factor
TE	Transitional element
ERGIC	ER/Golgi intermediate compartment
VSV	Vesicular stomatis virus
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
FRET	Fluorescence resonance energy transfer

Contents

Chapter One:

Introduction

1.1 Calcium Signalling

Intracellular calcium plays an important role in many cell signalling pathways. The level of cytosolic calcium ions affects a number of different cellular events in many different cells; from contraction of muscle cells to exocytosis in neurones (Berridge 2000). It has also been found to be important in many bacterial signalling pathways (Norris *et al.* 1996) including chemotaxis and the heat shock response.

Tight control is kept on cytosolic Ca^{2+} levels in eukaryotic cells. A number of intracellular calcium stores exist, and a host of pumps, channels and calcium binding proteins are involved in maintaining them (Pozzan *et al.* 1994).

1.2 Ca^{2+} as a second messenger

Calcium's effectiveness as a second messenger relies on two factors. The first of which is the ability to remove most of the free calcium ions from the cytosol and to keep the resting concentration at a low level. The second factor is that once the appropriate signalling pathway is activated, a rapid release of calcium is triggered. The cytosolic calcium concentration of a typical resting cell is estimated to be aproximately 100nM (Berridge 1997), compared to the 2mM found extra-cellularly (Beridge 2000).

This gradient is maintained by proteins that transport Ca^{2+} from the cytosol into the extracellular space or into intracellular stores such as the Endoplasmic Reticulum, and a variety of calcium permeable channels exist in the cell to facilitate calcium influx. Figure 1.1 summarises the variety of proteins that are involved in calcium signalling.

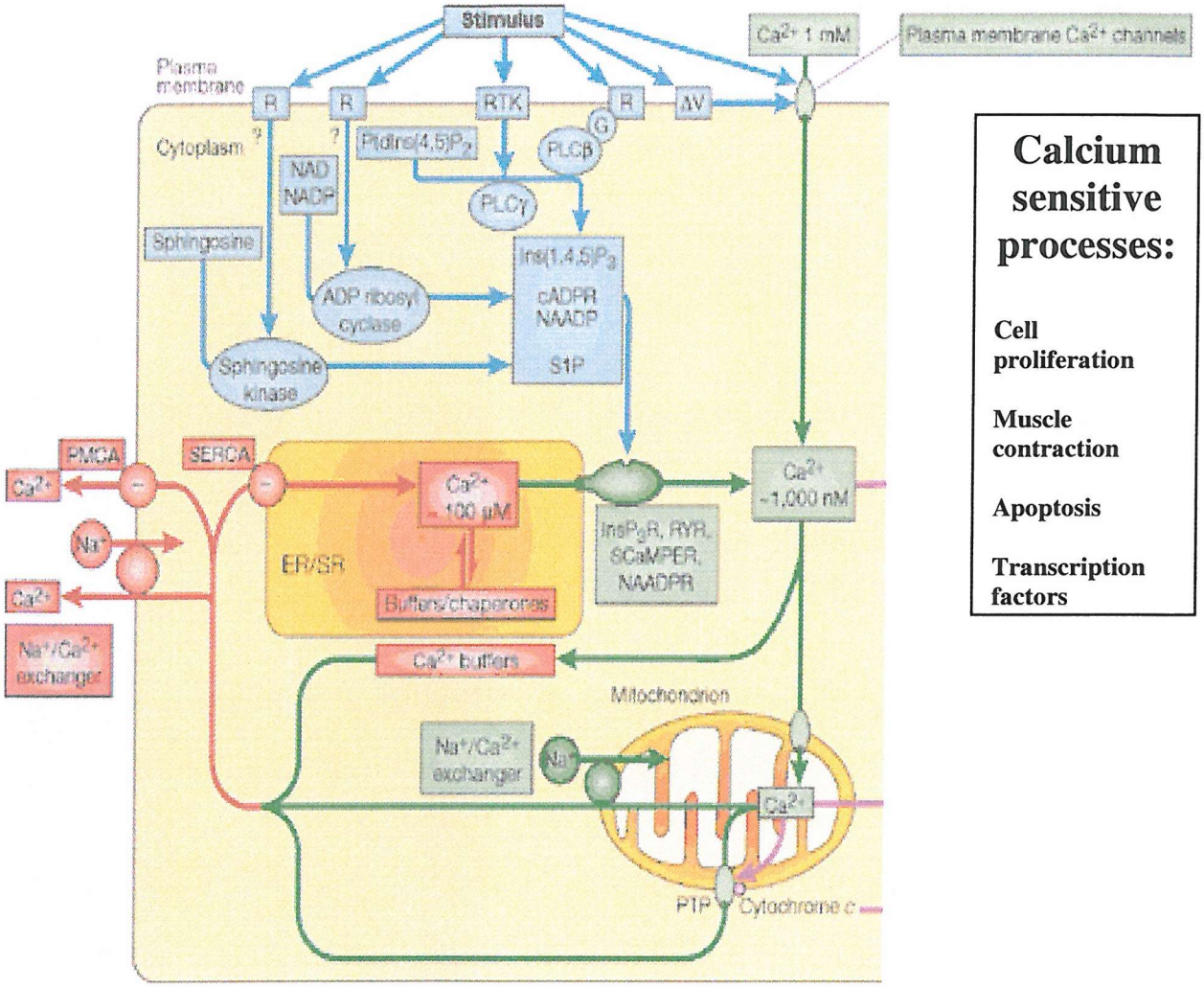

Figure 1.1 A summary of calcium signalling in the eukaryotic cell.

Calcium is released from the extra cellular space or intracellular stores like the ER into the cytosol through calcium channels (shown in green), and is triggered by an external stimulus (shown in blue). Various second messengers are known to activate calcium channels, such as $InsP_3$, and cADPR, as well as changes in the plasma membrane potential (blue). Proteins such as the calcium ATPases SERCA and PMCA, the plasma membrane Na+/Ca^{2+} exchanger and cytosolic buffering proteins help control the calcium signal (red). Once a threshold concentration of free calcium is reached in the cytosol, calcium sensitive processes are activated (pink). Modified from Berridge 2001.

1.2.1 Calcium transporters lower the concentration of cytosolic calcium

Ca^{2+} is transported from the cytosol into the extracellular space by a family of pumps known as Plasma membrane Ca^{2+} ATPases (PMCAs) (Carafoli *et al.* 1996). These pumps use the phosphorylation of ATP to transport calcium ions against the high concentration gradient. Also present in the plasma membrane is a Ca^{2+}/Na^{+} antiport that uses the high concentration of Na^{+} outside the cell to drive the transport of the Ca^{2+} ions out (Moonga *et al.* 2001).

The main store of calcium inside the cell is in the ER lumen, and another family of calcium pumps is present in the ER membrane. Sarco/Endoplasmic Reticulum Calcium ATPases are structurally related to the PMCAs, and have a similar mechanism of calcium transport. To enable even more calcium to be stored in the ER, a number of calcium binding proteins are present in the lumen. Examples of such proteins are calreticulin in the ER and calsequestrin which is found in high concentrations in the Sarcoplasmic Reticulum of muscle cells (Michalak *et al*, 1999 and Fliegel *et al* 1989). These sequestering proteins have a high capacity to bind calcium, allowing them to increase the total amount of calcium in the ER by reducing the free calcium concentration. They also have a low to moderate binding affinity so that when calcium influx into the cytosol is required, it is released quickly.

1.2.2 Calcium channels

A large variety of calcium channels exist, both in the plasma membrane in the ER membrane, and they facilitate the fast influx of calcium ions into the cytosol once signalling event has taken place. These are activated in different ways and most can be broadly classed onto three different types: Second messenger operated calcium channels (SMOCs), Receptor operated calcium channels (ROCCs), and Voltage operated calcium channels (VOCCs). Ryanodine receptors (RyR) are calcium

channels found in the ER/SR (especially in exhitable cells) and are triggered by a different mechanism.

1.2.2.1 Second messenger operated calcium channels (SMOCs)

The most well defined calcium channel that is stimulated to open by the binding of a second messenger is the IP_3 receptor in the ER. The production of inositol (1,4,5) trisphosphate (IP_3) is triggered by two main classes of receptors; G-protein linked receptors (GPLRs) and the receptor tyrosine kinases (RTKs). Both work by activating members of the Phospholipase C family (Berridge 1993).

GPLRs are large multi-spanning membrane proteins that activate small heteromeric GTPases in the cytosol (G-proteins). When the receptor binds a ligand, a conformational change causes GDP bound in the α-subunit of the $\alpha/\beta\gamma$ G-protein complex to be replaced by GTP. The α-subunit of the G-protein then binds to, and activates Phospholipase C_β (Kaziro *et al.* 1991). Examples of GPLRs that work through PLC_β activation are adrenergic and serotonergic receptors. RTKs activate PLC_γ, which is activated by phosphorylation (Fantl *et al.* 1993). Examples of ligands for RTKs include growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF).

Once activated PLC catalyses the production of inositol trisphosphate and diacyl glycerol (DAG) from phosphotidyl inositol bisphosphate (PIP_2). The IP_3 released then binds to the IP_3 receptor in the ER membrane. Binding of IP_3 causes the tetrameric IP_3 receptor to release calcium ions into the cytosol (Mikoshiba 1993).

1.2.2.2 Voltage operated calcium channels (VOCCs)

In excitable cells, a large number of calcium channels that are sensitive to changes in the membrane potential are present in the plasma membrane. Upon depolarisation, these voltage-gated calcium channels (VOCCs) undergo a conformational change, which makes them permeable to calcium ions. This quickly results in a large influx of Ca^{2+} into the cytosol.

There are many different types of VOCCs, which are heteromeric (allowing for even greater diversity in the subunits). These were originally classified by their different pharmacological and physiological properties when expressed in a variety of excitable cell lines (Nowycky *et al* 1985). Some VOCCs for example are sensitive to dihydropyridine and so are sometimes called dihydropyridine receptors (DHPRs)

1.2.2.3 Receptor operated calcium channels (ROCCs)

These have been described as "any plasma membrane calcium channel which is opened as a result of a ligand binding to a receptor" (Barritt 1999). As can be guessed from this rather vague definition, this is the least understood type of calcium channel. Second messengers such as cAMP (Lenz and Kleineke 1997), arachodonic acid (Munaron et al 1997), and even IP_3 (Kiselyov et al 1997) have all been shown to have a stimulatory effect on plasma membrane calcium channels. Heteromeric G-proteins (activated by G-protein linked receptors) are also thought to interact with calcium channels.

A sub-type of ROCCs has been termed store operated calcium channels (SOCCs). These are channels are activated by a depletion of calcium from the internal stores (the ER and SR). Again, the mechanism for this is not well understood and could rely direct interaction between the ER/SR and the channels in the plasma membrane or some kind of chemical signal (Venkatachalam *et al* 2002). A possible candidate for a SOCC is the TRP family of proteins which was first identified in Drosophila eye cells (Leung 2000), and has a number of vertebrate homologues (Clapham et al 2001)

1.2.2.4 Ryanodine receptors and calcium induced calcium release (CICR)

Ryanodine receptors are a calcium channel that is mainly found in excitable cells along with the IP_3 receptor. They are structurally similar to the IP_3 receptor, and are also tetrameric. There are three isoforms of the ryanodine receptor (RyR): RyR1,2 and 3. Isoform 1 is found in skeletal muscle and is triggered in a unique manner (see section 1.2.3.1), whereas RyR2 and RyR3 (predominantly found in cardiac, smooth muscle and neurones) are activated by what is known as calcium induced calcium release (CICR). Various calcium channels in the plasma membrane (see above) open briefly to raise the local $[Ca^{2+}]$ to 10-30μM. Ca^{2+} ions bind to the ryanodine receptor, which rapidly opens to release Ca^{2+} from the stores in the ER. (Zahradnikova *et al.* 1999).

In most cells that contain ryanodine receptors, high numbers of IP_3 receptors also exist (Cancella *et al.* 1999, Berridge 1998b). The ryanodine and IP_3 receptors are separated within the cell, leading to Ca^{2+} stores that are spatially and pharmacologically distinct (Golovina and Blaustein 1997).

1.2.3 Cellular processes that are modulated by calcium

Once calcium ions are released into the cytosol it can bind to a large number of calcium dependent proteins which are involved in a wide variety of cellular processes. Examples of enzymes include kinases like PKC (Wang H *et al* 2003, Kohout *et al* 2003, Zeng *et al* 2002), phospolipases (Lin *et al* 2001), and endonucleases (Urbano *et al* 1998). Transcription factors have also been found to be sensitive to calcium concentration: an increase in cell calcium has been found to be important in the activation of NFAT in a variety of cell types (Tomita *et al* 2002, Neal and Clipstone 2002).

A well-researched example of a calcium senstive protein is Calmodulin (CaM). CaM is a 17kDa protein with a calcium-binding motif known as an E-F hand motif. When calcium binds to CaM it is able to effect the activity of various enzymes. A good example of the wide range of effects CaM can have is in smooth muscle. It is known to activate certain classes of adenylate cyclase in smooth muscle, and can also decrease cAMP production by activating phosphodiesterases (Hinrichsen 1993). Smooth muscle contraction is directly affected by CaM binding to myosin light chain kinase (Gabella 1984) and an increase in CaM-Ca^{2+} in the cytosol has been directly linked with cell proliferation (Rasmussen and Means 1989). Another important example of CaM's action in the cell are the effects it has via a family of calmodulin binding kinases (CaMK's) important in the control of cell cycle (Patel et al 1999).

Such a wide range of effects means that it is vitally important to control calcium signalling tightly, and if that is not possible then the cell needs to be controlled itself Cell death (both necrotic and apoptotic) is triggered by a large and sustained concentration of free calcium in the cytosol (Trump and Berezesky 1995). Apoptosis in particular has been shown to be controlled by calcium dependent proteins, such as the calpain family of proteins and a variety of protein kinases. Calpains for example are known to play an important role in regulating cell survival protein p53 (Atencio *et al* 2000) and the phosphorylation of transcription factors which leads to expression of immediate early genes of apoptosis such as c-myc and c-fos is often calcium dependent (Zeng *et al* 2002).

1.2.3.1 Calcium signalling in skeletal muscle – a special case

Contraction in skeletal muscle fibres is also regulated by calcium release. Depolarisation of the plasmolemma, caused by a nerve signal results in a huge increase in cytosollic Ca^{2+} concentration, calcium ions then bind to calcium sensitive proteins in the cytoskeleton and a contraction of the muscle fibre occurs. This rapid response is known as excitation-contraction coupling. For excitation-contraction coupling to be effective, this process must be very rapid and result in a dramatic

influx of calcium ions; this is achieved due to the structure of the muscle fibre and the unique activation of the RyR1 calcium channel.

In muscle cells, a specialised sub-compartment of the ER exists: the sarcoplasmic reticulum (SR). The SR's function is to mediate the events involved in contraction, and is most pronounced in skeletal muscle myotubes. These polynuclear structures are made up of many microfibriles (see fig 1.2a). The SR forms a network of tubules around each microfibril. At regular intervals invaginations of the plasma membrane occur, called T tubules (DeMeis 1981). The SR tubules merge to form flattened sacs which are called terminal cisternae (TCs). These structures have a very close association with the T tubules and form what is known as a triadic junction (see figure 1.2b).

The close association is important for the method by which the ryanodine receptors expressed in skeletal muscle (RyR1) are activated. Because the time taken between excitation and contraction is too short for normal CICR from the stores, was thought that the DHPR in skeletal muscle actually interacted directly with the RyR1 tetramer. Instead of being a VOCC, the DHPR acts instead like a voltage sensor.

Depolarisation of the plasmolemma causes a comformational change in the DHPR, which would then be able to directly stimulate calcium release from the SR by RyR1 receptors very close by. This interaction is confirmed by electron microscopy showing alignment of DHPRs and RyRs in skeletal muscle (Protasi *et al* 1997) and the ability of short peptide sequences from the cytoplasmic loop of DHPRs to stimulate calcium release from RyR1 channels (O'Reilly and Ranjat, 1999).

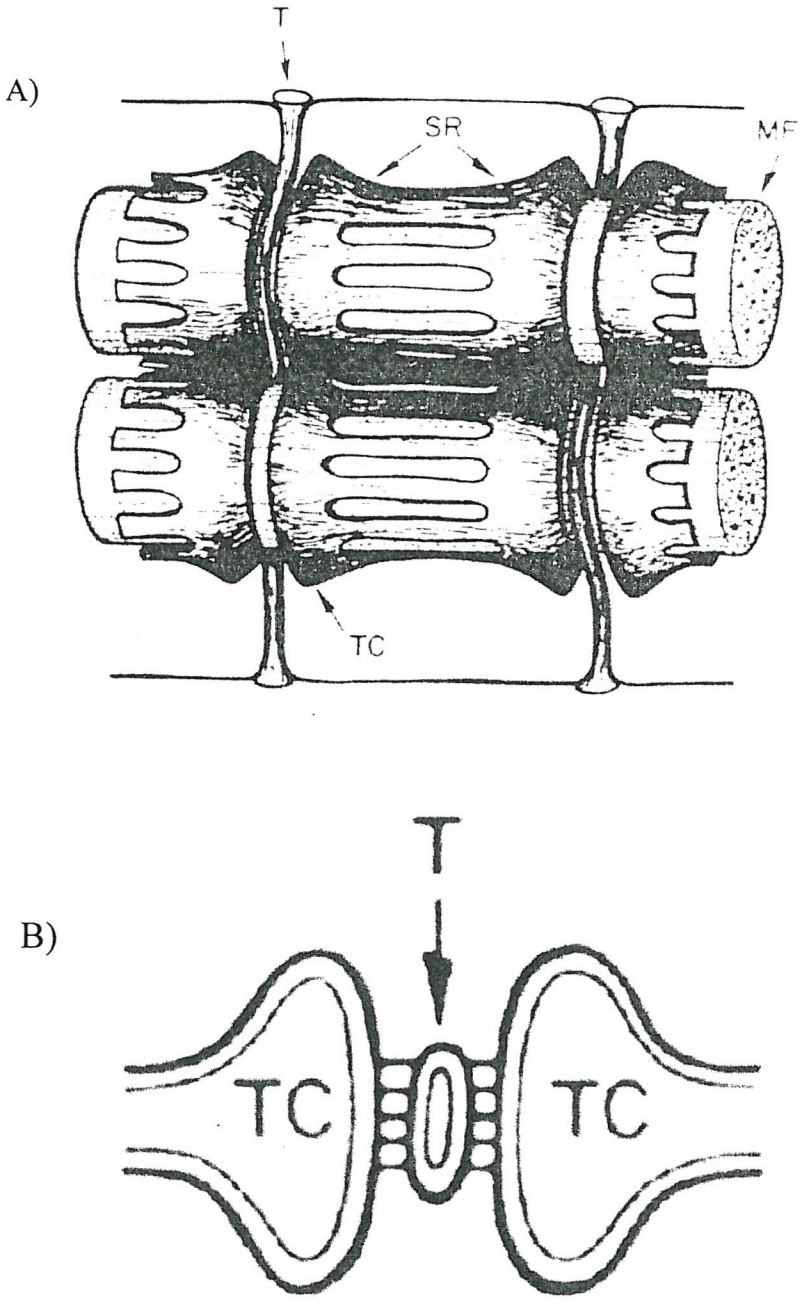

Figure 1.2: The arrangement of the Sarcoplasmic reticulum (SR) and microfibriles in a skeletal muscle fibre

a) The SR is found in a network around the microfibrils and is especially clustered at invaginations of the plasma membrane called T tubules (T). These flattened sections of SR at the T tubules are known as terminal cisternae (TC). These structures are closely associated and form a triadic junction where two sets of terminal cisternae meet one T tubule.

b) Shows a cross-section of a triadic junction.

Taken from DeMeis 1981.

1.3 The Pumps

Two families of related calcium pumps exist in the cell. One type, found in the ER/SR are called the sarco/endoplasmic reticulum calcium ATPases or SERCAs. The second family of calcium ATPases is found in the plasma membrane (plasma membrane calcium ATPases or PMCAs). Both types belong to a larger family of ion pumps called P-type pumps (Møller *et al.* 1996 and figure 1.3), the 'P' refering to the phosphorylated enzyme intermediate formed during catalysis (figure 1.4).

1.3.1 Sarco/endoplasmic reticulum calcium ATPases

Three Sarco/endoplasmic reticulum calcium ATPase (SERCA) genes have been identified, each coding for a multi-spanning membrane protein of approximately 110 kDa in size. The crystal structure of SERCA1a has recently been solved to a resolution of 2.6Å (Toyoshima *et al.* 2000).

Previous studies into the structure have identified important areas of the sequence. Hydropathy plots from the derived amino acid sequence predicted that there were ten transmembrane domains and a large cytoplasmic domain which can be described as a large hydrophobic 'head' and a 'stalk' of alpha helices (Toyishima *et al.* 1993). By comparing SERCA with other P-type pumps and by mutational analysis (Andersson *et al.* 1989), a number of important residues, and regions have been recognised. Ca^{2+} binding for example occurs at a number of negatively charged residues in the transmembrane helices labelled M4, M5, M6 and M8 (Clarke 1989).

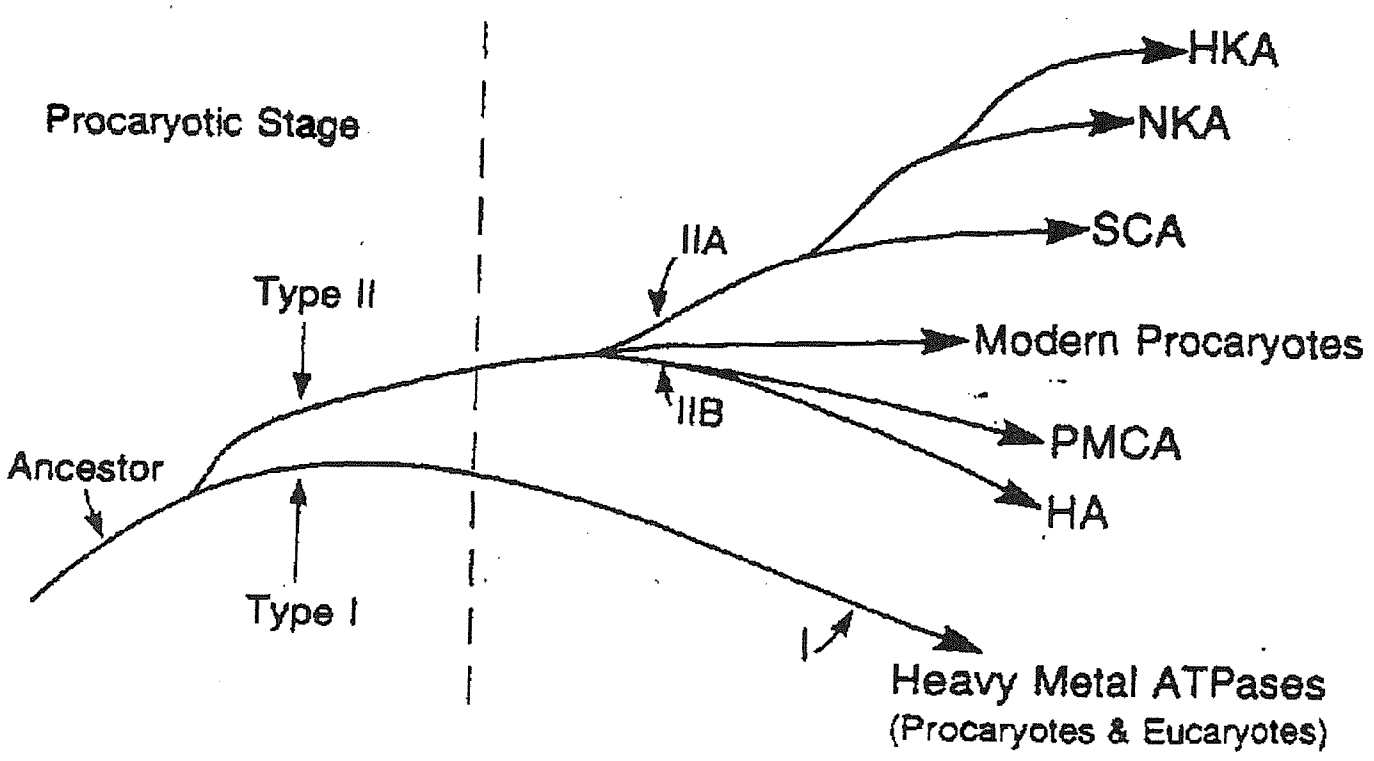

Figure 1.3: The evolution of P-type ATPases.

This figure shows the evolutionary relationship between the different types of P-type ATPases. It shows that the heavy metal pumps (type I) more distantly related to other P-type ion pumps (type II). Comparing the calcium pumps demonstrates that SERCAs (SCA) is more closely related to the H^+/K^+ ATPase (HKA) and the Na^{2+}/K^+ ATPase than to PMCA, which is closer related to the H^+ATPase (HA). Taken from Møller *et al.* 1996.

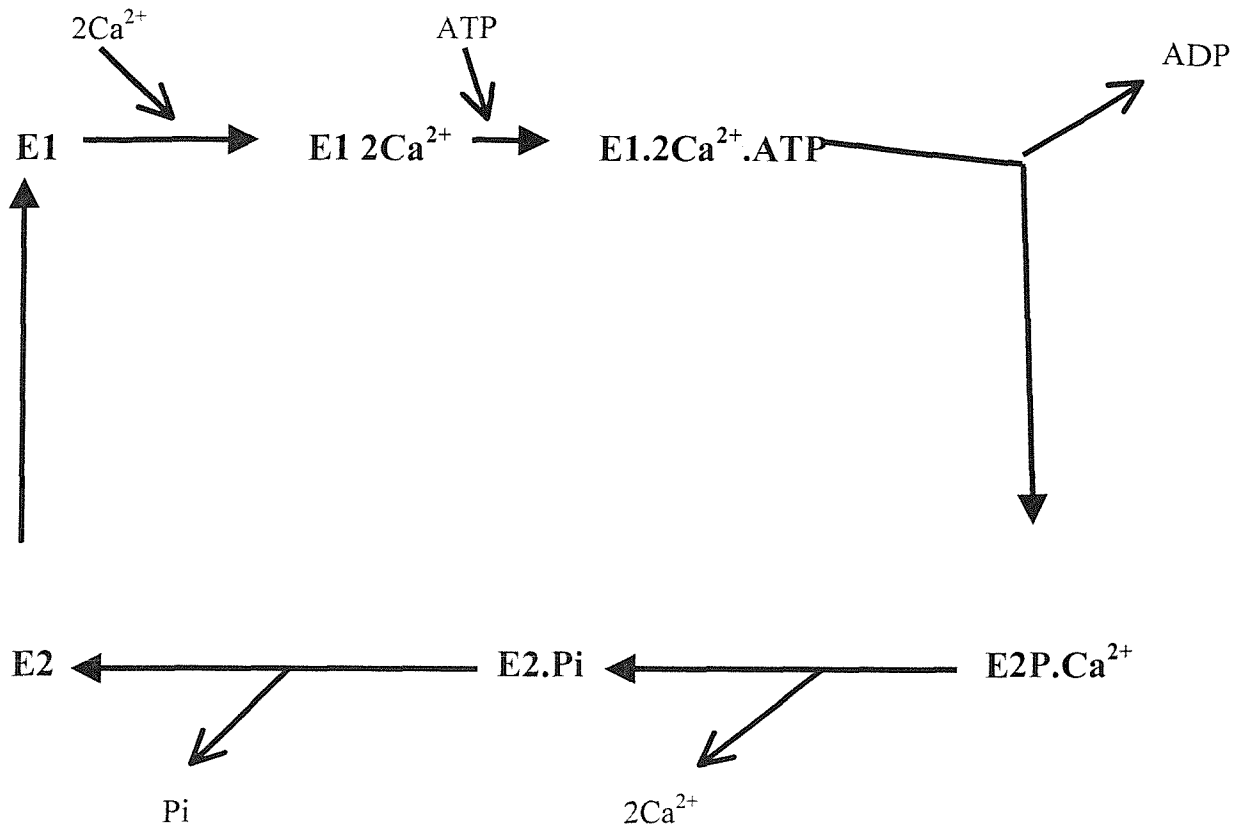

Figure 1.4: The proposed system for Ca2+ transport.

It has been suggested that there are two conformations. In the E1 conformation two Ca^{2+} ions are held in high affinity binding sites near the cytoplasmic side of the membrane. In the E2 conformation the Ca^{2+} ions are exposed to the lumen of the ER (DeMeis and Vianna 1979, modified by kinetic data obtained by Pickart and Jencks, 1982). The formation of the phospho-enzyme intermediate (E1-E2) and the release of inorganic phosphate, Pi (E2-E1), cause the large conformational change. Arrows indicate release or binding of Ca^{2+} ions, ATP and inorganic phosphate (Pi).

The cytoplasmic domain contains three separate regions, designated 'A', 'N' and 'P'. The 'N', or nucleotide-binding domain is the region responsible for binding ATP, and the 'P' or phosphorylation domain contains the residue (asp351) which gets phosphorylated as part of the catalytic mechanism. The 'A' domain, which is also known as the actuator or signal transduction domain is thought to be linked with transmitting the conformational changes required for calcium transport. A trypsin cleavage site (known as T2) residing within the actuator domain has been shown to be sensitive to the conformation of SERCA. Cleavage at the T2 site is enhanced by binding of calcium and inhibited by phosphorylation (Andersen *et al.* 1986), which suggests large conformational changes occur during transport

Figure 1.5 shows the 3-Dimensional structure of SERCA1a solved to 2.6 Å resolution (Toyoshima *et al.* 2000). This structure, to a large degree confirmed a lot of the work done already. Asp351, for example has been identified as the phosphorylated residue by mutagenesic analysis (Maruyama and MacLennon 1988) and is indeed shown to be positioned opposite the nucleotide binding domain, and the predicted Ca^{2+} binding residues in the transmembrane helices were seen to be clearly co-ordinated with the two Ca^{2+} ions.

However, there is much that was not predicted. The fifth transmembrane helix was seen to be a continuous 'mast', which extends a substantial distance into the cytoplasmic domain and M4 and M6 show a degree of unwinding near the centre of the membrane. The first transmembrane helix is particularly interesting as this now includes some negatively charged residues that were thought to form part of the 'stalk' region. It has been proposed that these residues could be part of a calcium entry site (Lee and East, 2001).

Another question that is highlighted by the crystal structure concerns the lack of any obvious Ca^{2+} channel within the transmembrane domain of the pump. This would mean that a conformational change is required to transport calcium ions across the membrane, since the Ca^{2+} ions are bound close to the cytoplasmic side of the membrane. The linking regions between the 'A', 'P' and N domains also indicate a large change in conformation is possible. These stretches display no rigid secondary structure and are thought therefore to be relatively flexible.

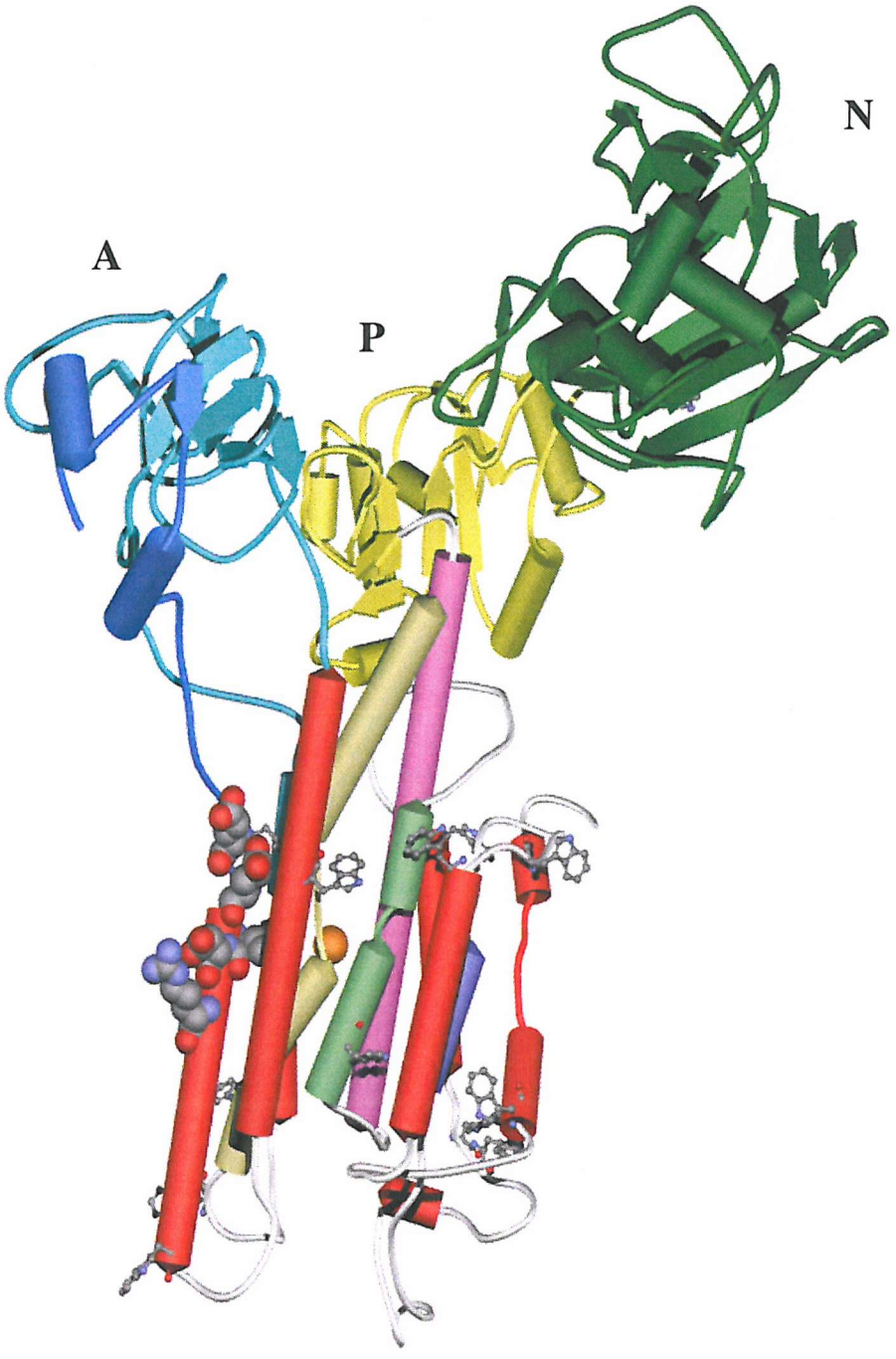

Figure 1.5: The 3-Dimensional structure of SERCA1a

This shows SERCA as a model elucidated from the crystal structure showing α-helices (cylinders) and β-pleats (arrows). It clearly shows the three domains: P – Phosphorylation domain, N – Nucleotide binding domain, and A – Actuator domain and the ten transmembrane α-helices. Taken from Toyoshima *et al.* 2000.

The magnitude of this change can be estimated by superimposing the high-resolution crystal structure (which is in the E1 conformation) over a previous 8 Å model (thought to be in the tighter E2 conformation). This shows that a 90° movement by the 'A' domain, and a 20° change in the position of the N domain are required between the conformations. However, it is possible only to speculate how exactly the structure relates to the transport mechanism until a sufficiently high-resolution E2 structure is acquired.

1.3.2 SERCA isoforms

As mentioned above, there are three SERCA genes and each of these has a number of different splice variants. These vary in expression pattern, both in a tissue specific manner, and according to developmental stage. They are still very homologous, and only vary widely in the C-terminus and N-terminus (see figure 1.6 for an alignment of the protein sequence of SERCA1a and SERCA2a).

The SERCA gene specific to skeletal muscle for example, is present as two splice variants; 1a and 1b. SERCA1a being the adult form, with SERCA1b expressed in neonatal muscle. The only difference is that the neonatal form contains a sequence (DPEDERRK) at its C-terminus instead of a glycine (Brandl 1986). No functional reason has been found for this difference.

SERCA2 can be found as two splice variants, but varies in tissue expression. SERCA2b is found in almost all cell types (Verboomen *et al.* 1995), whereas 2a is found in cardiac muscle, 'slow twitch' skeletal muscle and neurones. Both are regulated by phospholamban, but differ in activity; SERCA2a has a two-fold lower calcium affinity and turnover rate, with ten-fold higher vanadate sensitivity (Verboomen *et al.*1994). Another interesting difference between these two splice variants is that the SERCA2b protein has a putative eleventh C-terminal

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SERCA2a 1   MENAHTKTVEEVLGHFGVNESTGLSLEQVKKLKERWGSNELPAEEGKTLLELVIEQFEDL 60
            ME AH+K+ EE L +FGV+E+TGL+ +QVK+  E++G NELPAEEGK+L ELV+EQFEDL
SERCA1a 1   MEAAHSKSTEECLSYFGVSETTGLTPDQVKRHLEKYGPNELPAEEGKSLWELVVEQFEDL 60

SERCA2a 61  LVRILLLAACISFVLAWFEEGEETITAFVEPFVILLILVANAIVGVWQERNAENAIEALK 120
            LVRILLLAACISFVLAWFEEGEET+TAFVEPFVILLIL+ANAIVGVWQERNAENAIEALK
SERCA1a 61  LVRILLLAACISFVLAWFEEGEETVTAFVEPFVILLILIANAIVGVWQERNAENAIEALK 120

SERCA2a 121 EYEPEMGKVYRQDRKSVQRIKAKDIVPGDIVEIAVGDKVPADIRLTSIKSTTLRVDQSIL 180
            EYEPEMGKVYR DRKSVQRIKA+DIVPGDIVE+AVGDKVPADIR+ SIKSTTLRVDQSIL
SERCA1a 121 EYEPEMGKVYRADRKSVQRIKARDIVPGDIVEVAVGDKVPADIRILSIKSTTLRVDQSIL 180

SERCA2a 181 TGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAMGVVVATGVNTEIGKIRDEMV 240
            TGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKA+G+V  TGV+TEIGKIRD+M
SERCA1a 181 TGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAVGIVATTGVSTEIGKIRDQMA 240

SERCA2a 241 ATEQERTPLQQKLDEFGEQLSKVISLICIAVWIINIGHFNDPVHGGSWIRGAIYYFKIAV 300
            ATEQ++TPLQQKLDEFGEQLSKVISLIC+AVW+INIGHFNDPVHGGSW RGAIYYFKIAV
SERCA1a 241 ATEQDKTPLQQKLDEFGEQLSKVISLICVAVWLINIGHFNDPVHGGSWFRGAIYYFKIAV 300

SERCA2a 301 ALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQ 360
            ALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQ
SERCA1a 301 ALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQ 360

SERCA2a 361 MSVCRMFILDRVEGDSCSLNEFTITGSTYAPIGEVHKDDKPVKCHQYDGLVELATICALC 420
            MSVC+MFI+D+V+GD CSLNEF+ITGSTYAP GEV K+DKPV+  QYDGLVELATICALC
SERCA1a 361 MSVCKMFIIDKVDGDICSLNEFSITGSTYAPEGEVLKNDKPVRAGQYDGLVELATICALC 420

SERCA2a 421 NDSALDYNEAKGVYEKVGEATETALTCLVEKMNVFDTELKGLSKIERANACNSVIKQLMK 480
            NDS+LD+NE KGVYEKVGEATETALT LVEKMNVF+TE++ LSK+ERANACNSVI+QLMK
SERCA1a 421 NDSSLDFNETKGVYEKVGEATETALTTLVEKMNVFNTEVRSLSKVERANACNSVIRQLMK 480

SERCA2a 481 KEFTLEFSRDRKSMSVYCTPNKPSRTSM-SKMFVKGAPEGVIDRCTHIRVGSTKVPMTPG 539
            KEFTLEFSRDRKSMSVYC+P K SR ++ +KMFVKGAPEGVIDRC ++RVG+T+VP+T
SERCA1a 481 KEFTLEFSRDRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGVIDRCNYVRVGTTRVPLTGP 540

SERCA2a 540 VKQKVMSVIREWGSGSDTLRCLALATHDNPLRREEMNLEDSANFIKYETNLTFVGCVGML 599
            VK+K+MSVI+EWG+G DTLRCLALAT D P +REEM L+DSA F++YE +LTFVG VGML
SERCA1a 541 VKEKIMSVIKEWGTGRDTLRCLALATRDTPPKREEMVLDDSAKFMEYEMDLTFVGVVGML 600
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SERCA2a 600 DPPRIEVASSVKLCRQAGIRVIMITGDNKGTAVAICRRIGIFGQDEDVTSKAFTGREFDE 659
            DPPR EV  S++LCR AGIRVIMITGDNKGTA+AICRRIGIF ++E+V  +A+TGREFD+
SERCA1a 601 DPPRKEVTGSIQLCRDAGIRVIMITGDNKGTAIAICRRIGIFSENEEVADRAYTGREFDD 660

SERCA2a 660 LSPSAQRDACLNARCFARVEPSHKSKIVEFLQSFDEITAMTGDGVNDAPALKKSEIGIAM 719
            L  + QR+AC  A CFARVEPSHKSKIVE+LQS+DEITAMTGDGVNDAPALKK+EIGIAM
SERCA1a 661 LPLAEQREACRRACCFARVEPSHKSKIVEYLQSYDEITAMTGDGVNDAPALKKAEIGIAM 720

SERCA2a 720 GSGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAA 779
            GSGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAA
SERCA1a 721 GSGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAA 780

SERCA2a 780 LGFPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMNKPPRNPKEPLISGWLFFRYLAI 839
            LG PEALIPVQLLWVNLVTDGLPATALGFNPPDLDIM++PPR+PKEPLISGWLFFRY+AI
SERCA1a 781 LGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPLISGWLFFRYMAI 840

SERCA2a 840 GCYVXXXXXXXXXXXXXXXXXPRVSFYQLSHFLQCKDDNPDFEGVDCAIFESPYPMTMA 899
            G YV                    P VS++QL+HF+QC + NP+F+G+DC +FE+P PMTMA
SERCA1a 841 GGYVGAATVGAAAWWFLYAEDGPHVSYHQLTHFMQCTEHNPEFDGLDCEVFEAPEPMTMA 900

SERCA2a 900 LSVLVTIEMCNALNSLSENQSLLRMPPWENIWLVGSICLSMSLHFLILYVEPLPLIFQIT 959
            LSVLVTIEMCNALNSLSENQSLLRMPPW NIWL+GSICLSMSLHFLILYV+PLP+IF++
SERCA1a 901 LSVLVTIEMCNALNSLSENQSLLRMPPWVNIWLLGSICLSMSLHFLILYVDPLPMIFKLR 960

SERCA2a 960 PLNLTQWLMVLKISLPVILMDETLKFVARNYLE 992
             L+ TQWLMVLKISLPVI +DE LKF+ARNYLE
SERCA1a 961 ALDFTQWLMVLKISLPVIGLDELLKFIARNYLE 993
```

Figure 1.6 Protein sequence alignment of SERCA1a and SERCA2a

As determined by using Blosum alignment program. Sequences are aligned and middle consensus sequence shown (A letter signifies a direct match and '+' denotes a semi conserved change (i.e. two hydrophobic residues)

ransmembrane helix (Campbell *et al* 1992). This would have important consequences for any possible regulatory proteins.

Splice variants are documented for the SERCA3 gene (a,b,c) but these are found in a restricted number of cell types including platelets and cells of the lymphoid system (Wuytack *et al.* 1994, Møller *et al.* 1996). These isoforms are normally found in cells in combination with SERCA2b, and have a lower affinity for binding calcium although SERCA3a has a similar turnover rate to SERCA2b (Dode *et al.* 1998). Little was known about the function of SERCA3 isoforms until recently, but the wide variety of affinities and turnover rates is now thought to provide the complexity and control required for calcium signalling (Martin *et al* 2002).

1.3.3 Simarities and differences between calcium pumps: PMCAs and the yeast Golgi calcium pump

As mentioned above (section 1.3) there exists another family of calcium ATPAses that are located at the plasma membrane: the Plasma Membrane calcium ATPases (PMCAs). PMCAs are also P-type ion pumps, with a similar amino acid sequence and predicted topology (see figures 1.8 and 1.7). The most obvious structural difference is the large calmodulin-binding domain at the C-terminus present in all PMCAs (Carafoli *et al.* 1996), and the N-terminal cytosolic domain.

The transmembrane helices (especially the negatively charged residues present in the transmembrane domain which bind Ca^{2+} ions) are well conserved, as well as the different catalytic domains of the head region (Guerini *et al.* 1996). Although the catalytic mechanism is thought to be very similar, PMCAs are only able to pump one Ca^{2+} for every ATP hydrolysed. Like the SERCAs, the four PMCA genes produce a number of splice variants, with over 20 recognised forms (Carafoli 1994).

The PMCA isoforms vary in the first 70-80 residues, containing many target sequences for kinases; providing a method of varying the activity of the isoforms. The splice sites provide more variations; one at the calmodulin binding domain and another in an area allowing phospholipids to alter the activity of the pump. An example of the way in which the isoforms display different functional properties is seen with PMCA2, which has a much higher affinity for calmodulin (Hilfiker *et al.* 1994) than the other isoforms.

Another related calcium pump also exists, but is found in yeast, which do not have a native SERCA in their ER. The Pmr1 pump is located in the Golgi, and is known to be important in Calcium homeostasis in a similar way, with null mutants of the Pmr1 pump causing calcium ion build-up in the vacuole and lack of correct Golgi function (Halachmi *et al* 1996)

```
PMCA : 66  SPTEGLADNTNDLEKRRQIYGQNFIPPKQPKTFLQLVWEALQDVTLIILEVAAIVSLGLS 125
           S T GL  +   +++  + YG N +P ++ K+  +LV E  +D+ + IL +AA +S  L+
SERCA: 19  SETTGLTPD--QVKRHLEKYGPNELPAEEGKSLWELVVEQFEDLLVRILLLAACISFVLA 76

PMCA : 126 FYAPPGEESEACGNVSXXXXXXXXXXXXXXXXXXILLSVICVVLVTAFNDWSKEKQFRGL 185
           ++   GEE+                          ILL +I   +V  + + + E     L
SERCA: 77  WFEE-GEET---------------VTAFVEPFVILLILIANAIVGVWQERNAENAIEAL 119

PMCA : 186 QSRIEQEQKFTVIRNGXXXXXXXXXXXXGDIAQVKYGDLLPADGVL--IQGNDLKIDESS 243
           +    +  K                   GDI +V  GD +PAD  +  I+   L++D+S
SERCA: 120 KEYEPEMGKVYRADRKSVQRIKARDIVPGDIVEVAVGDKVPADIRILSIKSTTLRVDQSI 179

PMCA : 244 LTGESDHVRKSADKDP----------MLLSGTHVMEGSGRMVVTAVGVNSQTGIIFTLL 292
           LTGES  V K  D  P          ML SGT++  G    +V   GV+++ G I   +
SERCA: 180 LTGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAVGIVATTGVSTEIGKIRDQM 239

PMCA : 293 XXXXXXXXXXXXXXXXXXXXXMEMQPLKSAXXXXXXXXXXXXXXXXXXXXSVLQGKLTK 352
                                                              + LQ KL +
SERCA: 240 AATEQDK-------------------------------------------TPLQQKLDE 255

PMCA : 353 LAVQIGKAGLVMSAITVIILVLYFVIETFVVDGRVWLAECTPVYVQYFVKFFIIGVTVLV 412
              Q+ K   V+S I V + ++        V G  W            + +F I V + V
SERCA: 256 FGEQLSK---VISLICVAVWLINIGHFNDPVHGGSWFRGA--------IYYFKIAVALAV 304

PMCA : 413 VAVPEGLPLAVTISLAYSVKKMMKDNNLVRHLDACETMGNATAICSDKTGTLTTNRMTVV 472
            A+PEGLP  +T  LA   ++M K N +VR L + ET+G  + ICSDKTGTLTTN+M+V
SERCA: 305 AAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQMSVC 364

PMCA : 473 QSYL-----------------GDTHYKE---IPAPSALTPKILDLLVHAISINSAYTTKI 512
           + ++                  G T+  E   +     +     D LV   +I +
SERCA: 365 KMFIIDKVDGDICSLNEFSITGSTYAPEGEVLKNDKPVRAGQYDGLVELATICALCNDSS 424

PMCA : 513 LPPEKEGALPRQVGNKTECALLGFILDLKRDFQPVRE----------QIPEDQLYKVYT 561
           L   +   +  +VG  TE AL   +  +      VR             +    + K +T
SERCA: 425 LDFNETKGVYEKVGEATETALTTLVEKMNVFNTEVRSLSKVERANACNSVIRQLMKKEFT 484

PMCA : 562 --FNSVRKSMSTVIRMPDG-----GFRLFSKGASEILLKKCTNILNSNGELRGFRPRDRD 614
             F+  RKSMS             G ++F KGA E ++ +C  +    G  R
SERCA: 485 LEFSRDRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGVIDRCNYV--RVGTTRVPLTGPVK 542

PMCA : 615 DMVKKIIEPMAC--DGLRTICIAYRDFSAIQEP----DWDNENEVVGDLTCIAVVGIEDP 668
           + +  +I+      D LR + +A RD    +E     D     E   DLT + VVG+ DP
SERCA: 543 EKIMSVIKEWGTGRDTLRCLALATRDTPPKREEMVLDDSAKFMEYEMDLTFVGVVGMLDP 602
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PMCA : 669 VRPEVPEAIRKCQRAGITVRMVTGDNINTARAIAAKCGIIQPGEDFL--CLEGKEFNRRI 726
            R EV  +I+ C+ AGI V M+TGDN  TA AI  + GI    E+       G+EF+
SERCA: 603 PRKEVTGSIQLCRDAGIRVIMITGDNKGTAIAICRRIGIFSENEEVADRAYTGREFDDLP 662

PMCA : 727 RNEKGEIEQERLDKVWPKLRVLARSSPTDKHTLVKGIIDSTTGEQRQVVAVTGDGTNDGP 786
              E+ E  +             AR  P+ K  +V+ +         ++ A+TGDG ND P
SERCA: 663 LAEQREACRRAC--------CFARVEPSHKSKIVEYL-----QSYDEITAMTGDGVNDAP 709

PMCA : 787 ALKKADVGFAMGIAGTDVAKEASDIILTDDNFTSIVKAVMWGRNVYDSISKFLQFQLTVN 846
           ALKKA++G AMG +GT VAK AS+++L DDNF++IV AV  GR +Y+++ +F+++ ++ N
SERCA: 710 ALKKAEIGIAMG-SGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSN 768

PMCA : 847 VVAVIVAFTGACITQDSPLKAVQMLWVNLIMDTFASLALATEPPTESLLLRKPYGRDKPL 906
           V  V+  F  A +     L  VQ+LWVNL+ D   + AL   PP   ++ R P    +PL
SERCA: 769 VGEVVCIFLTAALGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPL 828

PMCA : 907 IS 908
           IS
SERCA: 829 IS 830
```

Figure 1.7 Protein sequence alignment of SERCA1 and PMCA3

As determined by using Blosum alignment program. Sequences are aligned and middle consensus sequence shown (A letter signifies a direct match and '+' denotes a conserved change (i.e. two hydrophobic residues)

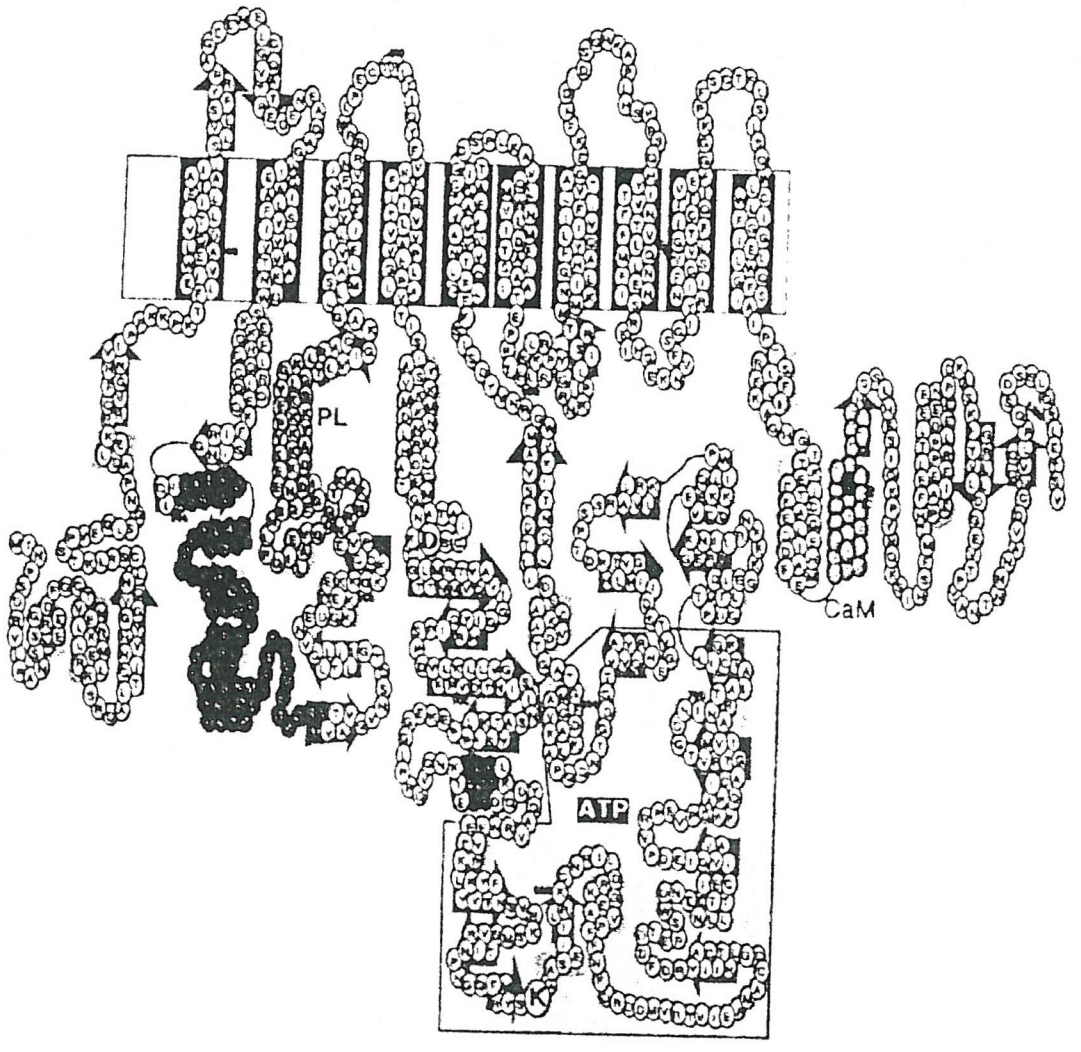


Figure 1.8: The secondary structure of PMCA as predicted from hydropathy plots of the amino acid sequence

The structure is very similar to that predicted for SERCA before any 3-D structure was obtained. ATP labels the part of the sequence that binds the nucleotide (corresponding to the N-domain in SERCA). The main difference in structure is the large calmodulin binding domain (CaM) at the C-terminus. PL shows a sequence known to interact with phospholipids to alter the activity of the pump. Taken from Carafoli *et al.* 1996.

1.3.4 Modulation of SERCA activity

To achieve the complexity of calcium signalling observed in mammalian cells, careful modulation of calcium pumps is maintained. PMCAs for example are controlled by a number of different protein kinases and are activated by calmodulin binding to a self-inhibitory sequence in their C-terminal cytoplasmic tail (Carafoli et al 1996). SERCAs are also controlled by a variety of proteins. For example, insulin response factor one (IRS-1) has been found to bind to SERCA3b, also regulating its' expression (Borge and Wolf 2003).

Two structurally similar proteins have been found to play an important role in SERCA regulation. Phospholamban and sarcolipin are small single spanning transmembrane proteins that decrease SERCAs calcium transporting activity when expressed in cells. Phospholamban is expressed at high levels in cardiac muscle and both are expressed in smooth muscle (Ferrington *et al* 2002). Interestingly, when sarcolipin is co-expressed with phospholamban, inhibition seems to increase (Asahi *et al* 2002), suggesting that sarcolipin has a role in breaking up the inactive phosholamban pentamers and creating 'super inhibition' of the SERCA pump.

Another two proteins that have found to regulate SERCA activity are the calcium binding chaperone proteins found in the ER, calreticulin and calnexin. The transmembrane protein calnexin, when co-expressed in oocytes with SERCA2b inhibits the calcium pump (Roderick *et al* 2000), with phosphorylation of the cytoplasmic domain being required for inhibition to occur. Direct binding does not seem to be involved in this regulation and is possibly controlled by subtle changes in the ER calcium concentration.

Calreticulin on the other hand has been demonstrated to bind directly with SERCA2a, and a specific peptide sequence has been identified by mutational analysis as important for this interaction. This is particularly interesting because this binding motif is not found in SERCA2a, and calcium uptake by SERCA2a is not inhibited by calreticulin (John *et al* 1998)

1.4 Protein Targeting

The eukaryotic cell consists of many different organelles, each with its own complement of resident proteins. One of the major questions addressed in cell biology is how these proteins are targeted to and then maintained in the appropriate organelle

Some proteins, like those destined for the mitochondria, peroxisomes and plastids are translated free in the cytosol, where they are recognised by selective transport machinery associated with the outer membrane of the appropriate organelle and then translocated through it. Other organelles, such as endosomes, lysosomes and secretary vesicles are supplied by what is known as the protein export pathway (figure 1.9). Proteins are passed through the ER membrane, in a co-translational manner and from the ER, delivered to organelles via the Golgi apparatus (Alberts *et al.* 1994).

1.4.1 The Translocon and SRP

Proteins that follow the export pathway are characterised by a short peptide sequence (16-30 residues in length) called a signal sequence. These sequences have one or more positively charged residues followed by 6-12 hydrophobic residues, but do not share any other sequence homology (Lodish *et al.* 2000). This signal is recognised by a complex of RNA and protein (comprised of 6 polypeptides and 1 strand of RNA) called the signal recognition particle (SRP). The SRP serves two functions; first it arrests elongation of the nascent peptide and second it targets the ribosome and protein to the ER (Walter and Johnson 1994)

The ribosome is targeted to a complex in the ER membrane called the translocon (see figure1.10). This consists of the two subunits of SRP receptor, and three other proteins

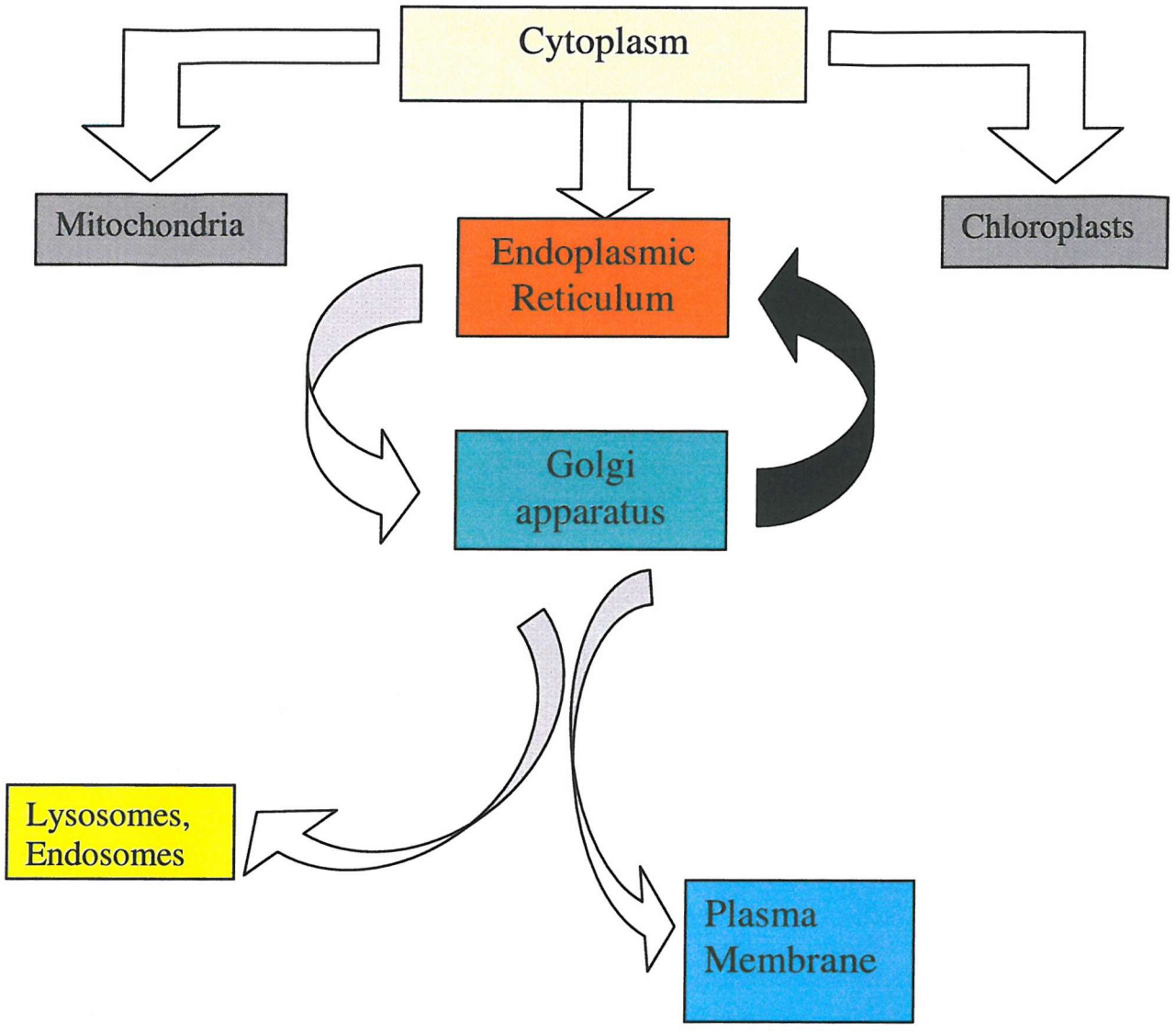


Figure 1.9: Schematic diagram of protein targeting and the protein export pathway

Arrows indicate transport from one location to another. Protein synthesis is initiated in the cytosol and proteins are then transported through the membranes of organelles (straight arrows). Once in the ER, the protein export pathway is mediated by a system of vesicles (curved arrows).

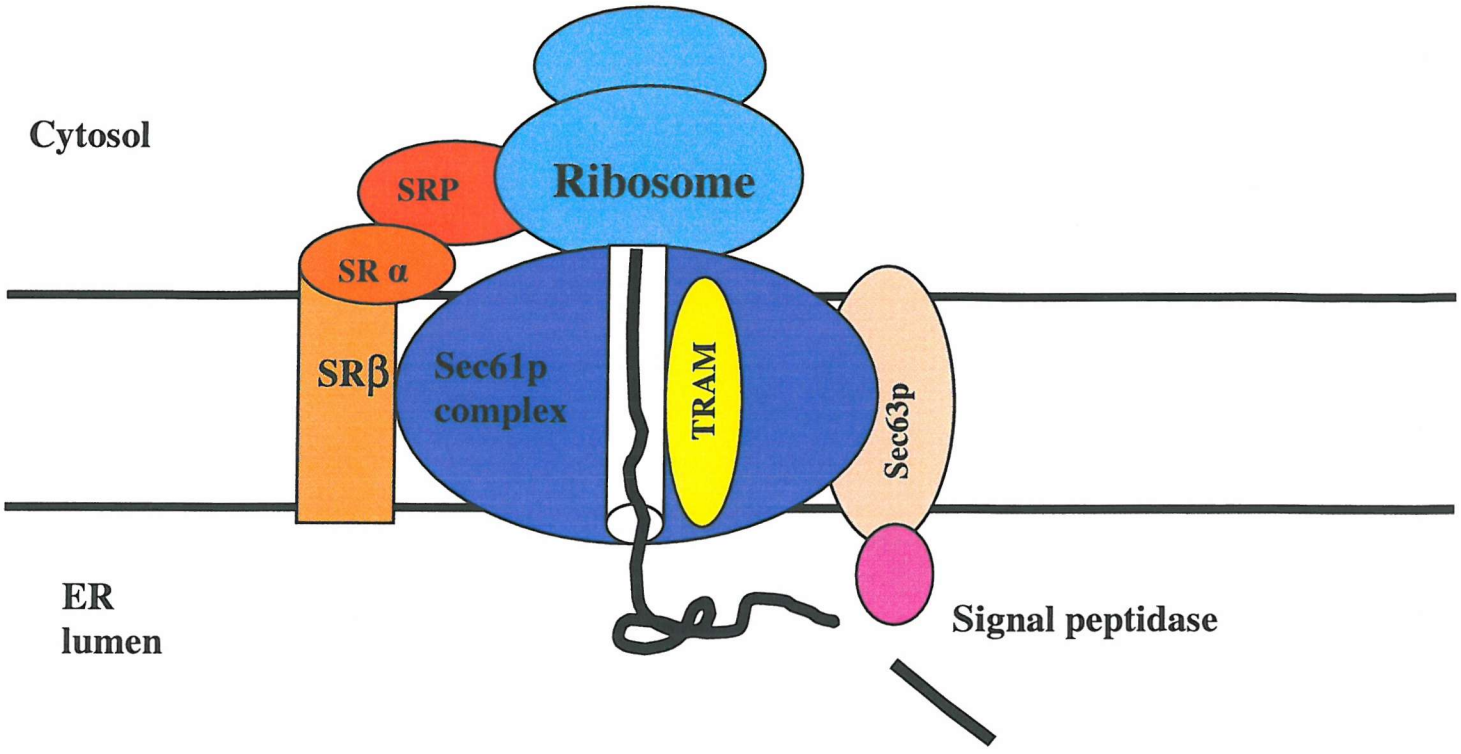


Figure 1.10: Synthesis of proteins destined for the Endoplasmic Reticulum and the export pathway

Signal sequences are recognised by the signal recognition particle (SRP), which halts elongation. SRP also targets the ribosome to the ER membrane via the SRP receptor (SRαp and SRβp). Once the ribosome 'docks' with the translocon (consisting of Sec61α, Sec61β, Sec61γ, TRAM and Sec63p) SRP is released and elongation continues. Signal peptides are cleaved in the ER lumen by signal peptidase (SPase). Proteins are then modified and chaperoned until a fully folded protein is formed. The ribosome is released into the cytosol once translation is complete. Taken from Agarraberes and Dice 2001

(Sec61α, β, and γ) (Gorlich and Rapoport 1993). Another protein sometimes required for membrane proteins is TRAM. The translocon forms a large gated pore, which is estimated to be 40-60 Å in diameter (Hamman *et al.* 1997). Once the SRP is bound with its receptor, the ribosome 'docks' with the translocon and forms a tight impermeable seal (Rapoport *et al.* 1996). SRP then hydrolyses GTP to GDP and is released into the cytosol again (Powers and Walter 1996); translation then continues.

1.4.2 Synthesis of membrane proteins

Integral membrane proteins are also targeted to the ER and translocated via the Sec61 complex, but are prevented from passing completely through to the lumen. The simplest form of membrane protein has a single membrane spanning α-helix, which can be described as either a type I membrane protein (this has a cytosolic C-terminus) or a type II membrane protein (with a C-terminus located in the lumen). Two types of signal are responsible for the correct insertion of membrane segments; these are called stop-transfer, and signal-anchor sequences.

Stop-transfer signals are sequences recognised by the translocation machinery in such a way that the transmembrane domain is stopped from progressing through to the ER lumen, effectively getting 'stuck' in the membrane. Signal-anchor signals are stop-transfer sequences, which also include an uncleaved SRP-binding signal. How the orientation of these transmembrane sequences is determined is not entirely understood, but the charge on the flanking residues is thought to play a role in some cases (Dalbey *et al.* 2000). (Figure 1.11)

The situation is slightly more complicated for membrane proteins like SERCA, which span the membrane many times. How such proteins are integrated into the membrane is still unclear, whether their topology is arranged once fully translated or whether

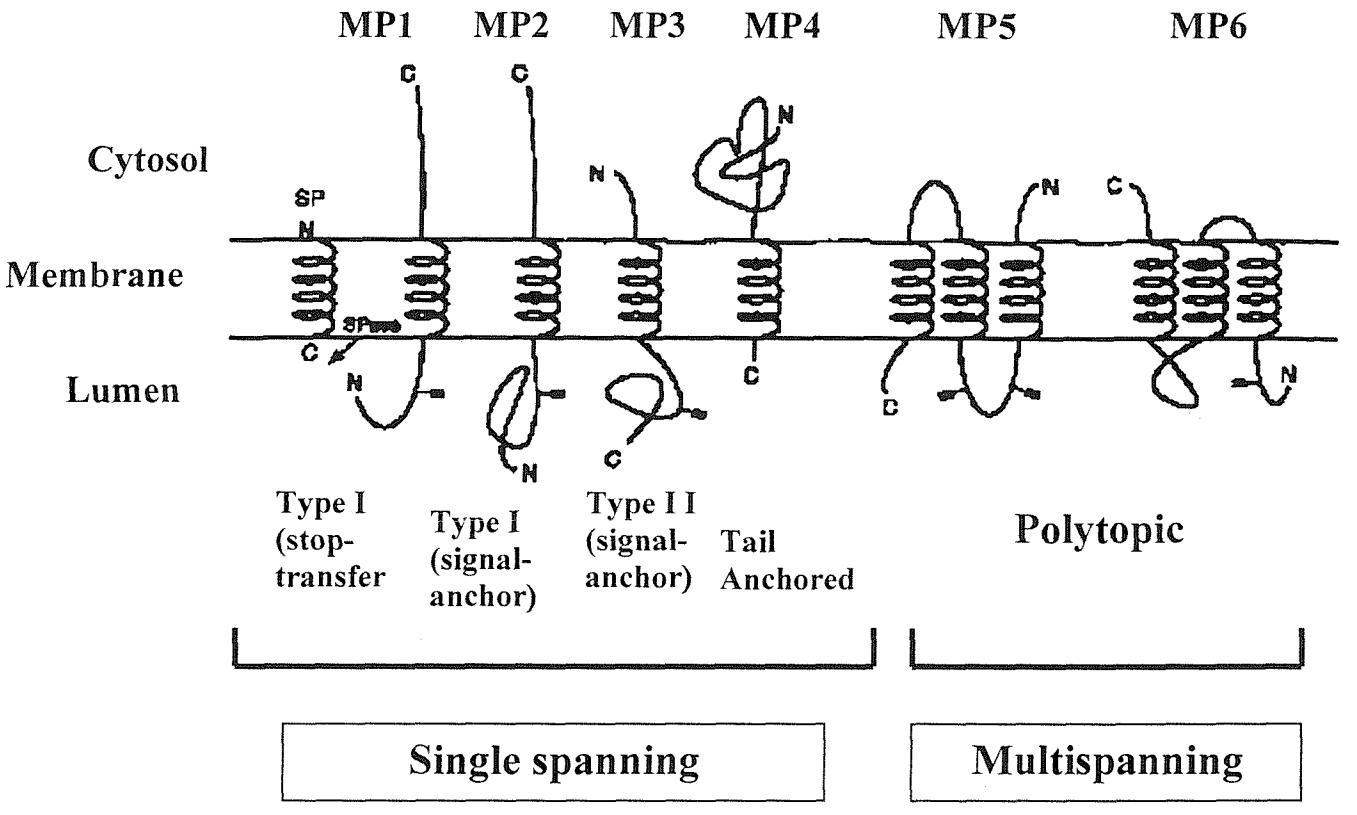


Figure 1.11: Membrane proteins

Showing different classes of membrane proteins. These are classed according to the positions of the N- and C- termini, whether the signal peptide is cleaved and how many times they span the membrane. Taken from High and Laird (1997).

they are inserted sequentially somehow. Recent opinion is inclined to think of the membrane helices being inserted one after the other (High and Laird 1997), almost literally being sewn into the membrane (see figure 1.12). It is not known either whether multispanning membrane are proteins gradually released into the membrane during translation or if they are held in the translocon complex until they are fully formed. Oligomers of Sec61 complexes are known to exist however, which could provide the large integration site for the latter scenario (Hanein *et al.* 1996).

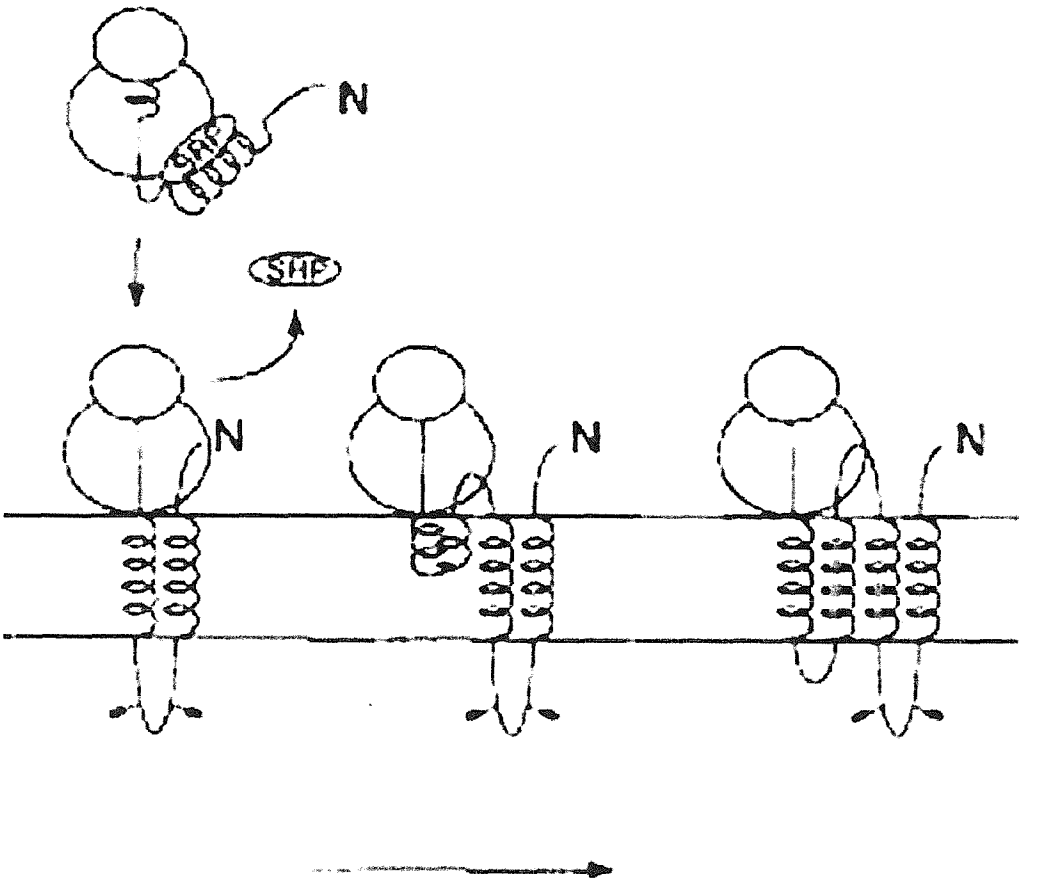


Figure 1.12: Insertion of Membrane Proteins

How multispanning membrane proteins are 'sewn' into the membrane. This shows a membrane protein that has a cytosolically exposed N-terminus. By binding to a signal which also is held in the membrane (signal-anchor), SRP positions the first two transmembrane helices in the membrane. Other transmembrane helices are organised as the protein elongates. Taken from High and Laird (1997).

1.5 Vesicles in targeting

Once in the export pathway, proteins are transported between the ER to the Golgi. They are then moved within the Golgi stack and from there to the plasma membrane or other internal organelles like endosomes. Transport in the Golgi occurs both in a forward manner, i.e. towards the plasma membrane (anterograde transport) and back towards the ER (retrograde transport). These events are mediated by a system of protein coated vesicles (Rothman and Wieland 1996). Each transport step is carried out by a different class of vesicle, distinguished by the protein content of the coat.

Although clathrin coated vesicles were the first to be discovered and can be clearly seen by electron microscopy (Kanseki and Kadota 1969), they cannot be reconstituted in vitro. As a result much of the mechanism of transport is not known.

Coat protein I (COP I) and Coat protein II (COP II) coated vesicles on the other hand have been reconstituted and the steps involved in budding, docking and fusion with target membranes are well understood. As both mechanisms are very similar the formation of COP I coated vesicles will be described in detail followed by a description of how COP II coated vesicles differ.

1.5.1 Budding

Budding of vesicles from their donor membranes is achieved by a stepwise addition of the protein coat, until a spherical shell is formed (Rothman and Wieland 1996). It was found that by isolating cis-Golgi stacks and adding GTP, a small cytosollic GTPase called ADP ribosylation factor (ARF), and a multimeric coat complex, termed coatomer this budding event could be triggered (Ostermann *et al.* 1993). Recently, a

family of membrane proteins has also been found to have a role in budding: the p24 family.

ARF has two conformational states. In its GDP bound form it is soluble and cytosollic. Upon encountering a guanine exchange factor (GEF), the GTP form is then able to partially insert into the membrane (Helms et *al.* 1993). The coatomer complex is then recruited by binding to ARFs cytosolically exposed domain.

The role of the p24 family in budding is less clear. Two mammalian genes; p23 and p24 have been found to be required for efficient Golgi to ER transport. They are present in COP I vesicles and their cytoplasmic domains can bind coatomer (Sohn *et al.* 1996). However, GTP is required for coatomer to bind to membranes, despite the presence of p24 family members. Wieland and Harter (1999) have suggested a mechanism that involves p24's ability to form hetero-oligomers with related proteins. They suggest that these oligomers are unable to bind coatomer, and the recruitment of ARF to the membrane stimulates homo-oligomers, which do then bind coatomer (see figure 1.13)

1.5.2 Docking and Fusion

Before being able to dock with their target membrane, the vesicles have to decoat. A slowly hydrolysed analogue of GTP called GTPγ-S blocks vesicle transport at this step, leaving the vesicles coated (Melancon *et al.* 1987). Therefore, de-coating is triggered by ARF hydrolysing GTP to GDP and withdrawing from the membrane. The coat, which is not fixed strongly to the membrane, then becomes unstable and disassembles. How these uncoated vesicles then dock with their target membrane is answered in part by the SNARE hypothesis.

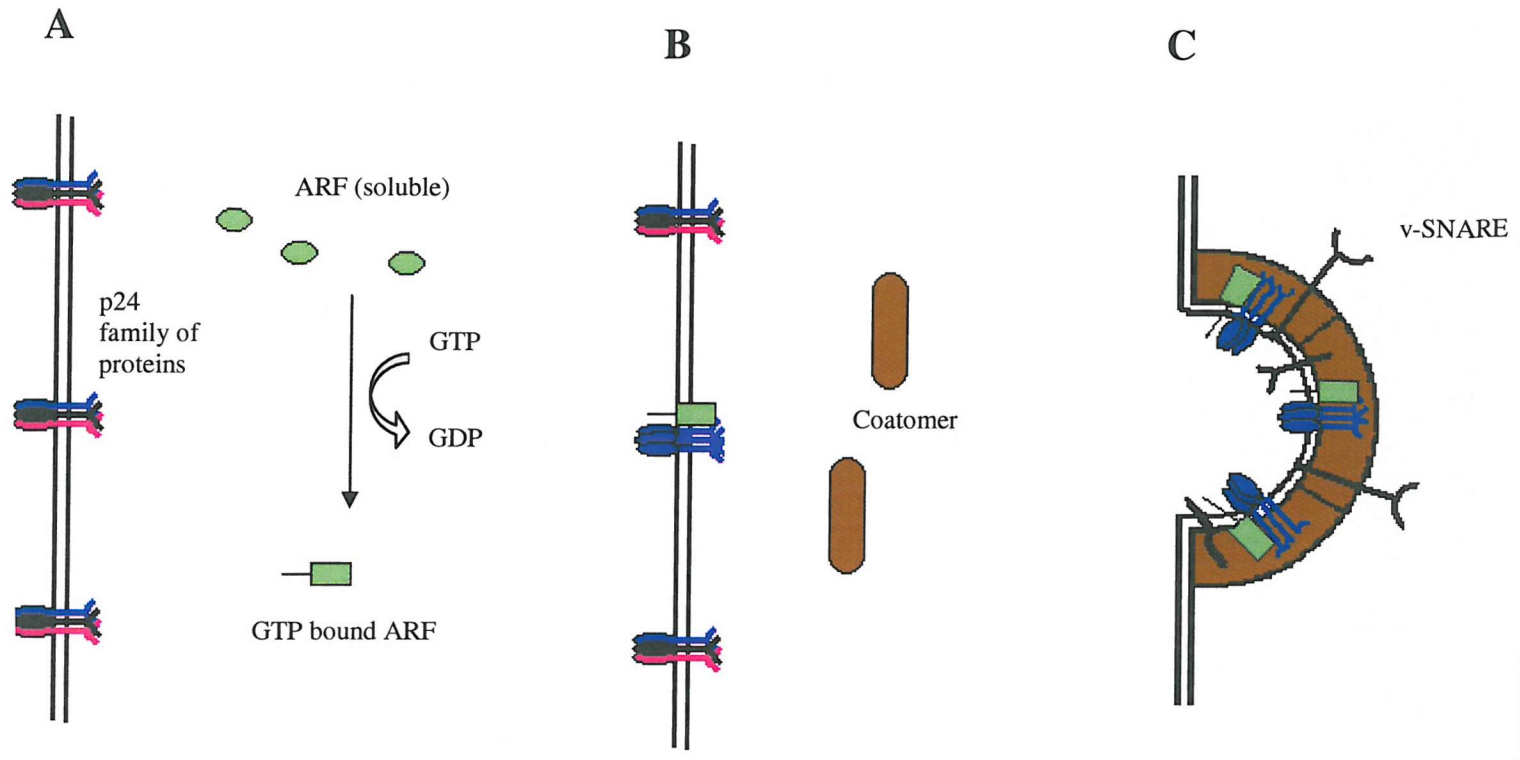


Figure 1.13: Proposed mechanism for recruitment of coatomer to the Golgi membrane.

a) P24 family members exist in the Golgi membrane as hetero-oligomeric complexes. ARF binds to the membrane by replacing bound GDP with GTP upon encountering a nucleotide exchange factor (NEF).
b) ARF.GTP interacts with p24 to form homo-oligomers.
c) Coatomer binds to the exposed regions of p24/ARF complexes to form a budding vesicle. Important proteins like SNARES and other transmembrane proteins are included in the coat complex

Modified from Wieland and Harter (1999).

SNAREs are a family of membrane proteins found in both the target membrane and in vesicles. The SNARE hypothesis states that for each fusion event i.e. ER to Golgi, or Golgi to Endosome, there is a specific pair of SNAREs. The SNAREs on the target membrane are known as t-SNAREs and those on the vesicles are called v-SNAREs (Jahn and Sudhod 1999).

The v-SNAREs 'dock' with the t-SNAREs in a specific manner, for example a Golgi to Endosome v-SNARE will not bind to an ER t-SNARE. Only paired SNAREs are meant to achieve the correct interaction and lead to membrane fusion (see figure 1.14) For a full docking event in biological membranes a number of other factors are also required for specificity and stability. One important family of proteins is the Rab family.

These are small GTPases, which are found in association with membranes and vesicles (Novick and Zerial 1997). They are thought to be involved in orchestrating events prior to a stable v-SNARE / t-SNARE complex being formed (Chavier *et al.* 1999). Other roles thought to involve Rabs include recruiting other proteins to form the docking complex (Guo *et al.* 1998) and maintaining interactions between vesicles and microtubules (Echard *et al.* 1998) Other factors involved in docking and fusion include an ATPase called NSF and an associated factor known as α-SNAP. The role of these accompanying factors is not clear; it is not known for example at what stage ATPase activity is used in the docking process, and what role it has (reviewed in Hay and Scheller 1997). Another area requiring further clarification is that of the fusion event itself. It is not clear what drives this process, but Ca^{2+} ions and the Ca^{2+} binding protein calmodulin have been implicated (Barlowe *et al.* 1993).

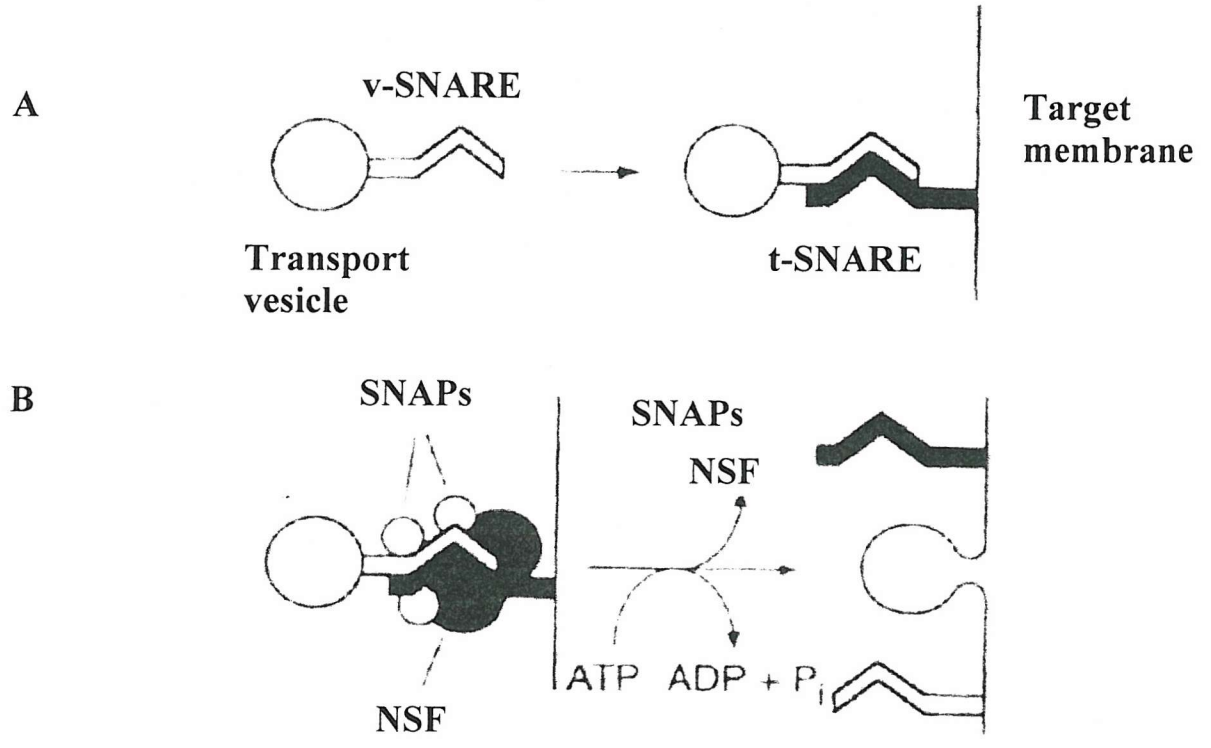


Figure 1.14: Simplified diagram showing docking and fusion of transport vesicles and illustrating the SNARE hypothesis

a) v-SNAREs on transport vesicles interact with corresponding t-SNAREs.

b) Associated factors like SNAPs and NSF interact with the docking complex and fusion with the membrane is initiated.

Taken from Rothman and Wieland (1996).

1.5.3 The transport system in COP II vesicles

The mechanisms of budding, docking and fusion in COP II coated vesicles are essentially the same as those of COP I coated vesicles. Sar1p, a small GTPase associated with COP II vesicles is related to ARF, and works in much the same way (Mayer 1999). It is activated by a GEF located in the ER membrane called Sec12p (Barlowe and Schekman 1993).

Instead of the coat proteins being already assembled in a single complex, like coatomer, there are two COP II coat complexes. The 700 kDa Sec31p/13p complex is recruited to the membrane first, followed by the Sec23p/Sec24p complex (~ 400 kDa) and this then forms a bud in the same way as coatomer.

1.5.4 Transport between the ER and the Golgi

At regions of the ER close to the Golgi apparatus there are stretches of membrane, about 350 μm in length. These ribosome free areas of the ER are known as transitional elements or TEs. TEs are coated with COP II coat proteins and COP II coated vesicles bud off from them and travel towards the cis-Golgi (Klumperman 2000).

At the very cis-side of the Golgi apparatus is a structure of tubules known as the Vesicular Tubular Clusters (VTCs) or the ER-Golgi intermediate compartment (ERGIC). The ERGIC is an important structure, which is recognised as the major recycling compartment in the export pathway (Griffiths *et al.* 1994). Any ER residents that have been packaged into COP II vesicles are sorted into COP I coated vesicles, which form at the rim of the ERGIC (Martinez-Menarguez 1999). The COP-I coated vesicles then travel back to the ER to recycle ER resident proteins.

1.5.5 Transport within the Golgi apparatus

Protein transport in the Golgi is a topic of much discussion (reviewed in Glick 2000). Two models by which proteins travel through the Golgi have been proposed: vesicular transport (or steady state model) and cisternal maturation.

Vesicular transport suggests that the Golgi stack is a fixed structure and proteins move between the cisternae by a system of vesicles (both anterograde and retrograde). In support of this, VSV-G protein has been found in COP-I (anterograde) transport vesicles (Ostermann *et al.* 1993) and proinsulin has also been localised to vesicles within the Golgi apparatus (Orci *et al.* 1997). Cisternal maturation is based on a more fluid model with proteins being packed into tubular vesicular structures, which fuse to form the cis-Golgi. The newly formed cisternae then moves towards the trans side of the Golgi i.e. maturing. The only vesicles that exist in this model are retrograde ones that maintain the Golgi resident proteins in their relative positions within the stack.

The idea of cisternal maturation comes from early observations that large structures such as algal scales and collagen are transported whole and are too big to enter transport vesicles. The question is whether this is the exception to the norm or the way all proteins are transported. Recent evidence indicates that the latter may be true.

VVSV-G protein has been shown to travel through the Golgi at the same rate as collagen aggregates and VSV-G tagged with GFP can be followed through the Golgi apparatus without any noticeable spreading to vesicles (Mironov *et al.* 2001). In addition to this Vesicular Stomatis Virus (VSV) G-protein is not as readily packaged into COP I vesicles as resident Golgi enzymes (Martinez-Menarguez *et al.* 2001

1.6 Methods of protein targeting

But how do resident proteins stay at their designated place in the protein export pathway? This is partly answered by the bulk flow hypothesis:

The idea of bulk flow is that proteins are carried along the pathway until their destination is reached. An internal signal sequence would then halt its further progress. Proteins without a signal sequence are therefore secreted, or expressed at the plasma membrane by default (Wieland *et al.* 1987).

Using the knowledge about the structure of the ER and the nature of vesicles, there are three main ways which vesicles can mediate targeting: Retention, Retrieval (recycling), and Active cargo selection (see figure 1.15).

1.6.1 Retention

Probably the easiest method to understand; the protein contains a retention signal which prevents them being packaged into anterograde transport vesicles. If a protein is not included in COP II coated vesicles for example, then it cannot travel to the Golgi.

One possible method of retention is by oligomerisation, also known as kin recognition (Fullerkrug and Nilsson 1998). The idea is that large oligomers of the proteins would form and somehow affect their ability to be included in transport vesicle, for example by being too large and bulky to enter outgoing transport vesicles (COP II). Kin recognition is thought to be common in Golgi enzymes which all have transmembrane anchors. Models using chimeric proteins suggest that transmembrane domains are

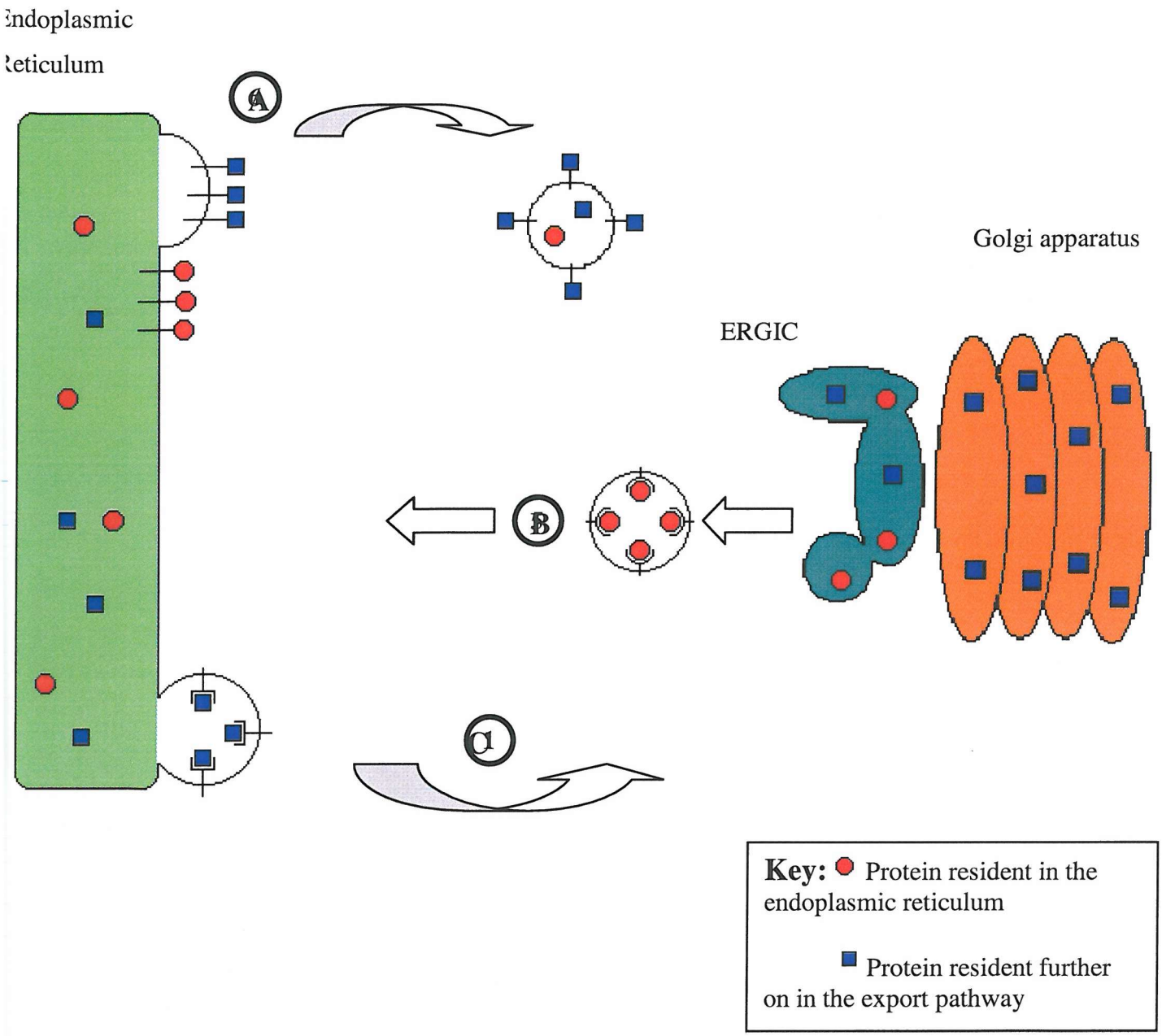


Figure 1.15: Simplified diagram showing the three types of targeting between the ER and the Golgi apparatus.

a) Retention: resident proteins are prevented from entering anterograde vesicles but non-ER residents are not.

b) Retrieval: ER residents that are present in post-ER compartments are recognised by proteins in retrograde vesicles and recycled back the ER.

c) Active transport selection: non-ER residents are recognised by proteins in **anterograde**

required for the formation of such oligomers, whilst the cytoplasmic domains are responsible for stabilising them (Sevier and Machamer 1998).

Oligomerisation is also present in ER resident proteins. Ribophorins are thought to be able to form large supramolecular complexes that are unable to enter COP II vesicles due to their size (Ivessa *et al.* 1992). Cytochrome p450 contains two distinct retention signals, one in the cytoplasmic domain and the other in a transmembrane segment (Szcezesnaskorupa *et al.* 1998).

The size of transmembrane domains in proteins also has some bearing on retention of membrane proteins at different parts of the export pathway. The width of the bilayer increases from ER to the plasma membrane due to the increasing amount of cholesterol and sphingolipids present. The longer the transmembrane domain is, the further it will be allowed to progress. A synthetic protein with a transmembrane domain of 22 residues is expressed at the plasma membrane, whereas one with a length of only 18 residues is retained in the ER (Honsho *et al.* 1998).

1.6.2 Retrieval

An early-recognised ER targeting signal was a tetrameric peptide: lys-asp-glu-leu (KDEL). This was found at the c-terminal end of various ER lumenal proteins, such as the chaperone BiP (Munro and Pelham 1987). If KDEL was tagged onto a protein which is normally secreted like Hen Egg Lysosyme (HEL), it results in it being re-localised to the ER. Modifications were present on such proteins however, suggesting that they had 'seen' enzymes located in the Golgi apparatus and had been retrieved back to the ER. The KDEL motif (also RDEL and HDEL in yeast) was therefore called a retrieval signal.

A component of retrograde COP I vesicles was found to be a KDEL receptor. This was known as erd2 in mammals and was located in the ER and the cis-Golgi (Lewis and Pelham 1990). Erd2 is especially concentrated in the ERGIC, the major recycling compartment of the export pathway (Tang *et al.* 1993).

Membrane proteins have also been found to contain retrieval signals. A C-terminal double lysine motif (KKXX) in type I membrane proteins (Schutze *et al.* 1994), and a RR- at the N-terminus of type II membrane proteins (Jackson *et al.* 1993) have been shown to work as retrieval signals for ER residents.

1.6.3 Selective secretion signals

Recently, the theory of bulk flow has been questioned. VSVG-protein is exported at five times its bulk concentration in the ER (Hammond and Helenus 1994), suggesting some kind of active selection or concentration step. It has been long known that clathrin coated vesicles have cargo receptors which recruit the coat via adaptor proteins (LeBorgne and Hoflack 1998). The search is now on for proteins of similar function in the COP II system.

Membrane proteins have been shown to exhibit selective transport by interacting with COP II coat proteins. Sed5p, a t-SNARE of the cis-Golgi is recruited into COP II coated vesicles by directly binding p24 of the p23/p24 complex. VSV-G has been shown to bind p24 (Peng *et al.* 1999) and even restricts COP II budding if held in the ER. Sec61 on the other hand is not seen to interact with coat proteins (Aridor *et al.* 1999).

A possible candidate for a soluble cargo receptor exists as well: the p24 family of transmembrane proteins. These are already known to interact with the coat proteins (Kuehn *et al.* 1998), and are also thought to recognise multiple internal signals in

transported proteins. There are no recognised transport motifs at the moment and the role of these multiple signals may be to function as a concentration or quality control step for properly folded proteins.

Targeting is clearly a complex process; with a number of types of signals involved, and more than one mechanism is involved in ensuring that proteins remain resident in their appropriate locations. Some proteins for example contain both retention and retrieval sequences, like calreticulin. A calreticulin mutant, which lacked its Ca^{2+} binding domain (containing the KDEL sequence) still did not fully escape the ER (Sonnichsen *et al.* 1994). Sec61, although not actively recruited into COP II coated vesicles is found in the ERGIC and therefore contains a retrieval signal (Greenfield and High 1999).

Even the double lysine motif has been found to contain some retention information as well as its recognised retrieval characteristics (Andersson *et al.* 1999). A dilysine signal on an ERGIC resident (ERGIC-53) was mutated to change the two residues immediately next to the KK signal. This mutant was observed to maintain its endo-A resistance – indicating that it never left the ER. In addition to this, the construct is not observed in the intermediate compartment using confocal microscopy.

Another area of interest in protein targeting is the targeting of large multi-spanning membrane proteins like SERCA and Sec61. This is not as well understood as targeting for soluble and simple membrane proteins and requires further analysis.

1.7 Aims

The aims of this project are to identify the region of SERCA responsible for ER localisation, and to identify the method(s) by which it is targeted to the ER/SR.

The question of where the targeting signal is will be addressed by constructing a set of chimeric proteins made up of SERCA1b and PMCA3, and tagged with enhanced Green Fluorescent Protein (eGFP). These chimeras will be expressed in COS-7 cells and observed using laser confocal microscopy. Studies into the activity of these constructs will also be carried out.

An investigation into whether SERCA is maintained in the ER by a process of retrieval or by retention will also be undertaken. This will by done by performing co-localisation studies of SERCA/GFP and ERGIC-53 (an ERGIC marker), to ascertain whether SERCA is found in a post-ER compartment. Cell fractionation studies; in conjunction with western blot analysis will be utilised to confirm the location of SERCA-eGFP.

Chapter two:

General Methods

2.1 Materials and Reagents

2.1.1 Reagents

Agarose NA (Amersham Pharmacia).

Antibodies (Serotech, Amershem Pharmacia.

BigDye™ terminator mix (Perkin Elmer).

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

Dried skimmed milk (Marvel).

Dubelcos Modified Eagles Medium – DMEM (GibcoBRL).

Easy gel Acrylamide/bisacrylamide mix (Scotlab).

ECL western blotting detection kit (Amersham Pharmacia).

Foetal bovine serum – FBS (GibcoBRL).

Fugene 6 transfection reagent (Roche).

Fungizone (GibcoBRL).

Gentamycin (GibcoBRL).

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

Nycodenz gradient medium (Calbiochem).

Protease Inhibitor Cocktail (Sigma).

Restriction enzymes (Promega, New England Bioscience).

Trypsin EDTA (GibcoBRL).

Wizard™ DNA clean-up kit (Promega).

Wizard™ Miniprep kit (Promega).

Wizard™ PCR clean-up kit (Promega).

2.1.2 Materials

0.2 µm disposable filters (Scheicher and Schuell).

13 mm diameter coverlips (Biorad).

14 mm diameter sterile tissue culture plates (Greiner).

Electroporation cuvettes (Biorad).

Hybond-N™ Nitrocellose paper (Amersham Life Sciences).

Kilobase DNA ladder (GibcoBRL).

Prestained protein molecular weight markers (Invitrogen).

Sterile 20 ml syringes (Becton Dickinson).

Sterile 50 ml centrifuge tubes (Falcon).

Sterile Disposable 3 ml pipettes (Alpha laboratories).

Tissue culture flasks (Falcon).

2.2 Bacterial growth media and vectors

2.2.1 Glycerol stocks

Clones were stored as glycerol stocks. These were made by adding 1 ml of culture to 500mls sterile glycerol, and stored at -80°C.

2.2.2 Growth media:

Growth media was sterilised by autoclaving at 121°C.

Lauri Broth (LB) Media:	10 g tryptone 5 g yeast extract 10 g NaCl made to 1L with distilled water, pH 7
LB agar:	4.5g Bacto Agar added to 300ml LB media
Antibiotic stocks:	ampicillin: 60 mg in 1ml sterile distilled water
SOB media:	20 g Tryptone 5 g Yeast extract 0.5 g NaCl made to 1L with distilled water
SOC media:	SOB media +20 mM glucose

2.3 DNA techniques

2.3.1 Vector:

Constructs were cloned into PcDNA3.1 vector (see figure 2.1). This vector contains, a multiple cloning site, a bacterial origin of replication for growth of the plasmid in bacterial cell, and three promoters (T7, CMV, and SV40) for expression of the cloned protein in mammalian cells. For selection in bacterial cells it contains an ampicillin resistance gene, and for selection in eukaryotes a neomycin resistance gene is also included.

2.3.2 Small scale DNA preparation

10 ml of LB media containing 60µl/ml ampiccillin was inoculated with single colonies of the DH5-α strain of *E.coli*. These cultures were then grown in a shaking incubator at 37°C for 12-16 hours at 200 rpm. Plasmid DNA was then extracted by using the Promega Wizard™ Miniprep Kit (a modified lysis by alkali procedure), in accordance with the manufacturers guidelines.

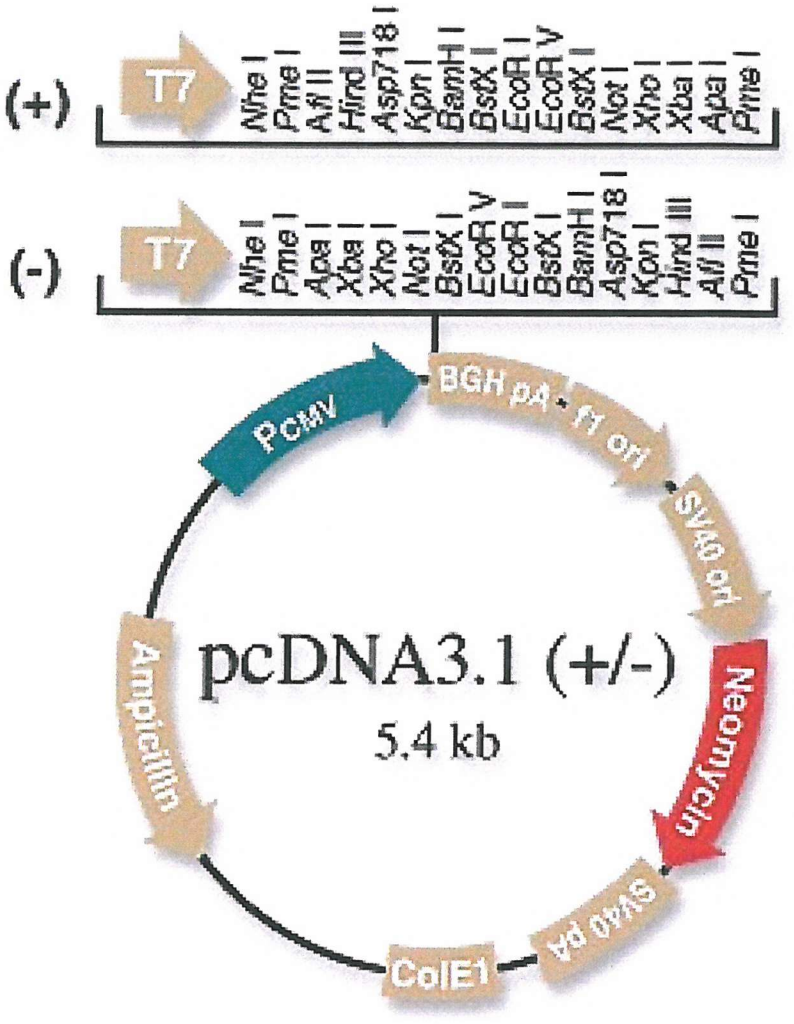


Figure 2.1: The vector used in the molecular biology work: pcDNA3.1+

This is a widely used vector which contains a CMV promoter for high levels of expression in mammalian cells, ampicillin resistance gene for selectability in E. coli, and a neomycin resistance gene for selection in mammalian cells. It also contains a SV40 pA, which triggers overexpression of constructs expressed in SV40 transformed cell-lines (eg COS-7).

2.3.3 Agarose Gels

1 litre TAE (50× solution):

242 g	Tris
57.1 ml	Glacial Acetic acid
1.9 g	NaEDTA
5 µl	Stock 10mg/ml Ethidium Bromide solution.

Gel loading buffer:

3 ml	glycerol
30 mg	bromophenol blue
1 ml	10×TE buffer
6 ml	sterile distilled water

For a 1% gel: 1 g of Agarose NA was added to 100 ml TAE (1×) and dissolved by heating. 5 µl stock ethidium bromide was then added to give a final concentration of 0.5 µg/ml ethidium bromide. After running, gels were observed using a UVP GDS 5000 illuminator and camera.

Different percentage gels were made according to the size of the DNA fragments to be observed.

2.3.4 Extraction of DNA from agarose gels

Restriction fragments were removed from agarose gels, by the 'squeeze-freeze' method as follows: Gels were viewed under UV illumination and the required restriction fragments were excised using sterile scalpels. The agarose gel pieces were

then placed in 0.2 ml tubes with siliconised glass wool plugs and snap frozen in liquid nitrogen. Holes were then made in the tubes with a 25-gauge needle, and they were then placed in 1.9 ml tubes and spun in a bench top centrifuge at 6000 rpm. The liquid was collected and treated by the Promega Wizard™ clean-up kit to remove unwanted aspects of the agarose.

2.3.5 Transformation of *E.Coli* DH5-α by electroporation

Clones were transformed into *E.Coli* by electroporation. Ligation reactions were dialysed on nitrocellulose discs placed on sterile 10% glycerol for 15 minutes. DNA from the ligation reactions (2-5 µl) was pipetted into 60 µl ice-cold electro-competent cells and left to equilibrate for 1 minute. The DNA/ *E.Coli* mixture was then pipetted into a 2mm electroporation cuvette and a Biorad state model electroporator was used to pass an electric current through the cuvette (state conditions). The cells were then immediately suspended in 1 ml SOC media and shaken for 30 minutes at 37°C. LB agar plates were spread with 10 µl, 100 µl and 950 µl of the culture and left in at 37°C for 12-16 hours. Making DNA preparations from resulting colonies and testing with diagnostic restriction digests identified the required clones.

2.3.6 PCR amplification

PCR reactions were set up in 0.2 ml tubes, containing 100 µl reaction mix.

PCR reaction mix:

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

The tubes were then placed in a thermal cycler and the amplification carried out:

Typical PCR cycle:

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

2.3.7 Sequencing

Automatic sequencing was carried out using the BigDye system (Perkin Elmer). DNA (100-250 ng) was placed in a reaction tube containing 4 µl BigDye terminator reaction mix, 1.6 pmol oligonucleotide primer, and made up to 10 µl with sterile distilled water. The reaction tubes were then placed in a state model thermal cycler and the following program carried out:

25 cycles of:	96°C for 10 seconds
	50°C for 5 seconds
	60°C for 4 minutes

The resulting DNA was dried by ethanol precipitation. The reaction mixes were made up to 20 µl by adding sterile distilled water and then added to a 1.5 µl tube containing 2 µl 3M Sodium Acetate (pH 4.6) and 200 µl 95% ethanol. The DNA was allowed to precipitate for 15 minutes then centrifuged at 14,000 rpm on a Eppendorf 5415C centrifuge for 20 minutes. The pellet was then washed with 70% ethanol and centrifuged at 14,000 rpm for 5 minutes and dried by placing in a heating block at 90°C for 1 minute. Samples were then sequenced using a ABI Prism DNA sequencer.

2.3.8 Preparation of electrocompetant DH5-α cells

A single colony of DH5-α *E. Coli* was used to inoculate 50 ml LB media and left in a shaking incubator for 12-16 hours at 37°C and 200 rpm. The resulting culture was then split between two 1L flasks each containing 500ml LB media, and the flasks were placed in a shaking incubator at 37°C and 200 rpm until a A600 of 0.5 was reached. The flasks were 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°C, and

the pellets were resuspended in 1 L ice-cold sterile distilled water. After a second centrifugation step at 4000 g the pellets were resuspended in 500 ml ice-cold sterile distilled water, span again at 4000 g and resuspended in 20 ml sterile 10% glycerol. A final centrifugation at 4000g was carried out and the resultant pellet was resuspended in 3 ml sterile 10% glycerol. Aliquots of 60 µl were snap frozen in liquid nitrogen and stored at -80°C.

2.3.9 Polyacrylamide gels for SDS-PAGE

A discontinuous buffer system was used for SDS-PAGE, with a stacking gel set onto a separating (or resolving) gel. For a 10% polyacrylamide gel, two gel mixtures were made up as follows:

Stacking Gel:

650 µl	Easygel acrylamide/ bisacrylamide mixture
1.25 ml	0.5M Tris (pH6.8)
150 µl	10% Ammonium Persulphate
5 µl	TEMED
2.85 ml	Distilled water

Resolving Gel:

3.3 ml	Easygel acrylamide/ bisacrylamide mixture
1.7 ml	1.5M Tris (pH8.8)
300 µl	10% Ammonium Persulphate
5 µl	TEMED
4.6 ml	Distilled water

Samples were dissolved in sample buffer (list buffer). Up to 40 µl of each sample was then loaded onto the top of the gels and electrophoresed for between 1 and 1.5 hours at 60 mA.

Chapter Three:

Identifying the ER targeting signal within SERCA

3.1 Targeting of SERCA

SERCA1 contains an uncleaved signal anchor sequence in its first transmembrane helix and is cytosolically exposed at both its N- and C- termini (Mata *et al.* 1992). The SERCA sequence does not contain any recognised retrieval signal like LL or RR at either end (Brandl *et al.* 1986), so the question arises: how is SERCA targeted to the endoplasmic reticulum?

One method of elucidating targeting signals within a protein is by creating chimeric proteins. Chimeras of SERCA and PMCA4 have been made, to isolate the sequence responsible for ER targeting (Foletti *et al.* 1995, Guerini *et al.* 1998). These seem to indicate that the first 28 residues of SERCA are important for ER localisation. None of these chimeras however have been shown to actively pump calcium ions and only one (containing the first 85 residues of SERCA, with the rest of PMCA4) was found to form the phosphoenzyme intermediate.

Active chimeras are important, because activity gives a good indication that the chimera is folded properly. Improperly folded proteins have been shown to be held in the ER as part of a quality control mechanism (Hammond and Helenus 1994). Such active chimeras of P-type pumps have been constructed, by using the highly conserved sequences of the two proteins as the linking regions between the two protein parts of the chimera. Luckie *et al.* (1991) created chimeras of Na^+/K^+ ATPase and SERCA were used to identify the area important for oubain resistance, which was located in the last 2/3rds of the Na^+/K^+ ATPase.

The starting point for this study is the six chimeras generated by Black (1998), comprised of PMCA3 and SERCA1b. A complete set of chimeras was constructed by dividing the SERCA1b and PMCA3 sequence into three sections. The first section includes the cytoplasmic N-terminus and the first two transmembrane α-helices. The second section codes for the large cytoplasmic 'head' region and transmembrane

helices 3 and 4. The final section includes the remaining six transmembrane α-helices and the C-terminus (see figure 3.1).

Chimeric proteins were then created by replacing a section of SERCA with the equivalent section of PMCA sequence, joining the sections at conserved regions (see figure 3.2). Replacing the same section of PMCA3 with SERCA1b sequence would then make a 'mirror image' chimera. This created the chimeras as a set of pairs. A chimera which consisted of the first two sections of SERCA sequence and the last section of PMCA sequence, for example would be paired with one consisting of the first two sections of PMCA sequence linked to the last section of SERCA sequence.

Unfortunately, these chimeras did not provide a satisfactory set of localisation pictures. This was attributed to the unpredictability of the antibodies used and the destructive nature of the fixation required. A possible solution would be to make a set of chimeras 'tagged' with Green Fluorescent Protein – which could be viewed in living cells.

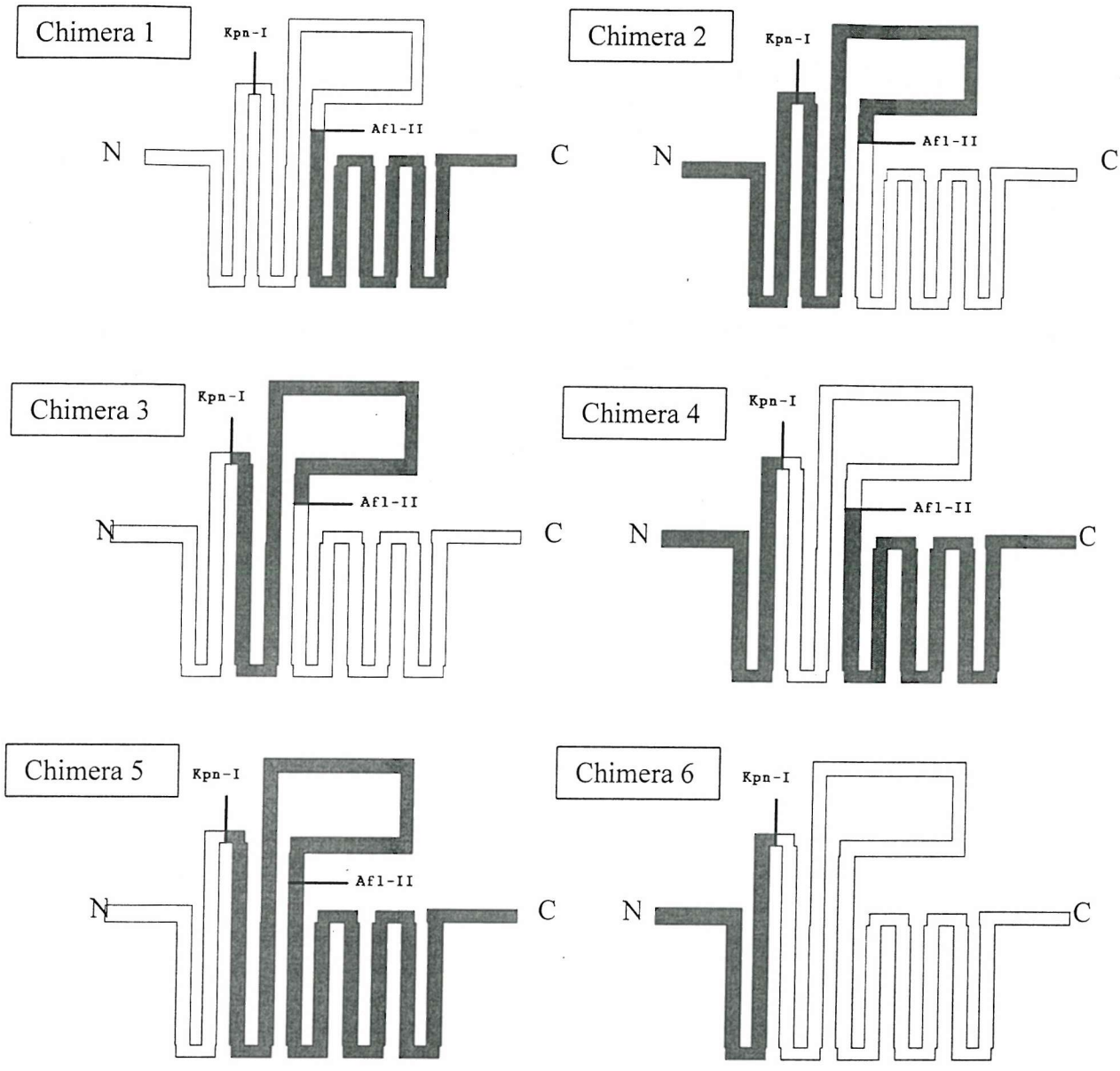


Figure 3.1: The set of chimeras previously made by John Black (thesis 1998).

The diagrams show the topology and amino acid sequence of the chimeric proteins in diagrammatic form. Each chimera has ten trans-membrane helices and a large domain made up of two cytoplasmic loops. Grey shading indicates PMCA3 sequence and white indicates SERCA1b sequence. Each chimera is paired with its 'mirror-image'. For example Chimera IV is paired with chimera V. Restriction sites within the cDNAs coding for each chimera are indicated to show their relationship to the protein sequence

First conserved region used for designing chimeras:

PMCA: **196** TVIRNGXXXXXXXXXXXXGDIAQVKYGDLLPADGVL--IQGNDLKIDESSLTGESDHVRK **253**

SERCA:**130** YRADRKSVQRIKARDIVPGDIVEVAVGDKVPADIRILSIKSTTLRVDQSILTGESVSVIK **189**

PMCA: **254** ----------SADKDPMLLSGTHVMEGSGRMVVTAVGVNSQTGIIFTLLXXXXXXXXXX **302**

SERCA:**190** HTEPVPDPRAVNQDKKNMLFSGTNIAAGKALGIVATTGVSTEIGKIRDQMAATEQDK--- **246**

PMCA: **303** XXXXXXXXXXXXMEMQPLKSAXXXXXXXXXXXXXXXXXXXXSVLQGKLTKLAVQIGKAGL **362**

SERCA:**247** ----------TPLQQKLDEFGEQLSK--- **262**

Second conserved region used for designing chimeras:

PMCA: **681** QRAGITVRMVTGDNINTARAIAAKCGIIQPGEDFLCLEGKEFNRRIRNEKGEIEQERLDK **740**

SERCA:**615** RDAGIRVIMITGDNKGTAIAICRRIGIFGENEE---VADRAYTGREFDDLPLAEQREACR **671**

PMCA: **741** VWPKLRVLARSSPTDKHTLVKGIIDSTTGEQRQVVAVTGDGTNDGPALKKADVGFAMGIA **800**

SERCA:**672** ---RACCFARVEPSHKSKIVEYLQSYD-----EITAMTGDGVNDAPALKKAEIGIAMG-S **722**

PMCA: **801** GTDVAKEASDIILTDDNFTSIVKAVMWGRNVYDSISKFLQFQLTVNVVAVIVAFTGACIT **860**

SERCA:**723** GTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAALG **782**

Figure 3.2: Protein sequence alignment of SERCA1b and PMCA3

Showing the two regions of SERCA and PMCA that contained conserved sequences, and were used as joining regions when designing chimeras designed by Black (1998). Highly conserved sequences are shown in blue.

3.2 Green Fluorescent Protein (GFP)

Discovered in 1962 after being isolated from the Jellyfish *Aequora Victoria*, GFP has recently developed into a major tool in molecular cell biology. Its fluorophore is created by a spontaneous nucleophillic attack of one of its amino acid residues. This means that it requires no jellyfish enzymes or extra cofactors for its formation, allowing it to be expressed in any other cell type (Tsein 1998).

Although potentially useful, the wildtype GFP had a few drawbacks. Its major excitation wavelength is at 395nm, whilst most laboratory microscopes are set up to excite FITC (another major fluorophore) at 475nm. Also, its quantum yield is relatively low, compared to other fluorophores, which provides a poor signal. Only when mutant GFPs were available did GFP's use really take off.

Enhanced GFP (eGFP) for example, contains mutations that increase its brightness 50-fold and increase the minor excitation peak at 475nm. This mutant and others have made eGFP an easily accessible fluorescent label. Table 2 shows a selection of available mutations and their spectral qualities.

3.2.1 Uses of GFP

Fluorescence Resonance Energy Transfer (FRET): FRET is a technique available due to the overlap present between the different excitation/emission wavelengths of the mutant GFPs. If a fluorophore (the acceptor) has an excitation wavelength which overlaps with the emission wavelength of another fluorophore (the donor), and if the fluorophores are close enough to each other, then energy transfer occurs. Experimentally this is seen by an increase in the acceptors' emission wavelength and a decrease in donor fluorescence. Because of the wide variety of GFP variants with different excitation/emission profiles, this technique can be carried out in live cells

Table 3.1: Commercially available fluorescent proteins

Colour	Variant Name	Ex/Em	Mutation
Green#	EGFP	488/507	F64L, S65T
Blue	BFP	387/450	F64L,Y66H,V163A
Cyan#	ECFP	434/475	K26R, F64L, S65T, Y66W, N146I, M153T, V163A, N164H, N212K
Yellow#	EYFP	514/527	S65G,V68L, S72A,T203Y
Red*	DsRed2	558/583	N/A

variants have Valine inserted at residue two (not counted in number designations) and a H231L mutation, which has no spectral effect.

table modified from Ellenburg et al, 1998 and Allan,2000.

*Not derived from GFP, but from a red fluorescent protein in coral.

using two proteins tagged with different GFP variants e.g. green fluorescent protein (GFP) and blue fluorescent protein (BFP).

This technique has been used to determine whether fusion proteins are interacting, and if so, how close to each other they are (Pollock and Heim, 1999). In order for FRET to gain any meaningful distance data, the dimers must position the fluorophores within 100 Å of each other. Proper controls must be also put in place to obtain FRET measurements when there is 100% interaction between dimers and for 0% interaction. Mahajan *et al.* (1998) observed interaction between Bcl-2 and Bax in the mitochondria of live cells using GFP and BFP as a FRET pair.

Another pair of GFP variants was used by Miyawaki *et al.* (1997) to measure the concentration of calcium within living cells. Two constructs, one with the N-terminus of calmodulin and CFP, and a YFP/C-terminus M13 chimera where expressed. Increase in Ca^{2+} causes the calmodulin to interact with the M13 chimera, thus inducing FRET. This was an especially versatile method of measuring Ca^{2+} concentration as the constructs could be expressed via a simple transfection and a specific targeting signal could be added to the constructs. This enabled easy measurement of the concentration anywhere within the cell (previously only possible in the extracellular space, and the cell as a whole).

PRIM: A recently observed effect termed PRIM (P.R.oximity I.M.aging) could provide yet another weapon in the molecular biologists arsenal. It uses the fact that wtGFP has two excitation peaks; at 395nm (major) and 475nm (minor). De Angelis *et al.* (1998) realised that the ratio of the peaks ($R_{395/475}$) changed when oligomerisation occurred. The effect however, is unpredictable. Chemically crosslinked transmembrane fusion proteins showed an increase in $R_{395/475}$, whereas a pharmaceutically induced oligomerisation state displayed the opposite effect. This unpredictability is not as bad a drawback as it first appears, as it is the change in ratio that is the evidence of oligomerisation. Although in it's early stages of development, PRIM could be a useful complimentary technique to the FRET method.

Protein Localisation: The major use of eGFP is in tagging proteins for localisation by fluorescence microscopy. GFP has obvious advantages over normal localisation methods like antibody detection, not least of which is that eGFP fluorescence can be observed in living cells. Normal fixation techniques are often lengthy and destructive, but when a fusion-tagged protein is expressed there are no such problems. This technique has been used to investigate the targeting of p230 (a trans-Golgi localised protein). A number of deletion mutants of p230 were created, and then 'tagged' with GFP to isolate the sequence responsible for targeting (Kjer-Nielson *et al.* 1999).

GFP fusion proteins are also much more versatile than using immunolocalisation techniques. Movement within the cell can be observed as well, with even horizontal movements of transmembrane proteins able to be observed. By bleaching an area of cell (like the Golgi membrane) and then observing the regain of fluorescence gives a measurement of fluidity in that region (White and Steltzer, 1999). With the wide variety of commercially available coloured GFP mutants transfecting cells with two separate GFP-chimeras can achieve dual labelling. Although there are problems with using two GFP variants, due to overlapping emission and excitement peaks it has been used successfully (Ellenburg *et al.* 1998).

In this chapter the chimeras originally produced by Black (1998) have been modified by the addition of eGFP to the C-terminus so that the problems encountered with fixing cells could be avoided. The eGFP tagged chimeras have been used to identify the segment of SERCA1 that contains the retrieval/retention signal responsible for the maintenance of SERCA1 in the endoplasmic reticulum.

3.3 Methods

3.3.1 COS-7 cells

COS-7 cells were used to express and view the GFP tagged constructs. These are a widely used cell line derived by transformation of another established cell line (simian CV-1 cells) by transformation with an origin defective SV40 virus, and contain large T antigen (Gluzman *et al.* 1981).

3.3.2 Transfection

COS-7 cells were grown to 60-80% confluency and transfected using Fugene-6 transfection reagent. Fugene-6 was diluted in DMEM without any antibiotics or foetal bovine serum and mixed with DNA to a ratio of 1 μg DNA/3 μl Fugene-6 as described in manufacturers instructions. The Fugene-6/DNA mixture was then left at room temperature for 15 minutes before being applied drop-wise to the COS-7 cells. Transfected cells were then treated to produce microsomes or viewed as described below.

3.3.3 Fluorescent microscopy

13 mm coverslips were placed into 24 well tissue culture plates and COS-7 cells were seeded onto to them and transfected with the construct to be observed. 48 hours after

transfection the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and left at 4°C overnight. The cells were viewed using a Biorad MRC-600 confocal microscope, or a Leica epifluorescent microscope. Images from the confocal microsope were manipulated using Confocal Assistant™ software.

3.3.4 Microsome preparation

Microsomes were prepared by using a modified method from that of Maruyama and MacLennon (1988). COS-7 cells were seeded onto 14 mm tissue culture dishes and transfected with the construct to be assayed. After 48 hours the cells were washed twice with ice cold PBS (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH 7), and harvested with 2x5 ml 5 mM EDTA in PBS, and centrifuged at 3000 rpm in a Heraeus labofuge 400e bench top centrifuge. The harvested cells were then washed with 5 ml PBS at 3000 rpm and the pellets resuspended in 1ml of a hypotonic 'swelling' solution (solution A – 10 mM Tris-HCl pH 7.5, 0.5 mM $MgCl_2$). After being left in for 10 minutes at 4°C, 50 µl of protease inhibitor cocktail was added and the cells were homogenised with 30 strokes of a Dounce homogeniser. An equal volume of microsome preparation solution (solution B - 0.5 M sucrose, 6 mM β-2-mercaptoethanol, 40 µM $CaCl_2$, 300 mM KCl, 10 mM Tris HCl), was added to the homogenate, and the suspension was centrifuged at 10,000 g for 20 minutes. The supernatant was made up to 0.6 M KCl by the addition of 0.45 ml of a 2.5 M KCl solution, and then spun at 100,00 g for 1 hour. Microsome preparation solution was diluted 1:1 with 10 mM Tris-HCl (pH 7.5) to produce solution C, and this was used to resuspend the resultant pellet. The suspension was then centrifuged at 100,000 g for 1 hour, and the final pellet was resuspended in 100 µl solution C. After 3 strokes with a micro-homogeniser (0.1 ml), the microsome suspension was divided into 15 µl aliquots, snap frozen in liquid nitrogen and stored at -80°C.

3.3.5 Calcium uptake assay

The ability of the constructs to transport calcium was assayed, by using the calcium transport assay in Maruyama and MacLennan (1988). Microsomes were added to 1ml of calcium uptake solution (20 mM Mops-KOH (pH6.8), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.45 mM $CaCl_2$ (containing ^{45}Ca at a specific activity of 10^6 cpm/μM, 0.5 mM EGTA, 5 mM potassium oxalate), to start the time course. Duplicate samples of 100 μl were taken at 0,2,4,8 and 10 minutes after addition of microsomes. Samples were immediately pipetted onto damp nitrocellulose discs, and washed twice with 150 mM KCl/20mM MOPS (ph 6.8). The nitrocellulose discs were then placed into scintillation tubes, and covered with scintillation fluid. Radioactive counts were measured using a Beckman LS 6500 automatic scintillation counter.

3.4 Constructs

In order to create a set of chimeras fusion-tagged with eGFP, two initial constructs had to be made: one with the C-terminal third of PMCA linked to eGFP, and a similar C-terminal SERCA/eGFP construct.

3.4.1 Making a construct containing the C-terminal of SERCA1b linked to enhanced Green Fluorescent Protein (ctSERCA/eGFP)

A SERCA1b-GFP already existed and is known as pPA50 (courtesy of Phil Adams). This consists of the sequence coding for SERCA1b fused to the eGFP sequence at the 3' end via a short myc epitope linker (shown in figure 3.3). Melanie Logan-Smith has provided this construct cloned into the Xho-1 and Nhe-1 sites of vector pcDNA3.1 (pPA50/3.1), with an Afl-II site introduced at residues 711-712. Digesting SERCA1b-eGFP with Xho-1 and Alf-II created a 1.7 kb fragment (containing residues 712-1001 of SERCA linked to eGFP by the myc epitope linker). This fragment was then cloned into pcDNA3.1 (see Fig 3.4) and the resulting construct contained the C-terminal segment of SERCA linked to eGFP (ctSERCAeGFP).

Figure 3.5 shows an agarose gel on which the digested vector (pcDNA3.1) and the insert (1.7 kb fragment from Afl-II/Xho-1 digest of SERCA-eGFP) used in the creation of ctSERCAeGFP were run. A set of ligation reactions were set up using 2 μl of vector and between 4 μl and 14 μl of insert. The required construct was screened for by digesting putative recombinants with Afl-II and Xho-I, and then sequenced.

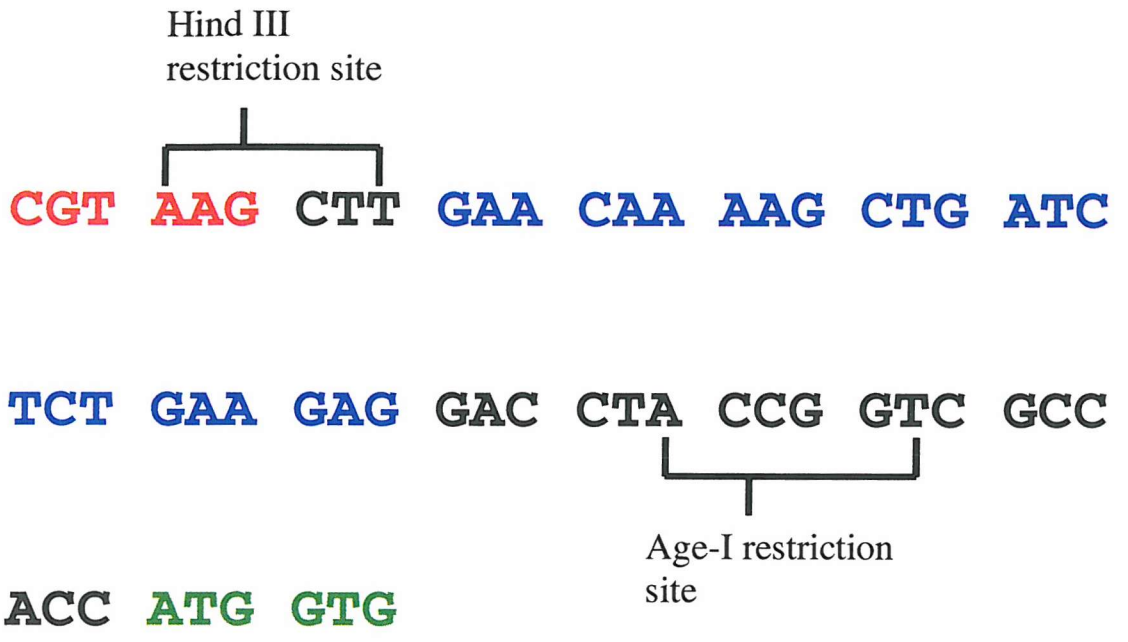


Figure 3.3: The DNA sequence encoding the myc-epitope 'linker between SERCA1b and eGFP in SERCA-eGFP

Bases shown in blue show the myc epitope region of the linker, whereas red bases are to show where the SERCA1b sequence joins into the linker (with bases changed to alter the stop codon) and green bases denote the start of the eGFP sequence.

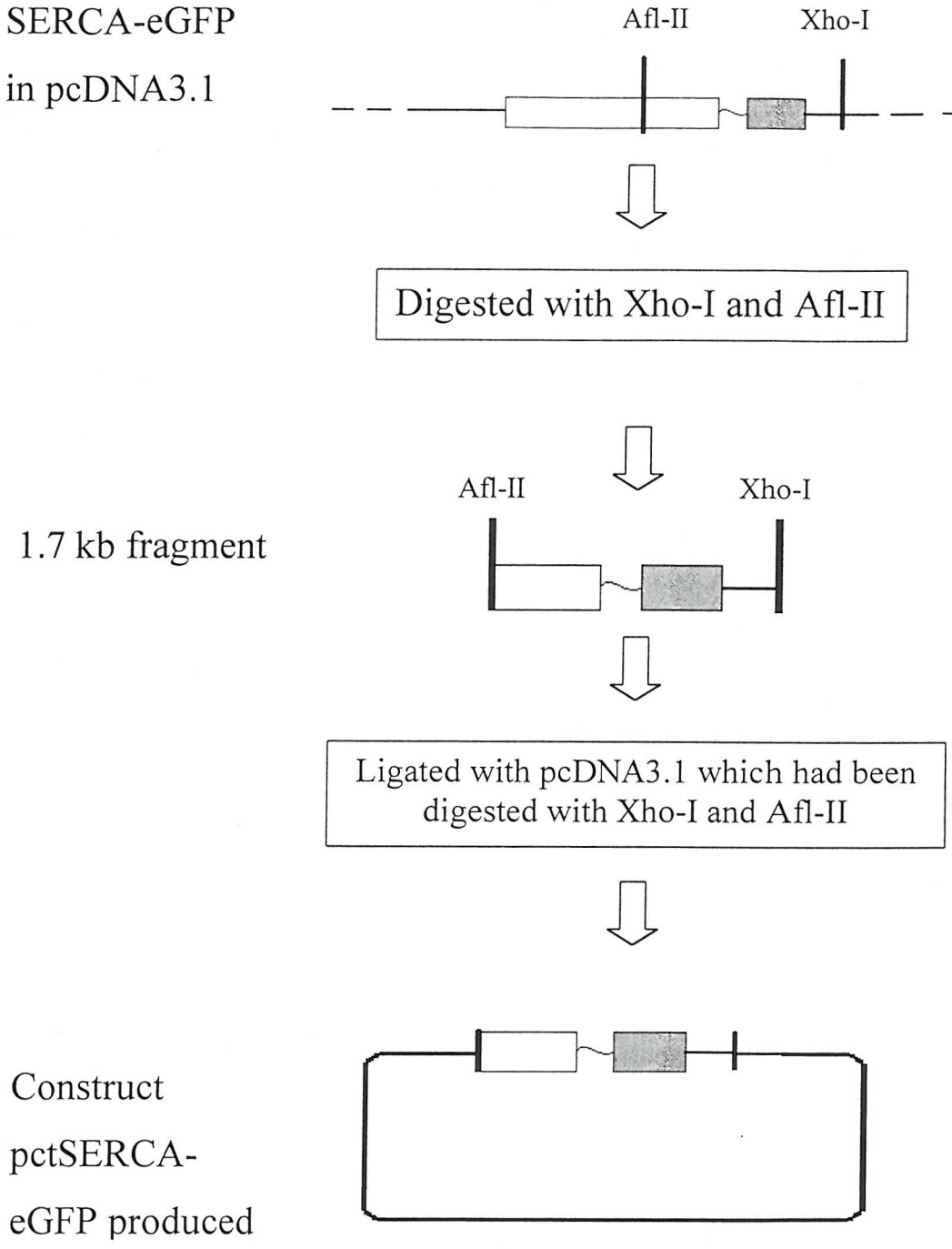


Figure 3.4: Simple diagram showing the protocol for the cloning of construct pctSERCA-eGFP.

pSERCA-eGFP was double digested with the restriction enzymes Xho-I and Afl-II to produce a 1.7 kb DNA fragment with Xho-I and Afl-II compatable 'sticky ends'. This DNA fragment was then ligated into the Afl-II and Xho-1 sites of the vector; pcDNA3.1 to create pctSERCAeGFP.

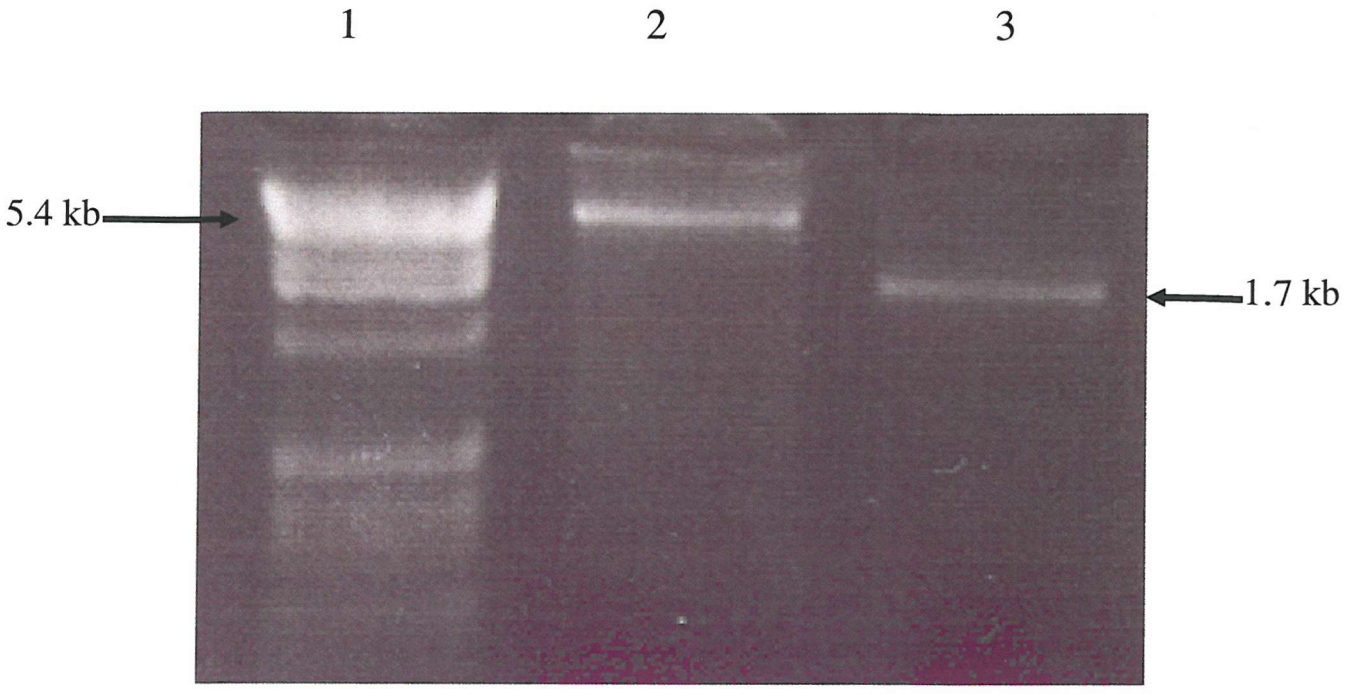


Figure 3.5: Cloning of ctSERCA-eGFP

An agarose gel used during the production of pctSERCA-eGFP: the C-terminus of SERCA1b linked to enhanced Green Fluorescent Protein, after Afl-II/Xho-I restriction digest. 1 μg vector (pcDNA3.1) was digested and then cleaned up using Wizard clean-up kit, 10μg SERCA-eGFP was digested, a 1.7 kb fragment was excised from a 1% agarose gel and then cleaned up to provide the insert. Lane one is a marker lane (sizes of fragments shown by arrows). Lane two shows 2 μl of the digested vector, lane three shows 2 μl of the digested insert. A range of ligation reactions were then set up with a variation of vector:insert ratios.

3.4.2 Cloning a construct consisting of the C-terminal domain of PMCA linked to enhanced Green Fluorescent Protein (ctPMCA-eGFP)

The C-terminal PMCA/GFP construct (ctPMCA-eGFP) was made in two stages: firstly to create a construct which contained the C-terminal segment of PMCA3 (pctPMCA) and then linking it with eGFP with an identical 'myc-linker' to that of cSERCAeGFP (pctPMCA-eGFP). See figures 3.6 and 3.8

pctPMCA

The sequence encoding the c-terminal third of PMCA3 was amplified from PMCA3 in pcDNA3.1 to produce a fragment of 1.1 kb. PCR primers tn1 (5'-CCA GCC CTC GAG CGG AAT GCT TTC ACC ACT-3') and tn2 (5'-CCA GCC CTT AAG AAG GCA GAT GTG GG-3') introduced an Afl-II site at the 5' end and a 3' Xho-I site. The construct ctPMCA was constructed by cloning this PCR fragment into pcDNA3.1 after a Xho-1/Afl-II digest. Figure 3.7 shows an agarose gel on which the digested vector (pcDNA3.1) and the insert (1.2 kb PCR fragment after Xho-I/Xba-I digest) used in the creation of pctPMCA were run. A set of ligation reactions were set up using 2 μl of vector and between 4 μl and 14 μl of insert. The required construct was screened for by digesting putative recombinants with a diagnostic double-digest with Afl-II and Xho-I, and then sequenced.

pctPMCA-eGFP

The full pctPMCA-eGFP construct was made my amplifying a PCR fragment of approximately 800 bp, encoding the linker sequence and eGFP from pPA50/3.1. Primers GFP5' (5'-TGC GCA CTC GAG CAA AAG CTG ATC TCT GAA GAG-3') and GFP3' (5'-TGC GCA TCT AGA TTA CTT GTA CAG CTC GTC CAT-3') introduced a 5' Xho-1 site and a 3' Xba-I site. This fragment was cloned into

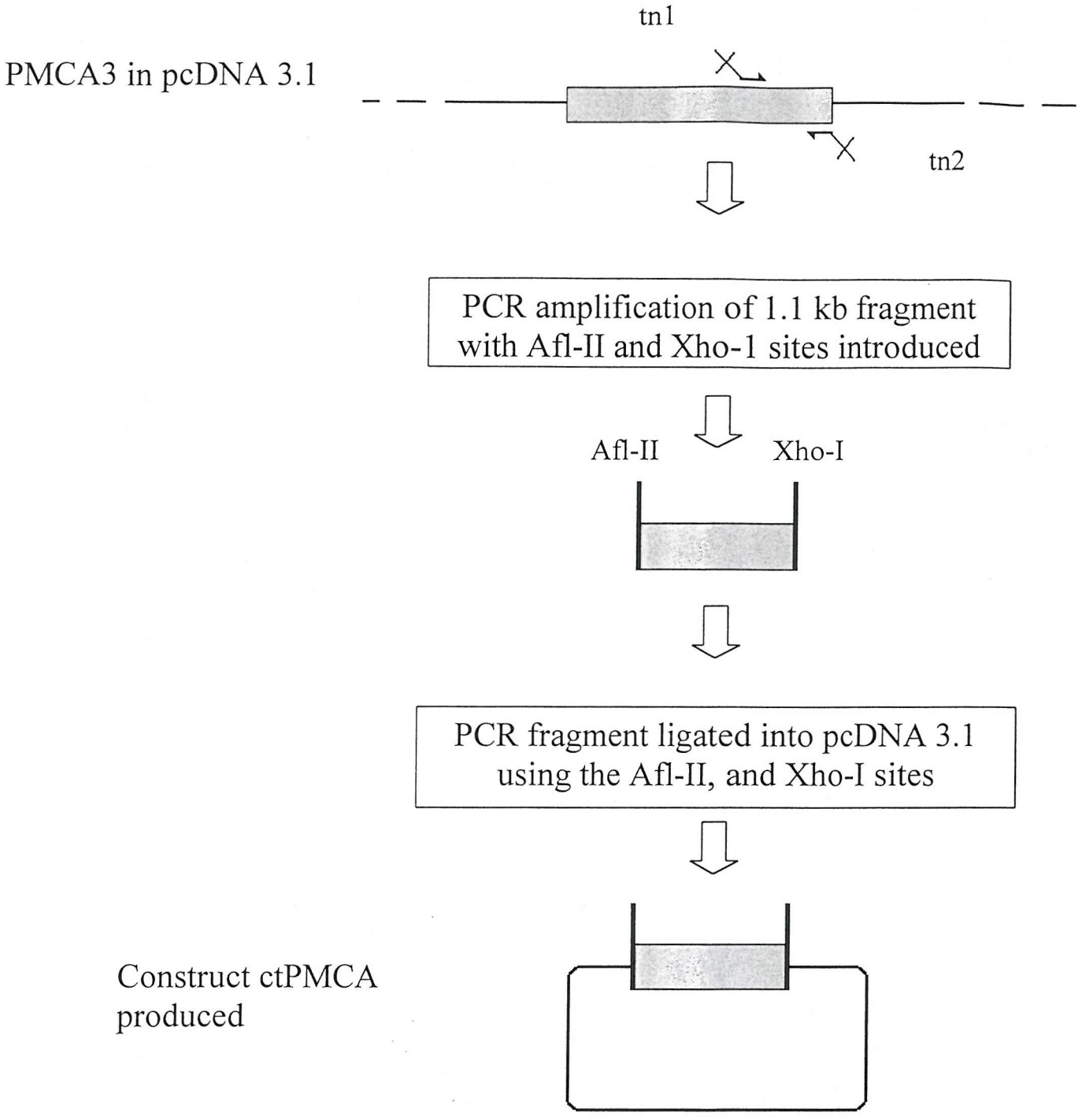


Figure 3.6 Simple diagram showing the protocol for the cloning of construct pctPMCA.

PCR amplification using primers; tn1 and tn2 produced a 1.1 kb fragment. This PCR fragment was then digested with the restriction enzymes Afl-II and Xho-I. The digested PCR fragment was then ligated into the Afl-II and Xho-1 sites of the vector; pcDNA3.1 to create pctPMCA.

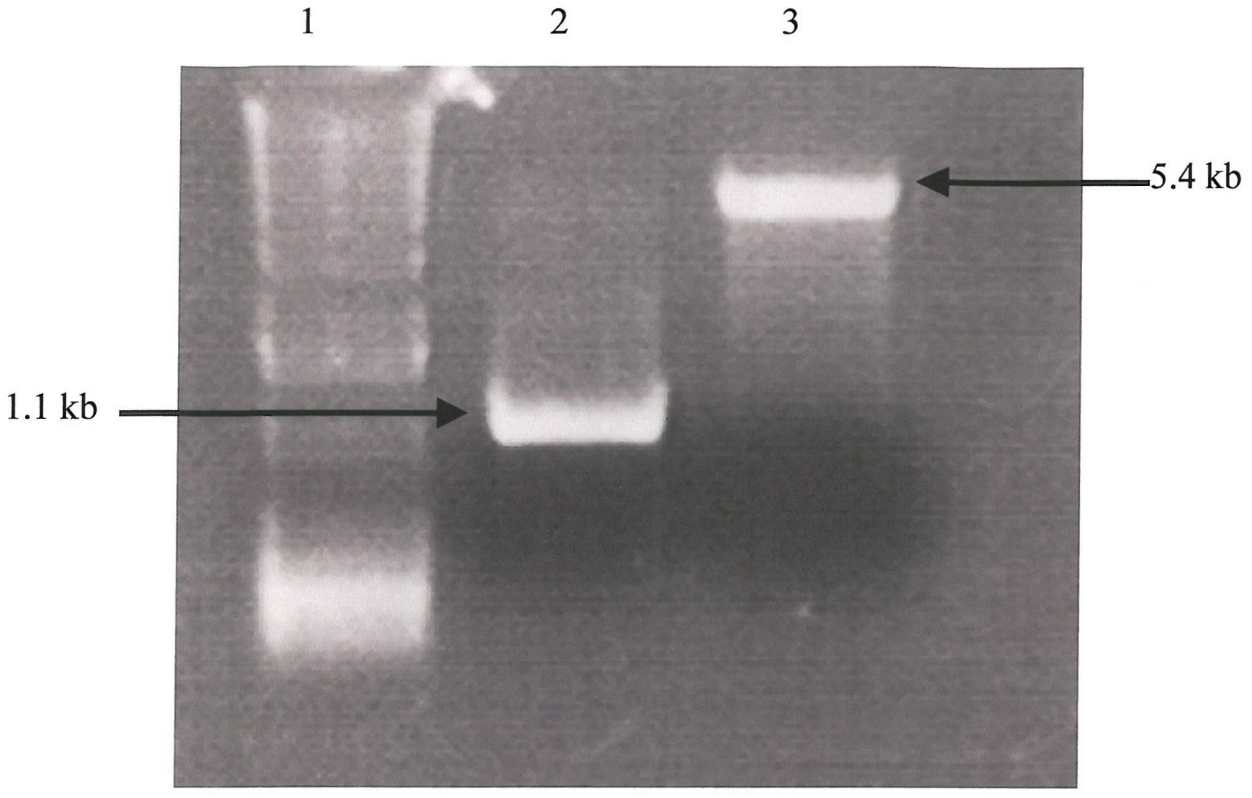


Figure 3.7: Cloning of ctPMCA

An agarose gel used during the production of pctPMCA: the C-terminus of PMCA, after Afl-II/Xho-I restriction digest. 1 μg vector (pcDNA3.1) was digested and then cleaned up using Wizard clean-up kit, the PCR fragment was cleaned up using Wizard PCR clean-up kit, digested and then cleaned up to provide the insert. Lane one is a marker lane (sizes of fragments shown by arrows). Lane two shows 2 μl of the digested insert, lane two shows 2 μl of the digested vector. A range of ligation reactions were then set up with a variation of vector:insert ratios.

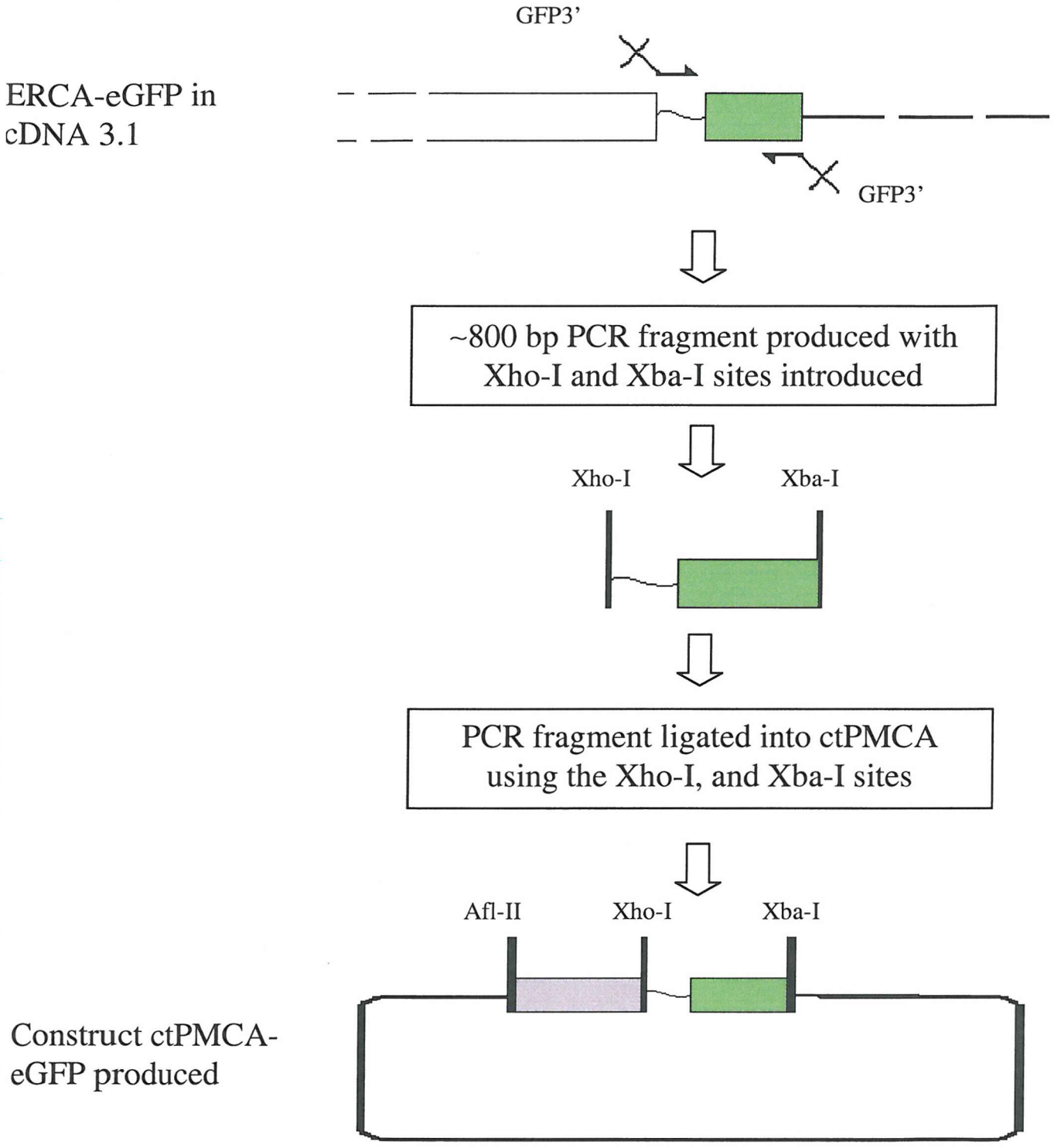


Figure 3.8: Simple diagram showing the protocol for the cloning of construct pctPMCA-eGFP

PCR amplification using primers; GFP5' and GFP3' produced a 800 bp fragment. This PCR fragment was then digested with the restriction enzymes Xho-I and Xba-I. The digested PCR fragment was then ligated into the Afl-II and Xho-I sites of the vector; pcDNA3.1 to create pctPMCA-eGFP.

pctPMCA after a Xho-I/Xba-I digest to create pctPMCA/GFP. The vector (pctPMCA) and insert (800 bp PCR fragment) are shown on an agarose gel after a Xho-I/Xba-I digest in figure 3.9. A set of ligation reactions were set up with 2μl of Vector was used and between 4 μl and 14 μl of insert. The required construct was screened for by digesting putative recombinants with a diagnostic double-digest with Xba-I and Xho-I, and then sequenced.

3.4.3 Tagging the chimeras and PMCA with enhanced Green Fluorescent Protein

After creating pctPMCA-eGFP and pctSERCA-eGFP, the whole set of chimeras and PMCA3 could be fusion-tagged with enhanced Green Fluorescent Protein, using a system of Nhe-I/Afl-II 'cassettes'. By selecting the appropriate C-terminal construct as the vector, and introducing a DNA fragment made from digesting a selected non-tagged chimera with Afl-II and Nhe-I all of the required combinations can be made.

For example: Chimera 2 consists of PMCA3 in the first two segments and a C-terminal SERCA1b segment. Digesting with Afl-II and Nhe-I produces a fragment of approximately 2kb in size, which contains the first four transmembrane helices and the cytoplasmic domain of PMCA3. This 'cassette' can then be cloned into pctPMCA-eGFP (containing the last five transmembrane helices and the C-terminal domain of PMCA3 linked to eGFP) to create a construct, which has the entire sequence of PMCA3 linked to eGFP.

Figure 3.10 shows a schematic diagram of how PMCA-eGFP was produced. Figure 3.11 shows an agarose gel on which the digested vector (pctPMCA-eGFP) and the insert (2.4 kb fragment from Afl-II/Nhe-I digest of SERCA-eGFP) used in the creation of PMCA-eGFP were electrophoresed. A set of ligation reactions were set up using 2 μl of vector and between 2 and 14 μl of insert. The required construct was

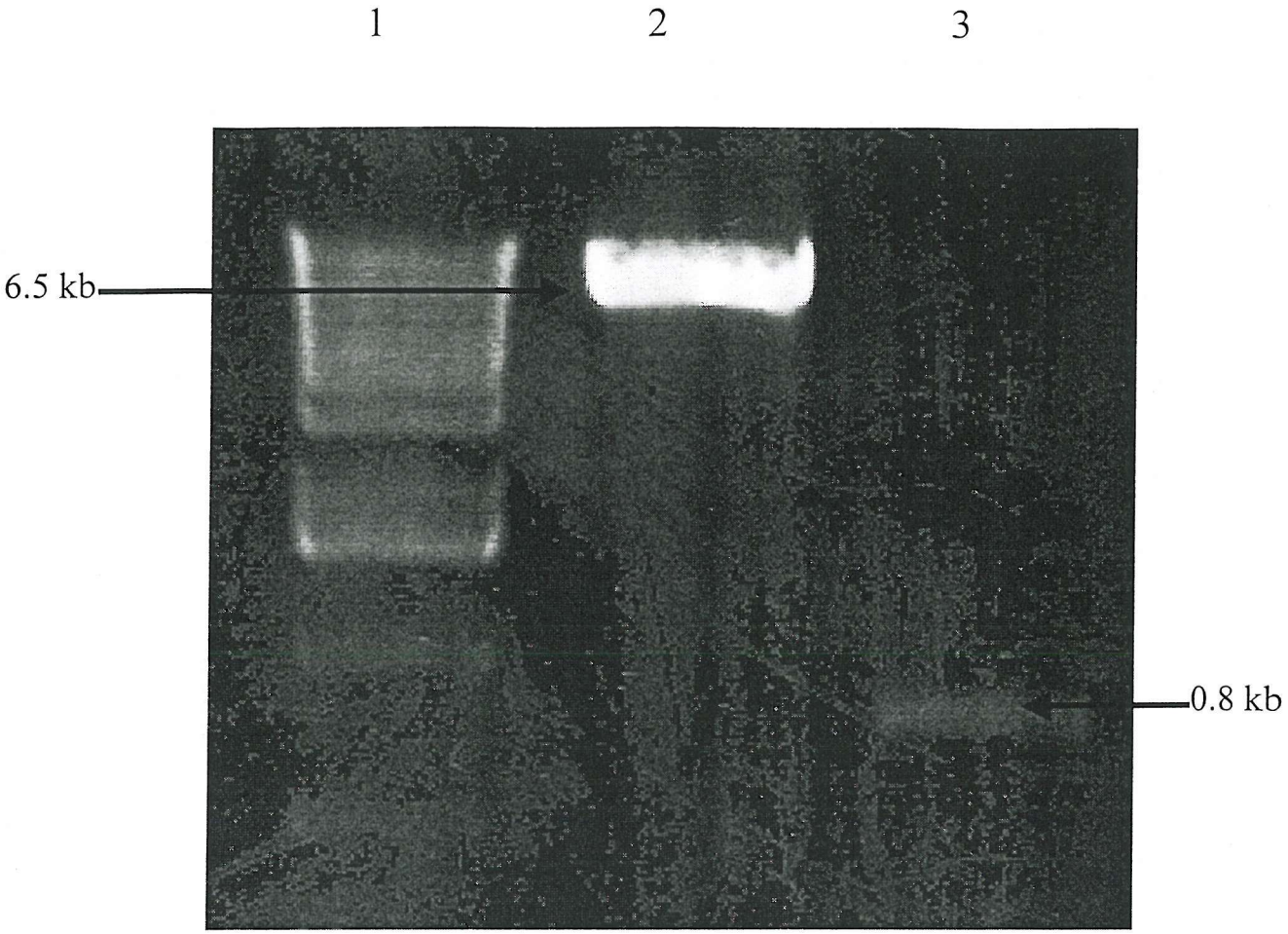


Figure 3.9: Cloning of ctPMCA-eGFP

An agarose gel used during the production of pctPMCA-eGFP: the C-terminus of PMCA linked to enhanced Green Fluorescent Protein, after Xho-I/Xba-I restriction digest. 1 μg vector (pctPMCA) was digested and then cleaned up using Wizard clean-up kit, the PCR fragment was cleaned up using Wizard PCR clean-up kit, digested and then cleaned up to provide the insert. Lane one is a marker lane (sizes of fragments shown by arrows). Lane two shows 2 μl of the digested insert, lane two shows 2 μl of the digested vector. A range of ligation reactions were then set up with a variation of vector:insert ratios.

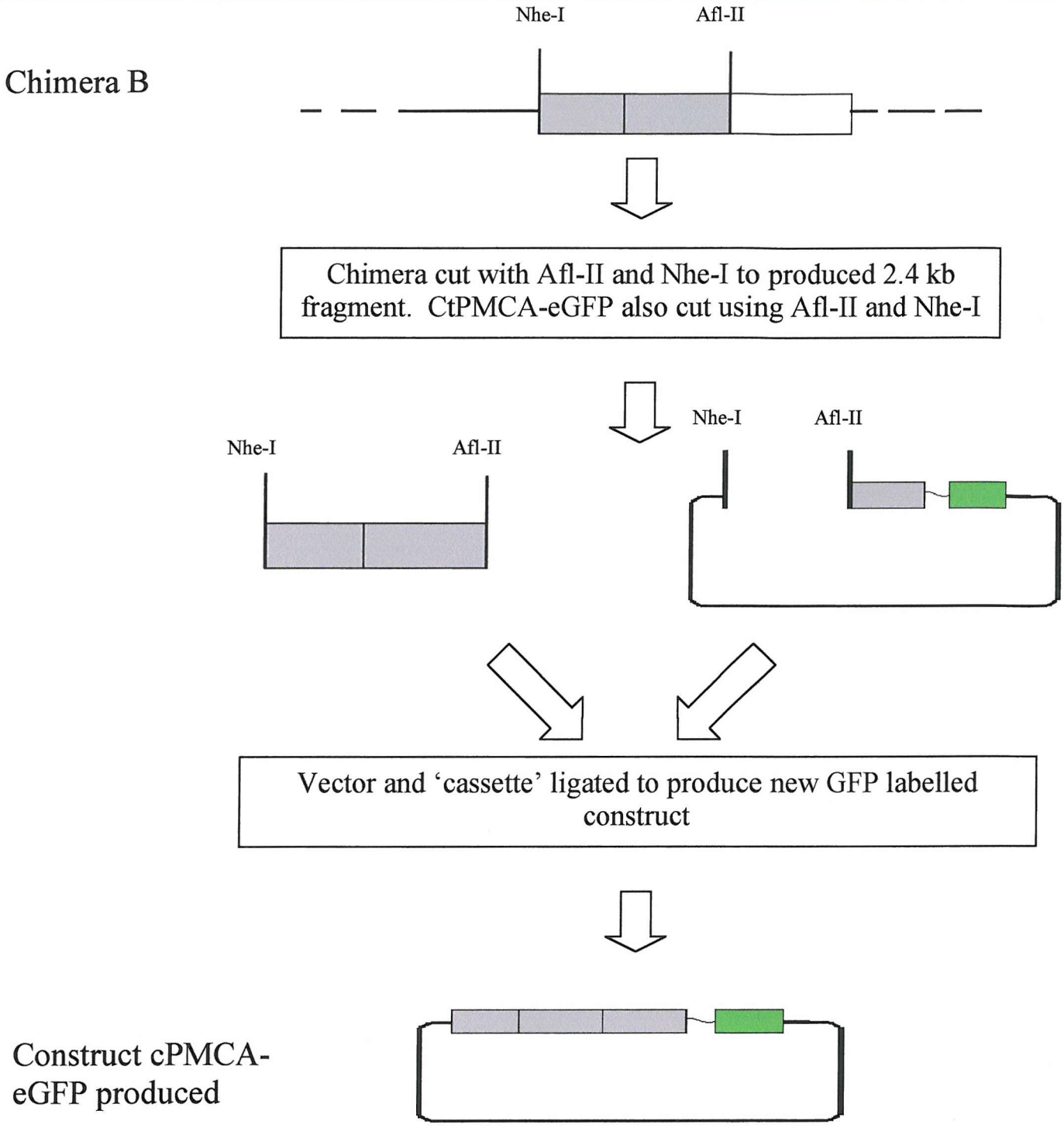


Figure 3.10: Simple diagram showing the protocol for the cloning of construct pctPMCAeGFP.

Chimera 1 was double digested with the restriction enzymes.
Nhe-I and Afl-II to produce a 2.4 kb DNA fragment with Nhe-1 and Afl-II compatable 'sticky ends'. This DNA fragment was then ligated into the Afl-II and Xho-1 sites of pctPMCAeGFP to create PMCA-eGFP.

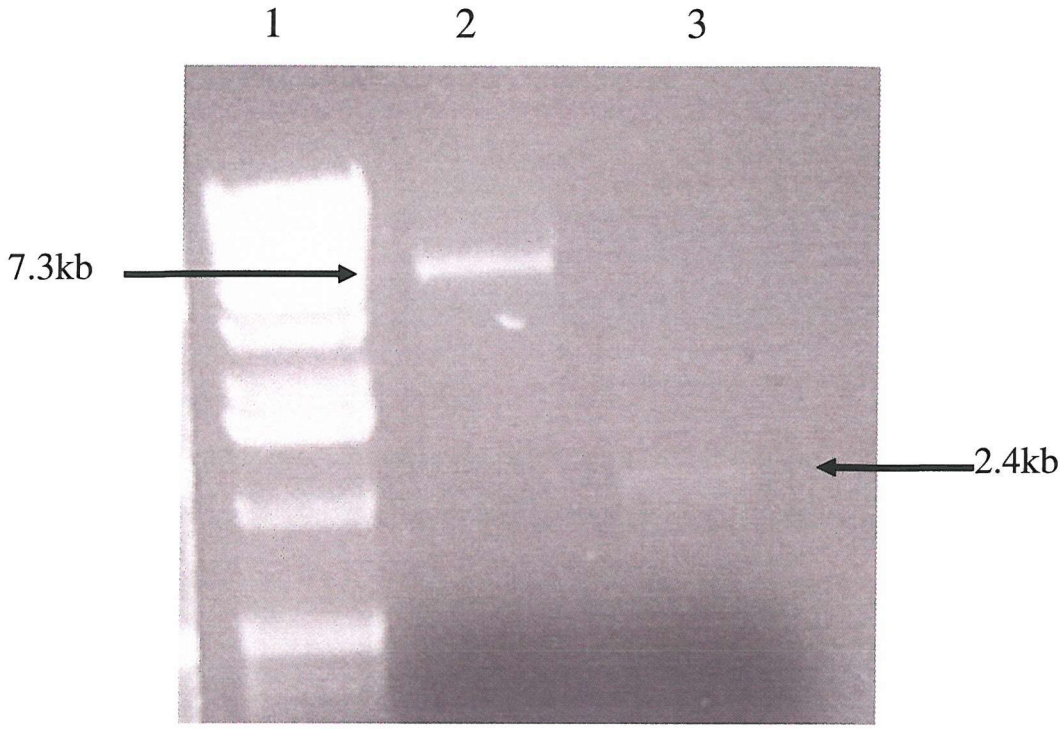


Figure 3.11: Cloning of PMCA-eGFP

An agarose gel used during the production of pPMCA-eGFP: the sequence of PMCA linked to enhanced Green Fluorescent Protein, after Nhe-1/Afl-II restriction digest. 2 µg of pctPMCA-eGFP was digested, then cleaned up using the Wizard clean-up kit. 5 µg of chimera 2 was digested, and a 2.4 kb fragment was excised from a 1% agarose gel and then cleaned up to provide the insert Lane one is a marker lane (sizes of fragments shown by arrows). Lane two shows 2 µl of the digested insert, lane two shows 2 µl of the digested vector. A range of ligation reactions were then set up with a variation of vector:insert ratios.

screened for by digesting putative recombinants with Afl-II and Nhe-I, and then sequenced.

In contrast, if the same sequence was cloned into pctSERCA-eGFP then chimera 2 tagged with eGFP would be produced. Table 3.1 summarises the vectors and 'cassette' vectors that were used to produce the six eGFP tagged chimeras and PMCA-eGFP.

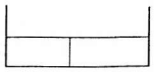
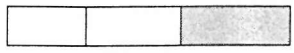
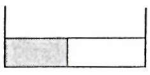
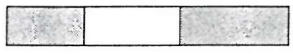
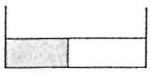
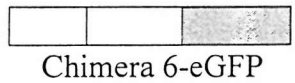
Nhe-I/Afl-II 'Cassette' used	'Cassette' cloned into :	Resulting construct
 From Chimera 2	pctPMCA-eGFP	 PMCA-eGFP
 From Chimera 1	pctPMCA-eGFP	 Chimera 1-eGFP
 From Chimera 2	pctSERCA-eGFP	 Chimera 2-eGFP
 From Chimera 3	pctSERCA-eGFP	 Chimera 3-eGFP
 From Chimera 4	pctPMCA-eGFP	 Chimera 4 -eGFP
 From Chimera 5	pctPMCA-eGFP	 Chimera 5-eGFP
 From Chimera 6	pctSERCA-eGFP	 Chimera 6-eGFP

Table 3.2

Summarising the methods of creating a set of GFP tagged SERCA/PMCA chimeras. The make-up of the chimeras is displayed as simple diagrams in which the white denotes SERCA1b sequence and the grey denotes PMCA3

3.5 Results

3.5.1 Sequencing results

The constructs were sequenced as described in the materials and methods chapter (section 2.3.7). Figures 3. to 3. show the sequencing results for chimera 4-eGFP which consists of the first section of PMCA, a middle section of SERCA sequence and a C-terminal PMCA section. Other sequences are shown in the appendix.

3.5.2 SERCA-eGFP expressed in COS-7 cells

Figure 3.15 shows the typical eGFP fluorescence of a single cell expressing SERCA-eGFP. The nucleus can be clearly seen in the middle of the picture, as there is strong perinuclear fluorescence in the centre of the cell with the brightest fluorescence present to the top of the nucleus. Vesicular fluorescent patterns can also be seen around the nucleus, with one of the most obvious localised to the bottom right of the nucleus. The rest of the fluorescence is seen throughout the cell as a reticular structure spreading from the nucleus. Although the reticular structure does seem to take up most of the cell's surface area, there does not seem to be a clear outline to the cell. This interconnected network can be seen clearest to the left of the cell in the picture. The reticular structure can be seen with a high degree of resolution, and the cell has a distinct shape but no fluorescence can be seen at the plasma membrane.

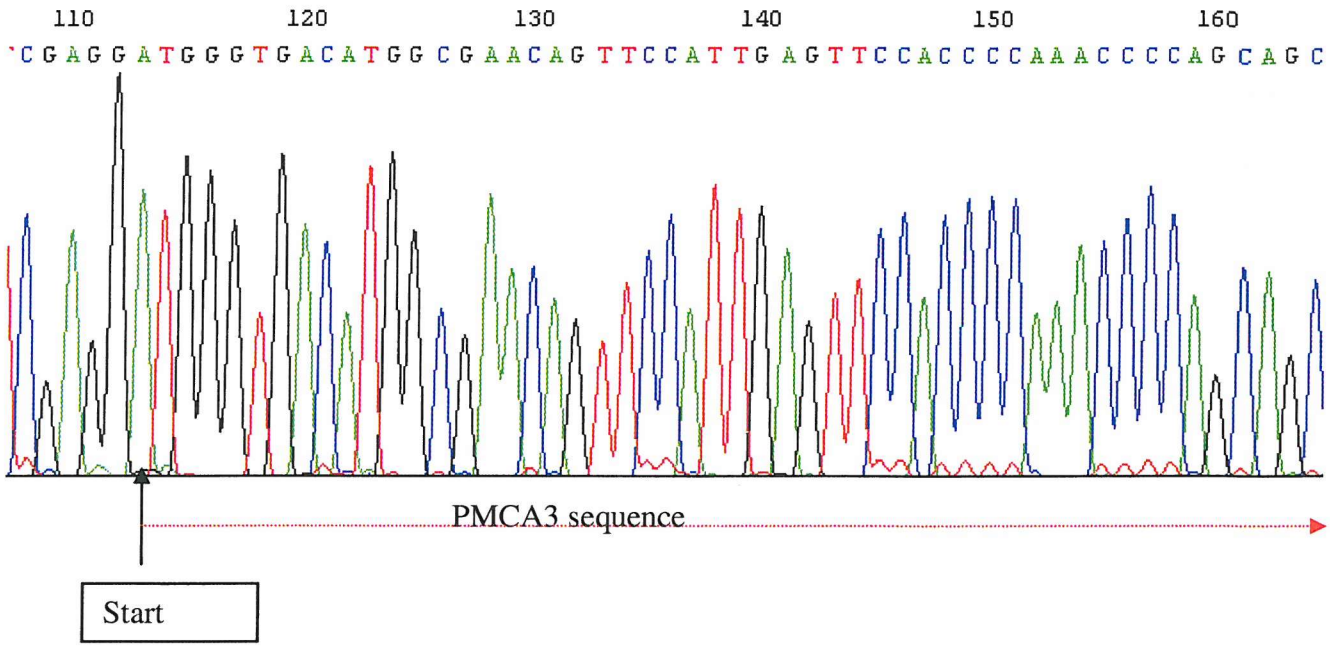


Figure 3.12

Chromatogram showing the sequence data for chimera 4-eGFP using sequencing primer 3.1 Fwd. The primer binds to a sequence within the vector which allows the sequencing of the joining region between chimera 4-eGFP (PMCA3 sequence) and the vector (pcDNA3.1) at the 5' end of the sequence.

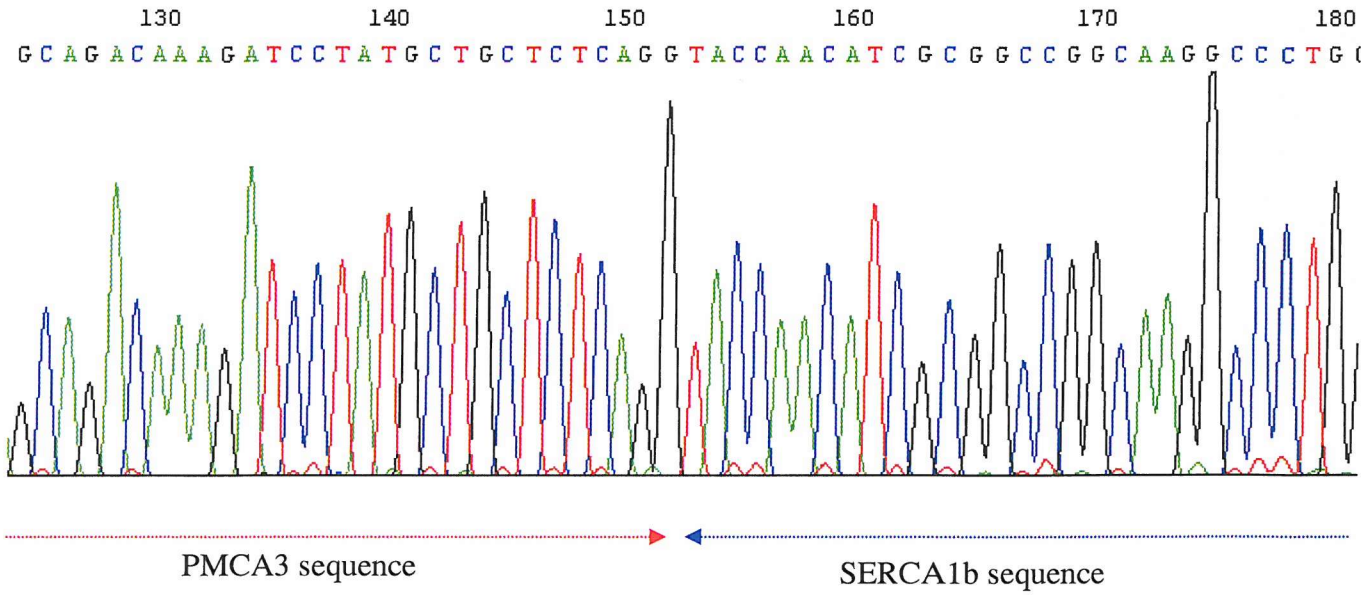


Figure 3.13

Chromatogram showing the sequence data for chimera 4-eGFP using sequencing primer SP3. The primer binds to a sequence within PMCA which allows the sequencing of the joining region between the first segment of chimera 4-eGFP (PMCA3 sequence) and the middle segment (SERCA1b).

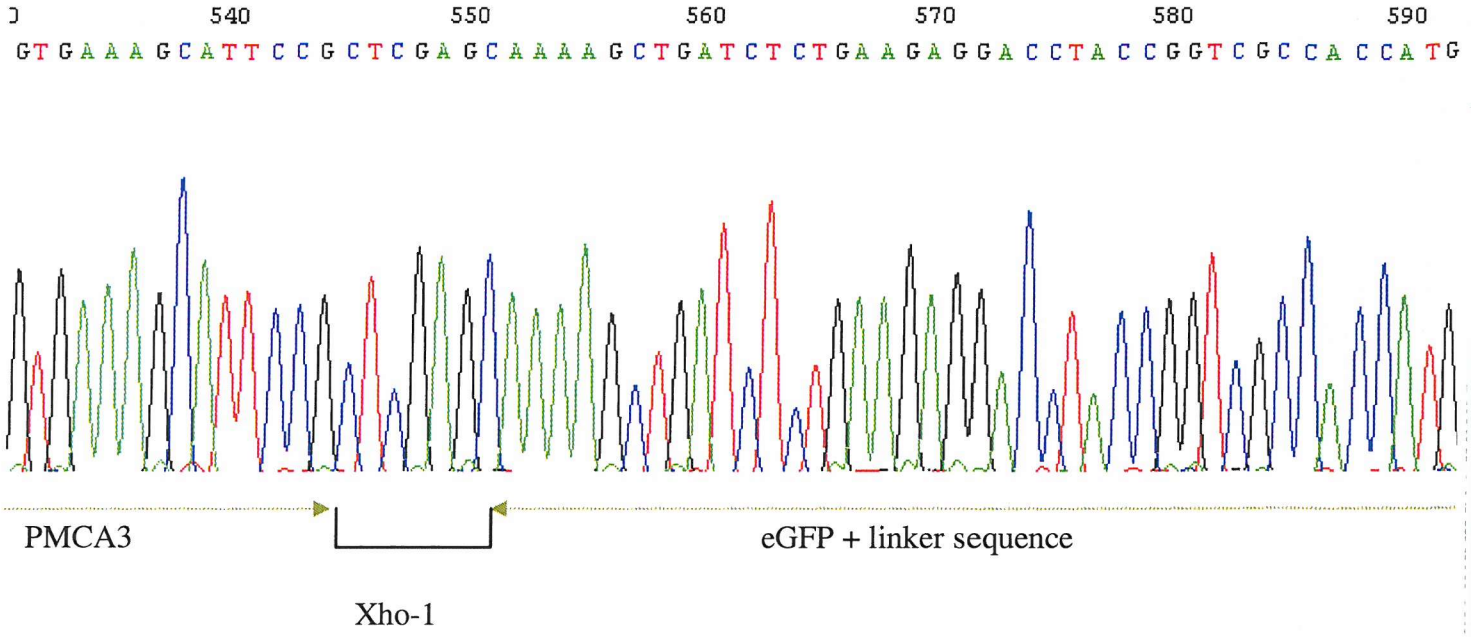


Figure 3.14

Chromatogram showing the sequence data for chimera 4-eGFP using sequencing primer SP5. The primer binds to a sequence within eGFP which allows the sequencing of the joining region between chimera 4-eGFP (PMCA3 sequence) and the 3' eGFP.

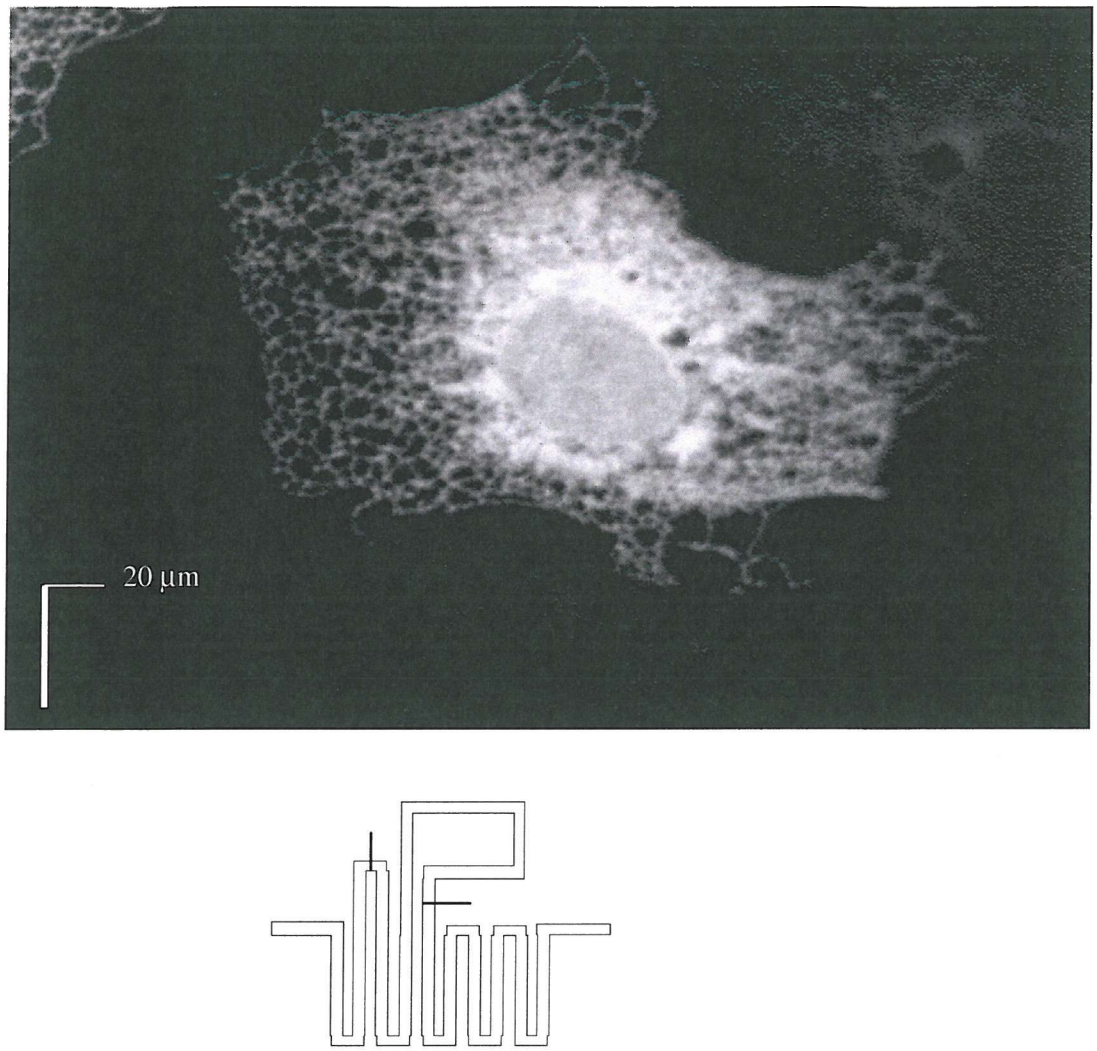


Figure 3.15: eGFP fluorescence in a single COS-7 cell transfected with SERCA-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of SERCA.

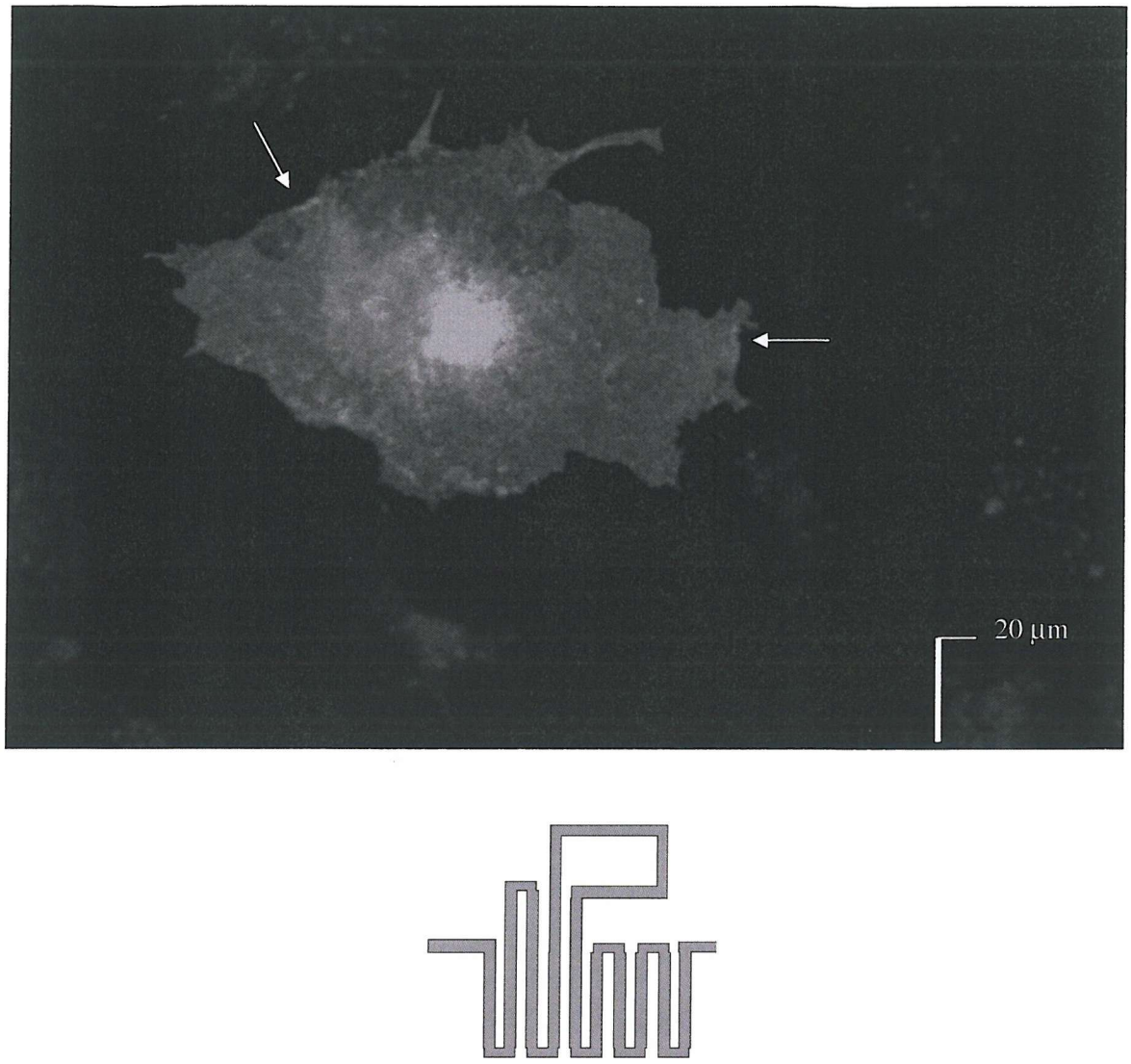


Figure 3.16: eGFP fluorescence in a single COS-7 cell transfected with PMCA-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of PMCA.

3.5.1 Chimera 1-eGFP expressed in COS-7 cells

Figure 3.17 shows the typical eGFP fluorescence of cells expressing Chimera 1-eGFP. Two cells are shown in the diagram, showing similar fluorescence patterns. The larger cell (on the right) has a clear perinuclear fluorescence pattern in the centre-left of the cell. There is a high concentration of fluorescent material to the right of the nucleus and a reticular network extends out from this central structure. The smaller cell (on the left) is not quite so regular in structure with a similar sized nucleus in the middle of the cell, with a high fluorescent signal to the left of it. Fluorescent reticular networks extend into the top, left, and bottom of the left-hand cell The reticular structure is extremely clear throughout both of the examples shown, but the clearest example of this inter-connecting 'spider-web' like structure is seen to the right of the large cell. This structure stretches out across the cytoplasm of the cell and provides a definite shape to the cell, but lacks any distinctive accumulation at the plasma membrane. This chimera consists of the first two thirds of SERCA1b and the last third of PMCA3.

3.5.2 Chimera 2-eGFP expressed in COS-7 cells

Figure 3.18 shows a typical COS-7 transfected with Chimera 2-eGFP. The eGFP fluorescence is diffuse throughout the whole of the cell and there is a bright fluorescence in the centre of the cell. Although there is no reticular structure stretching out throughout the cell like in SERCA, a feint pattern can be seen spreading out through the centre of the cell. Small vesicles can be seen in the cell where no fluorescent material is present and a distinct plasma membrane can be seen. The edge of the cell can be seen best where there is a high concentration of fluorescent material at the plasma membrane. Arrows on the figure show where the plasma membrane is

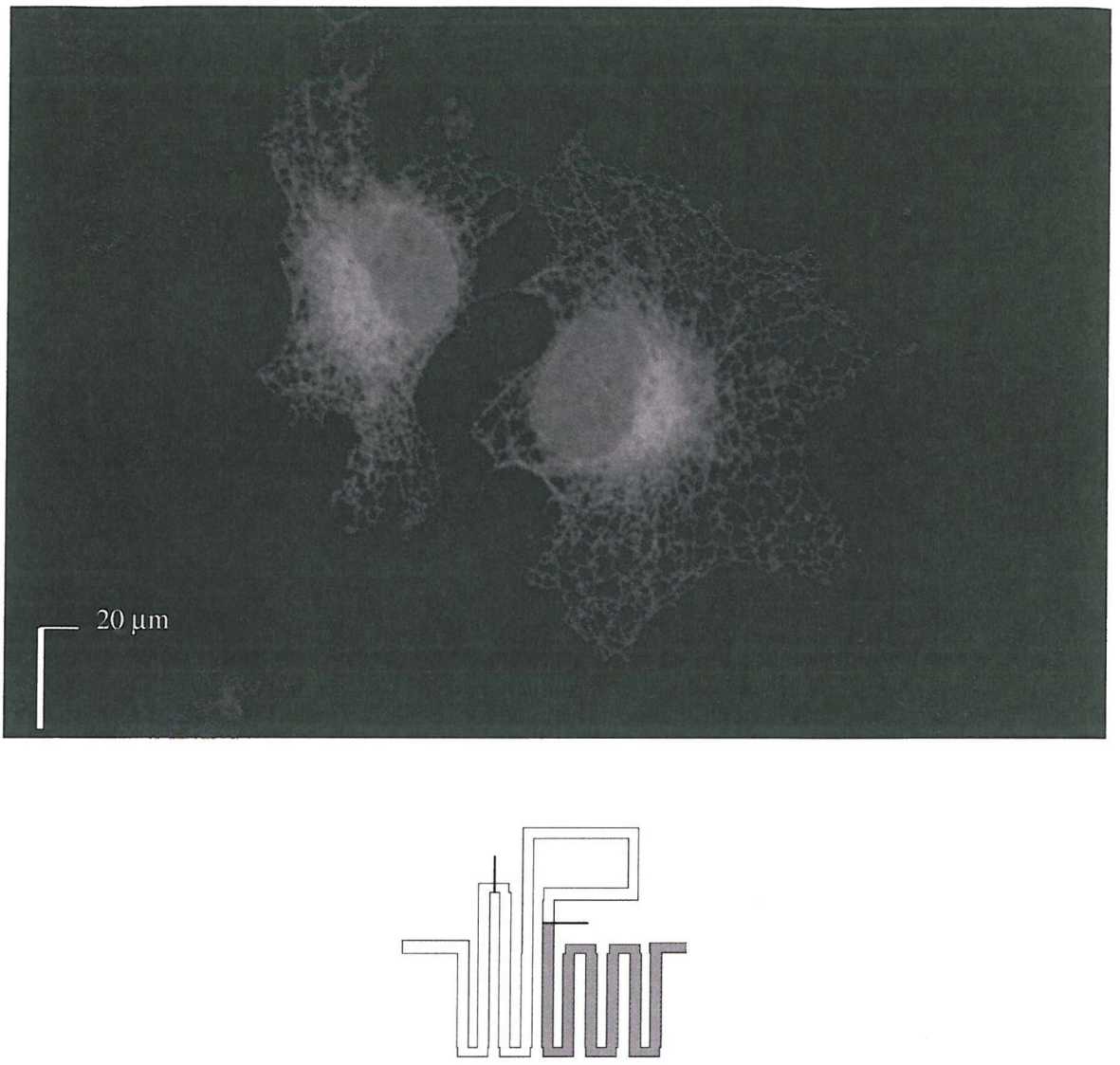


Figure 3.17: eGFP fluorescence in two COS-7 cells transfected with chimera 1-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera and the composition: black indicates PMCA sequence, white is SERCA sequence.

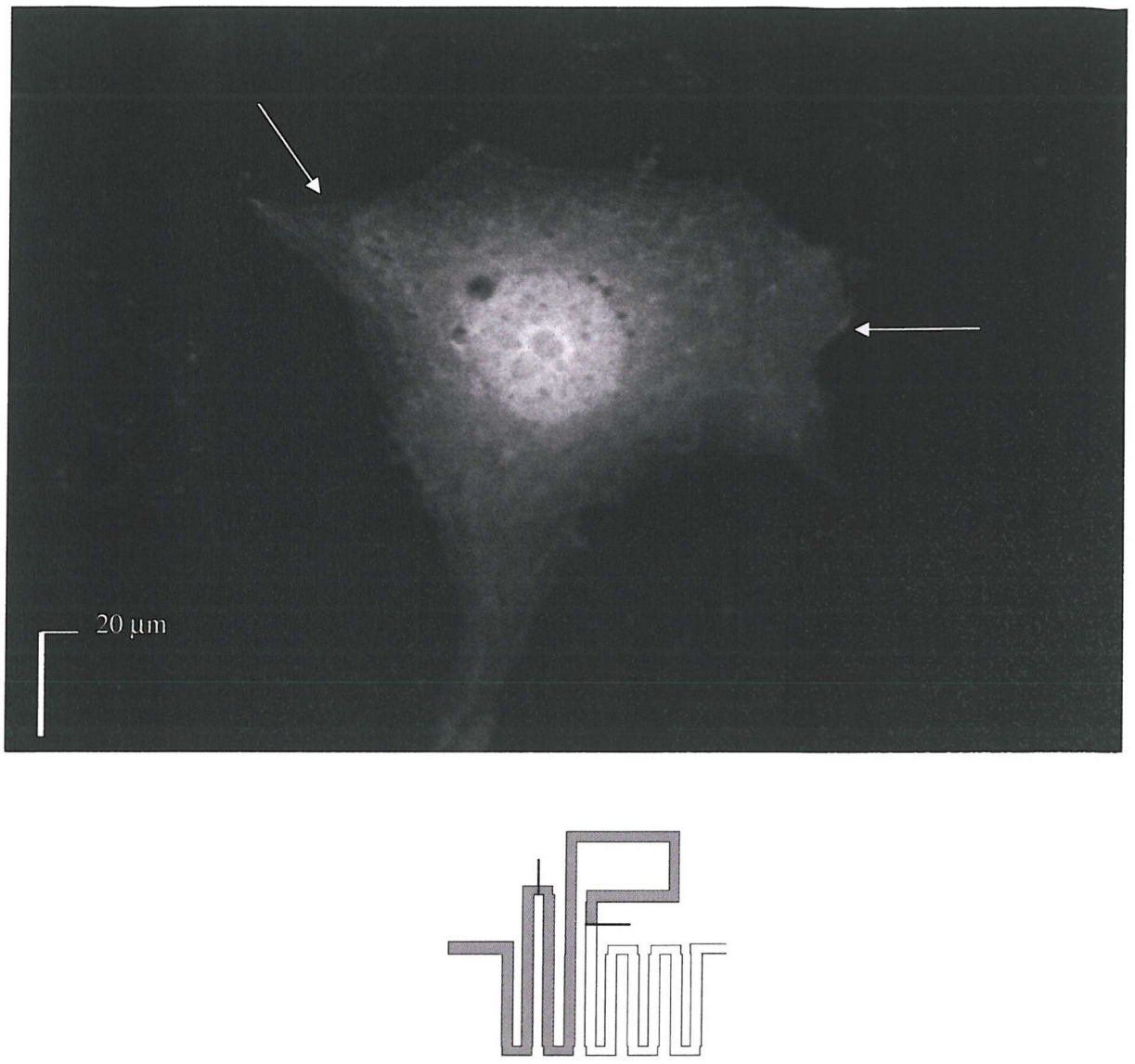


Figure 3.18: eGFP fluorescence in a single COS-7 cell transfected with Chimera 2-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera: black indicates PMCA sequence, white is SERCA sequence.

particularly clear. Chimera 2 consists of the first two sections of PMCA joined to the last section of SERCA.

3.5.3 Chimera 3-eGFP expressed in COS-7 cells

Figure 3.19 shows two COS-7 cells typical of those expressing Chimera 3-eGFP, both of similar size and showing a similar localisation pattern. In the top cell there is strong perinuclear fluorescence at the bottom of the cell, with a high concentration of fluorescent material aggregated at the top of the nucleus (in the middle of the cell). Surrounding the nucleus of the top cell is a clear reticular network, which is vesicularised to the left of the bright fluorescence. The rest of the network is highly interconnected and can be easily seen to the right of the cell. The bottom cell has a very round nucleus at the top of it. A patch of bright fluorescence can be seen at the bottom of the nucleus and a reticular structure takes up the rest of the cell. This reticulation can be clearly noted at the bottom right of the cell and to the right of the nucleus. Although this network appears to occupy most of the surface area of the cell, no edges can be distinguished. Chimera 3 consists of the central domain of PMCA3 linked to the N- and C-terminus of SERCA1b

3.5.4 Chimera 4-eGFP expressed in COS-7 cells

Chimera 4 consists of the first third of PMCA3 and the last two thirds of SERCA1b. Figure 3.20 shows the eGFP fluorescence of two typical cells expressing Chimera 4-eGFP. Both cells show a similar fluorescent pattern, with a very diffuse fluorescence around a central nucleus. There is a blurred central fluorescence present in both cells (brightest in the bottom cell) but no reticular network can be defined. Several vesicles

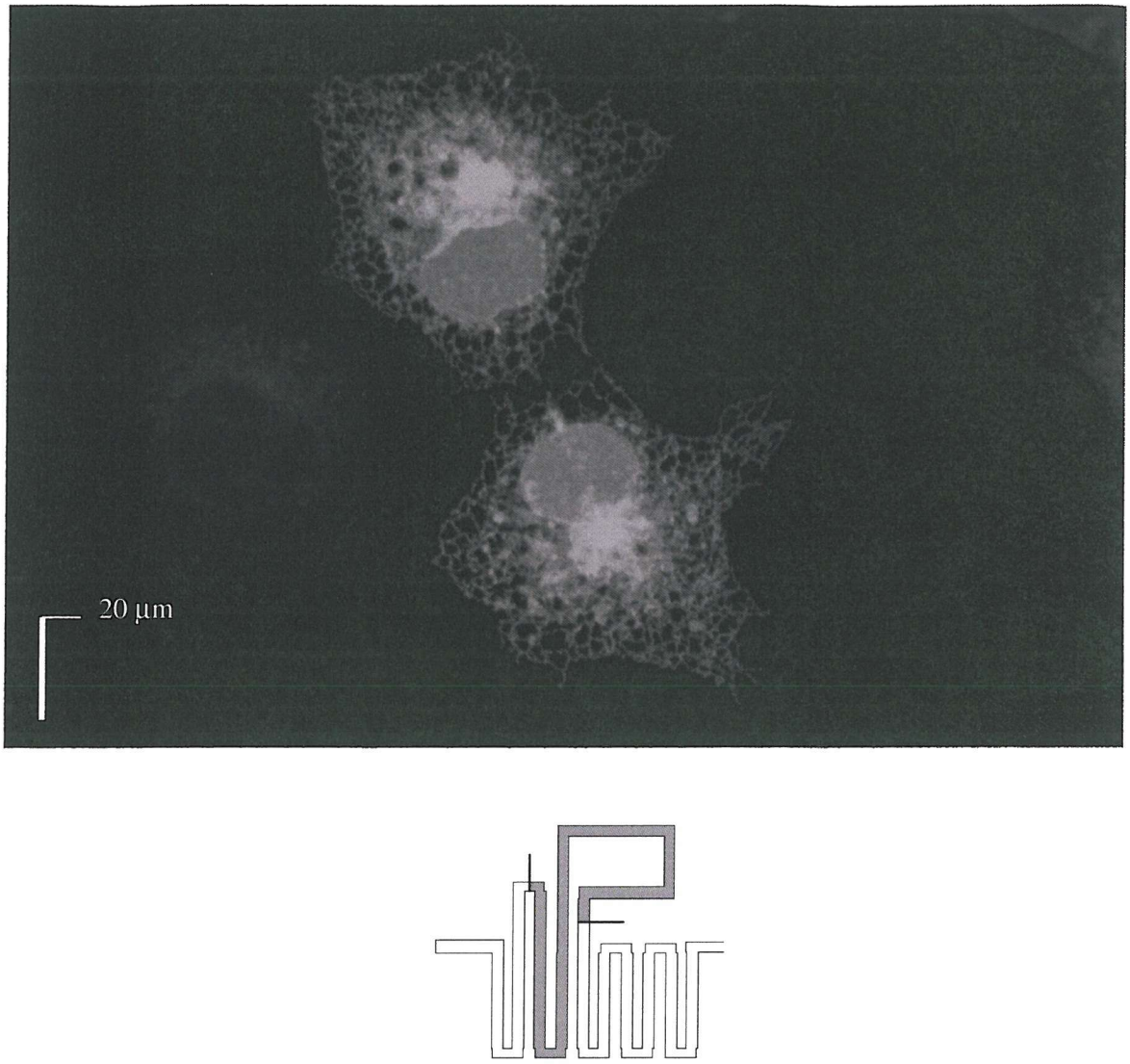


Figure 3.19: eGFP fluorescence in two COS-7 cells transfected with Chimera 3-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera: black indicates PMCA sequence, white is SERCA sequence.

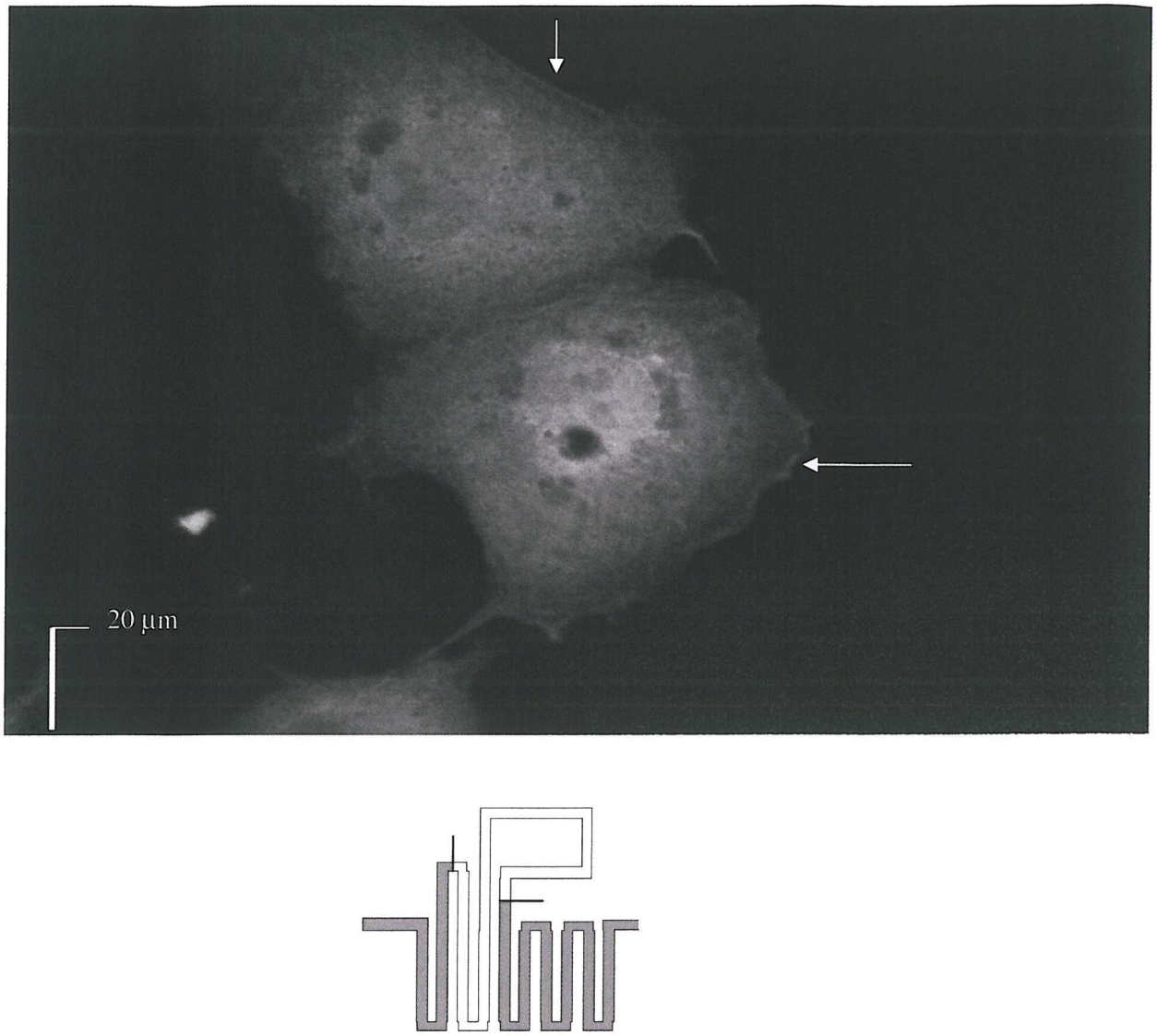


Figure 3.20: eGFP fluorescence in two COS-7 cells transfected with Chimera 4-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera: black indicates PMCA sequence, white is SERCA sequence.

are present around the nuclei and a distinct edge is clearly visible. Although both cells have well defined plasma membrane fluorescence (shown by the white arrows), this is more evident in the bottom cell's left-hand and bottom edge.

3.5.5 Chimera 5-eGFP expressed in COS-7 cells

Figure 3.21 shows two typical cells expressing Chimera 5-eGFP, which consists of the first third of SERCA1b and the last two thirds of PMCA3. Both cells have a relatively high concentration of fluorescent material surrounding the nucleus and the accumulation to the side of it. Despite this bright fluorescence the nucleus can be seen clearly in both cells. The cell on the right has a nucleus to the right-hand side of it and a very bright fluorescence to the left of this nucleus. The cell on the left has a nucleus to the bottom side of the cell with a large build up of fluorescent material at the top of the nucleus. This displays perinuclear fluorescence typical of ER localisation, although it does not show as distinct a reticular network as seen in SERCA-eGFP.

3.5.6 Chimera 6-eGFP expressed in COS-7 cells

Figure 3.22 shows the eGFP fluorescence of a typical cell expressing Chimera 6-eGFP, which is made up of the first section of PMCA linked to the last two sections of SERCA. There is a strong but diffuse fluorescence throughout the whole of this cell, with a very bright fluorescent area in the centre of the cell. A less bright fluorescence can be seen surrounding this bright spot, but because of the diffuse fluorescent pattern, no reticular structure can be identified. There is a clearly defined edge to the cell but the plasma membrane is not as clearly separated from the diffuse fluorescence

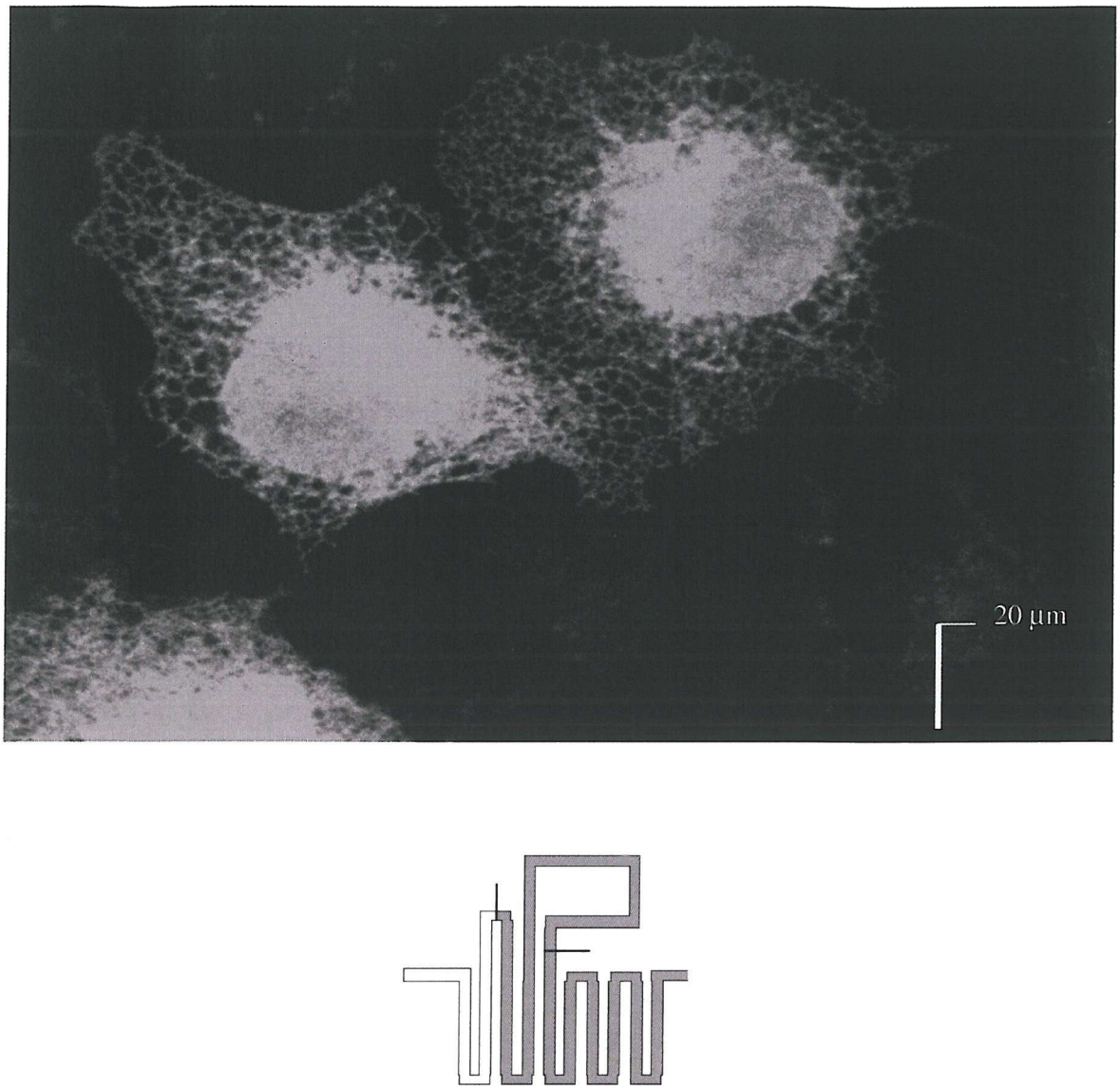


Figure 3.21: eGFP fluorescence in two COS-7 cells transfected with Chimera 5-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera: black indicates PMCA sequence, white is SERCA sequence.

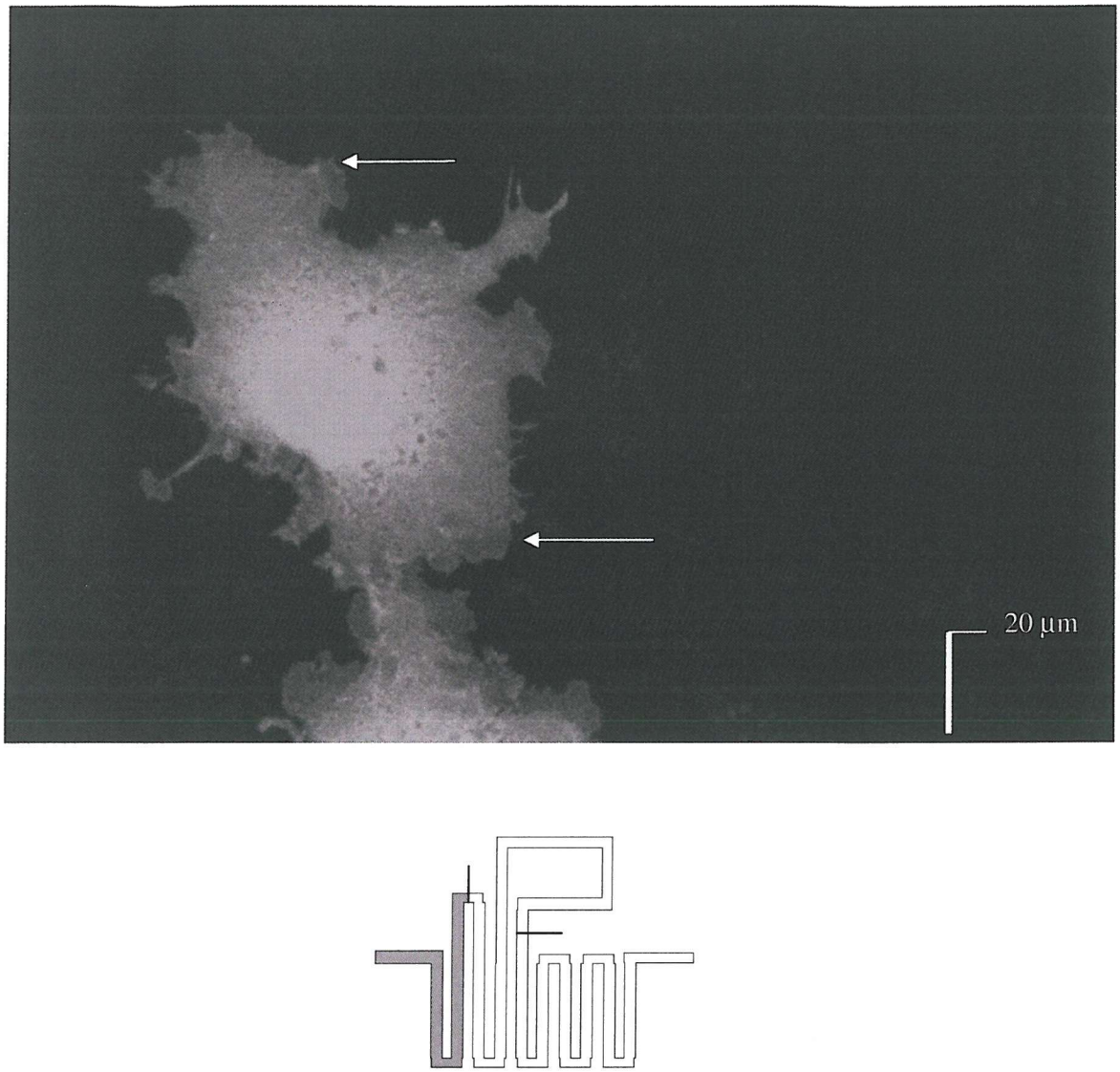


Figure 3.22: eGFP fluorescence in a single COS-7 cell transfected with Chimera 6-eGFP

Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope. The schematic diagram under the picture shows the layout of the transmembrane domains of the chimera: black indicates PMCA sequence, white is SERCA sequence.

as in PMCA-eGFP (figure 3.16) for example. There are parts of the cell surface however that display clear plasma membrane localisation of fluorescent material (white arrows in the diagram).

3.5.1 Transfection efficiency

In COS-7 cells seeded at 50-80% confluency, expression of the constructs was seen in 10-20% of the cells viewed.

3.5.2 Calcium Uptake Assay

Calcium uptake was used as a method of ascertaining whether the GFP constructs were folded properly and functional within the ER. Figure 3.23 shows the uptake of calcium over time for purified sarcoplasmic reticulum, and figure shows the results, using the same method for microsomes produced from COS-7 cells transfected with SERCA1b and SERCA-eGFP. No other results for PMCA or any of the eGFP-tagged chimeras could be obtained.

The calcium uptake curve for purified SR shows a high rate of calcium uptake between 0 and 5 minutes of the assay. This initial rate is approximately 4,270 nmoles calcium/mg protein/ minute. After 5 minutes the rate slows down and the amount of calcium taken up eventually levels off, reaching about 19,000 nmoles calcium per mg protein.

The results from the microsomes are considerably lower in magnitude than the SR. The curves shown in figure 3.24 do not plateau off at all and there is a discernible lag observed before reaching the linear rate (most obvious for the set of data for SERCA-eGFP). The linear rates for SERCA1b and SERCA-eGFP are 26.6 and 18.8 nmoles calcium/ mg protein/ minute respectively.

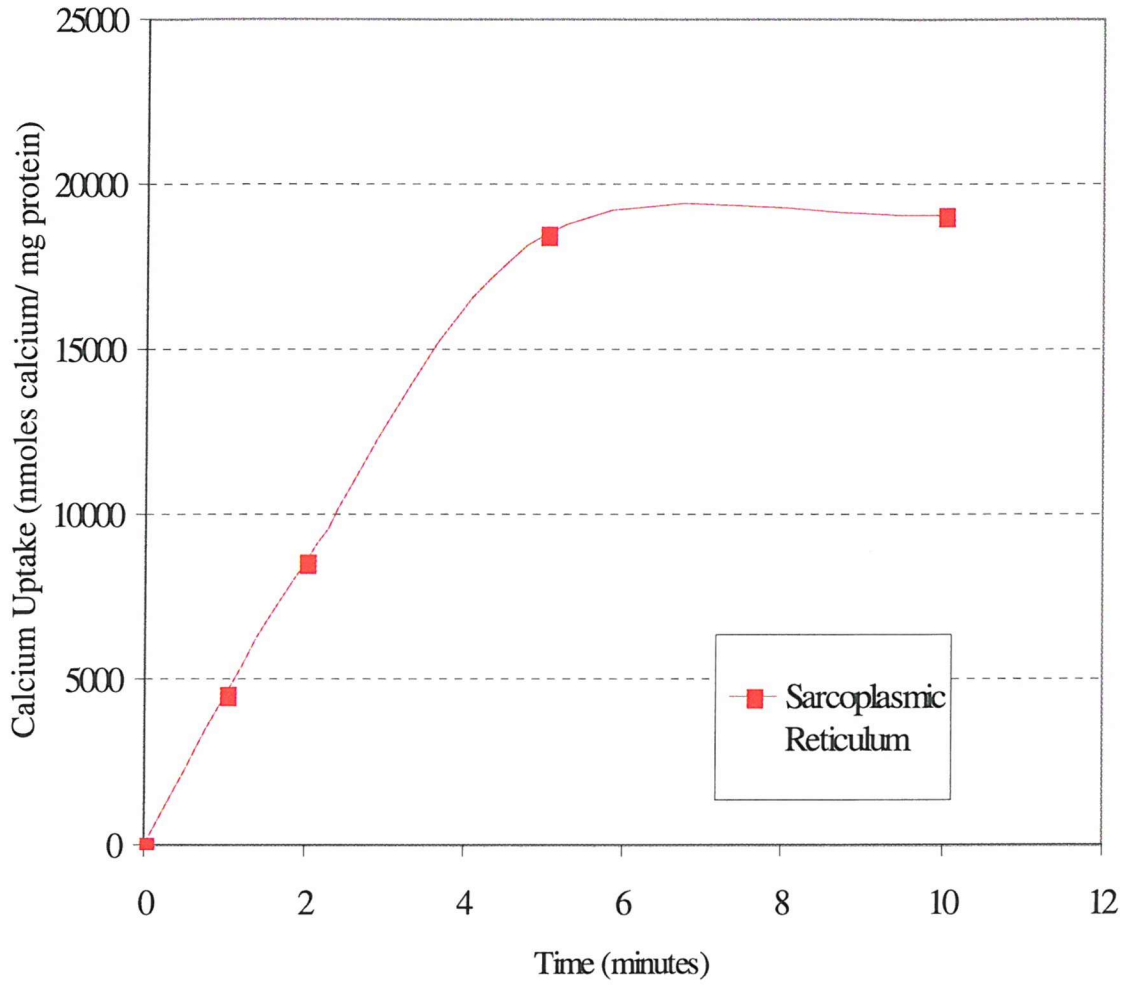


Figure 3.23 Graph to show the uptake of calcium into SR vesicles over time (see section 3.5.9).

Time is shown in minutes on the x-axis, and calcium uptake measured in nmoles/ mg of protein is shown on the y-axis.

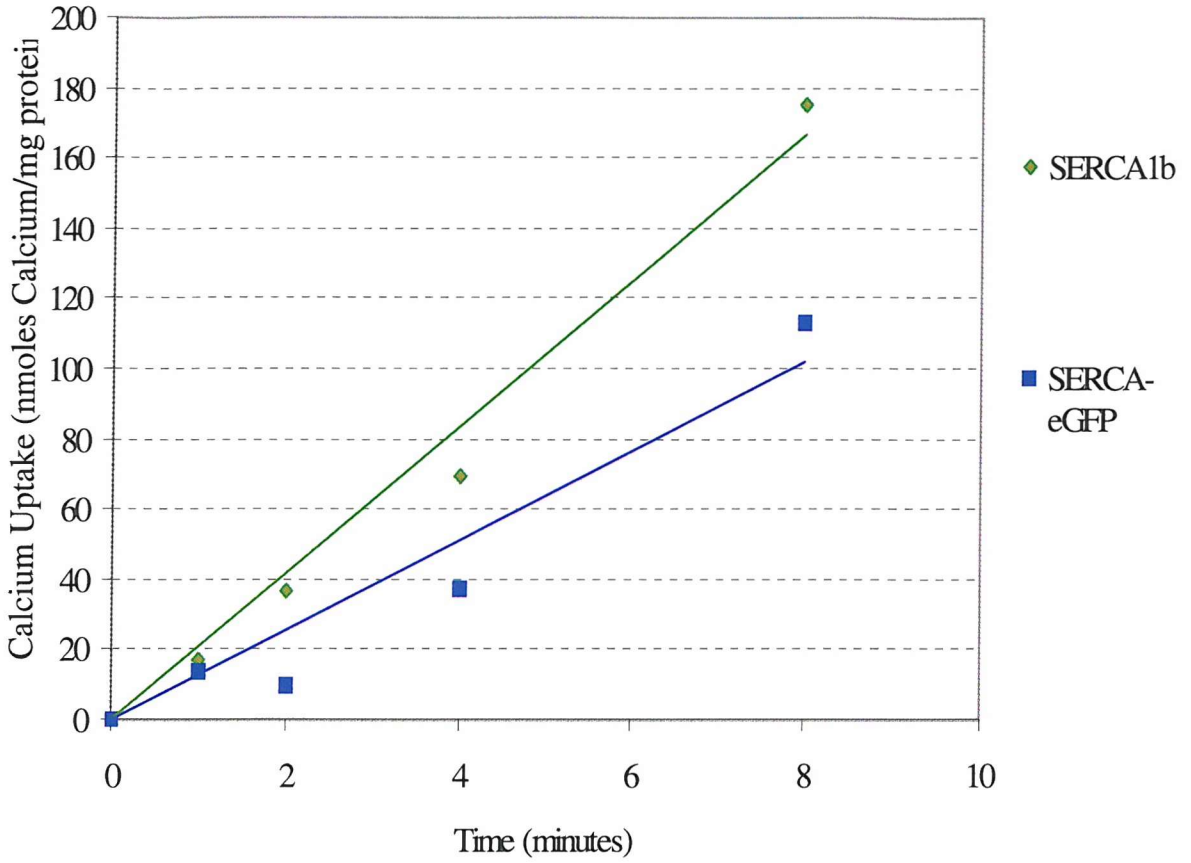


Figure 3.24: Graph to show the uptake of calcium into microsomes over time (see section 3.5.9).

Time is shown in minutes on the x-axis, and calcium uptake measured in nmoles/ mg of protein is shown on the y-axsis. Diamonds indicate data points for microsomes produced from cells transfected with SERCA1b and squares denote data from microsomes from cells transfected with SERCA-eGFP.

3.5.7 Western Blot Analysis

Figure 3.25 shows the results of a western blot on an SDS-PAGE gel on which COS-7 cells transfected with SERCA-eGFP, PMCA-eGFP and all of the eGFP tagged chimeras were analysed using an anti-GFP anti-body. It is clear that there is a wide variance in the level of protein expressed for each construct.

The lane containing cells transfected with SERCA-eGFP has by far the highest concentration of material that reacts with the anti-GFP antibody. The most abundant protein can be seen just above the 148 kDa marker protein. Other protein bands can be seen at about 98 kDa and between the 98 kDa and the 148 kDa marker. A number of weaker bands can be seen at other molecular weights, notably at just below 148 kDa and at the bottom of the gel (below 36 kDa). The other lanes contain bands at equivalent weights to some or all of these examples. PMCA-eGFP provides a much weaker signal than SERCA-eGFP, with its strongest band at approximately 150 kDa. The lane showing cells transfected with PMCA-eGFP also has lesser bands at just below the 148 kDa marker and about 50 kDa.

Of the chimeras shown, only cells transfected with chimera 3 did not have any detectable bands at the higher molecular weights (although fainter bands can be seen at approximately 50 kDa and below 36 kDa). Chimeras 1,2,4,5 and 6 all have bands at around 150 kDa and just below the 148 kDa marker. Chimera 5 has the strongest bands at these molecular weights but also contains a strong signal just above the 98 kDa molecular weight marker. Chimera 6 has a strong band just above the 148 kDa marker but a feinter band just below 148 kDa than the other chimeras and chimera 4 has a relatively strong band at 150 kDa compared to the overall anti-eGFP signal. Chimeras 1, 3 and 6 have strong bands at the bottom of the gel, and chimera 6 has a series of bands between 50 kDa and 36 kDa.

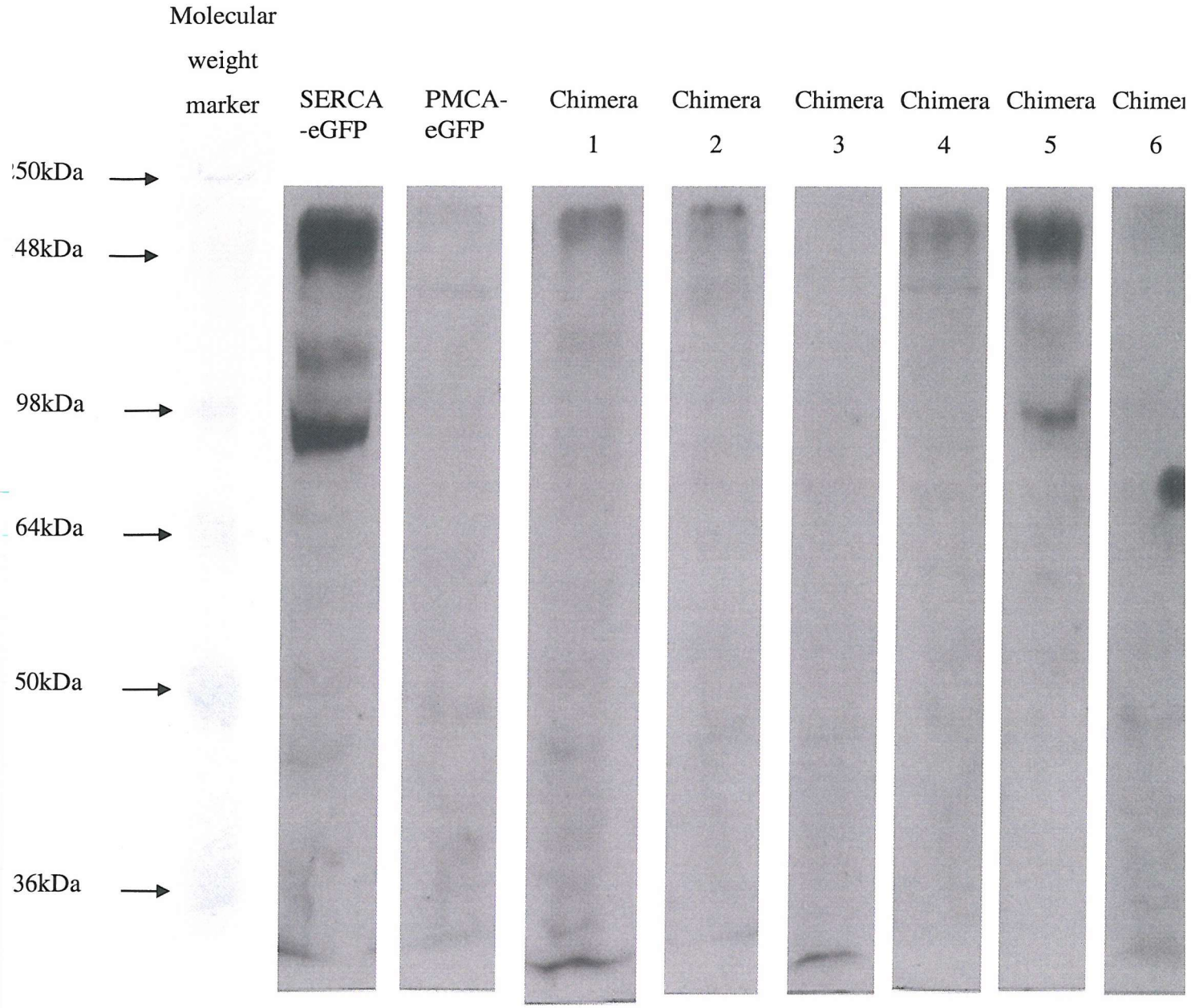


Figure 3.25: Western Blot Analysis of COS-7 cells transfected with SERCA-eGFP, PMCA-eGFP and all of the GFP tagged chimeras.

Molecular weight markers transferred onto the nitrocellulose are shown on the left, all of the lanes are labelled with the construct used to transfect the COS-7 cells.

3.5.3 Using SERCA-BFP for double labelling

Double labelling would have been a useful technique to establish whether SERCA-eGFP and PMCA3-eGFP were being targeted to separate locations within the cell. A construct consisting of SERCA1b fusion-tagged to blue fluorescent protein (BFP) was created. Both PMCA-eGFP and SERCA-BFP could then be viewed in the same cell and a clear distinction of their locations would have been possible. Further experiments involving FRET could have also been possible.

The SERCA-BFP construct could be viewed using an epifluorescent microscope (see figure 3.26) using a DAPI filter set. Unfortunately, BFP has a low intensity of fluorescence, which bleaches very rapidly and this would not be practical for double labelling or FRET experiments.

3.5.4 Using truncated sequences to identify SERCAs targeting signal

Further narrowing down of the sequence required for ER targeting could involve constructs that were made up of the first two transmembrane helices and the N-terminal region of the calcium pumps. Chimeric proteins could then be made using the same approach that was used with the set of chimeras used in this chapter. This would have the advantage of being able to focus on the targeting sequence and not have to worry about the rest of the protein folding properly, but could have a disadvantage for a similar reason (for example, the constructs might not properly insert into the membrane).

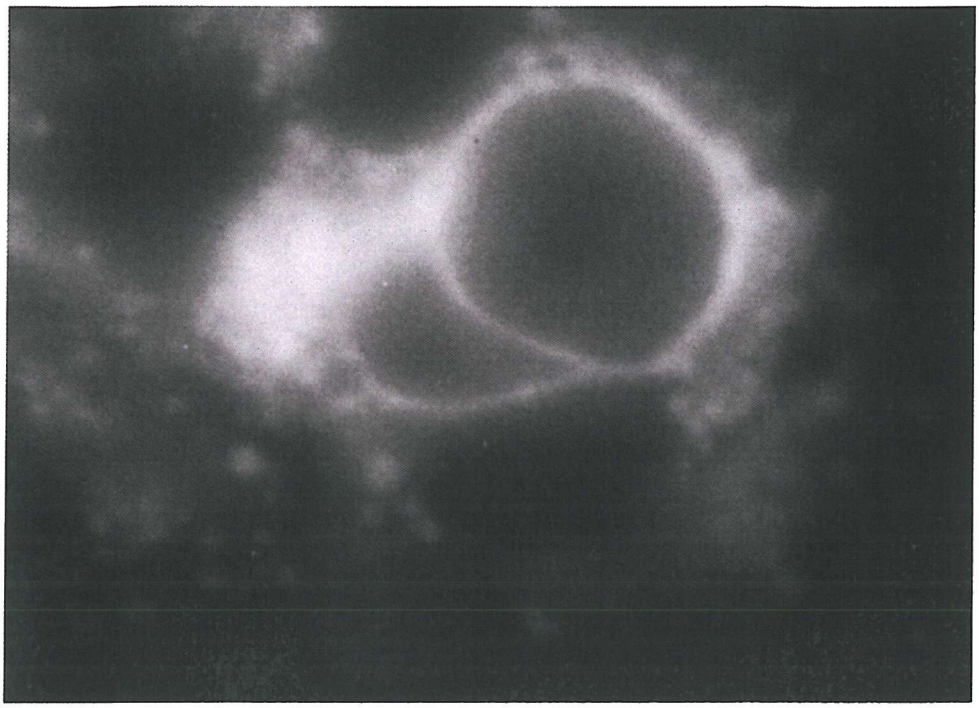


Figure 3.26: SERCA-BFP expressed in a single COS-7 cell

SERCA-BFP was made by cloning a SERCA sequence joined to a *myc* linker into pcDNA3.1 using the EcoRI and EcoRV cloning sites and then introducing a PCR fragment encoding BFP at the C-terminal end.Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides in PBS and viewed using a Leica epifluorescent microscope.

To test the practicality of this approach, a construct consisting of the first 212 residues of SERCA1b and tagged with enhanced green fluorescent protein (ntSERCA-eGFP) was expressed in COS-7 cells. Figure 3.27 shows the eGFP fluorescence of cells expressing ntSERCA-eGFP. This construct displays an extremely widespread fluorescence throughout the whole of the cytoplasm.

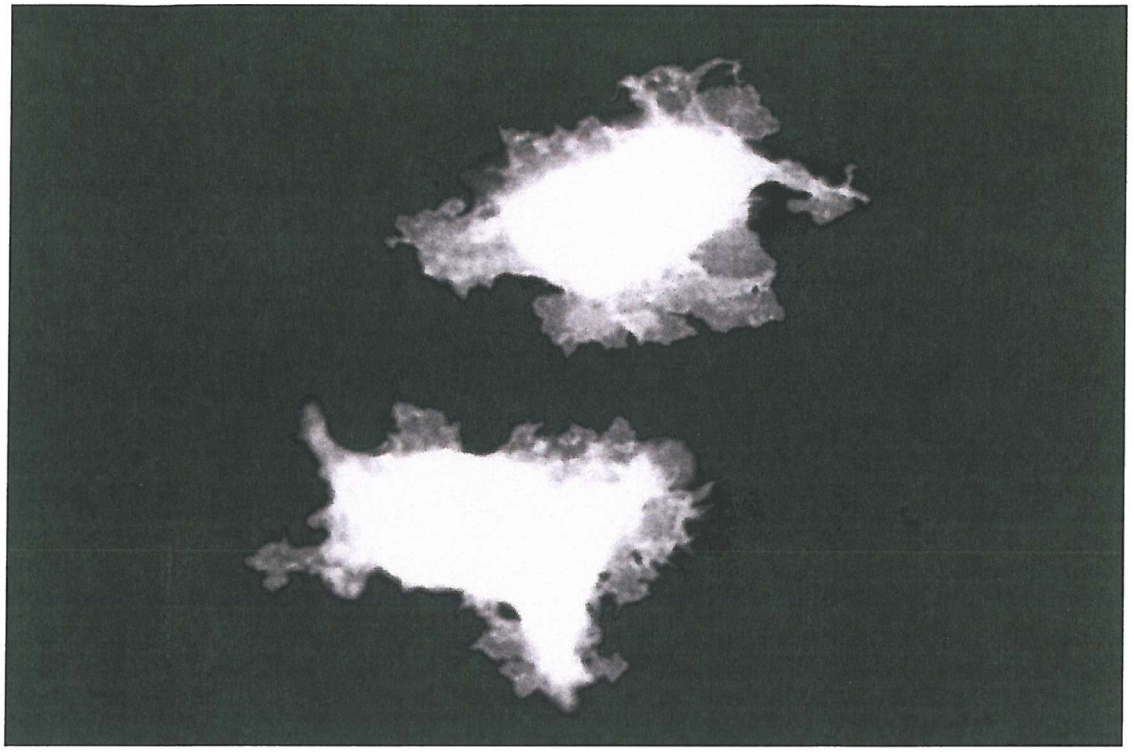


Figure 3.27: ntSERCA-eGFP expressed in COS-7 cells

Shows the eGFP fluorescence of two cells expressing ntSERCA-eGFP. The N-terminal region of SERCA was linked to eGFP by digesting chimera 1-eGFP with Kpn-I and Age-I and replacing the excised fragment with a short linker sequence made by annealing two oliginucleotides. Cells were seeded onto 13 mm coverslips at a confluency of 30-60%. They were then transfected using Fugene-6 reagent. The cells were then left for 3 days and the coverslips were mounted onto microscope slides using Mowiol mountant +0.1% citifluor and viewed using a Biorad MRC-600 confocal microscope.

3.6 Discussion

The work in this chapter was concerned with the identification of the signal sequence within SERCA that targets the protein to the Endoplasmic Reticulum. A set of chimeric proteins consisting of SERCA1b and PMCA3 sequences was tagged with enhanced Green Fluorescent Protein, and their location within COS-7 cells was observed. The creation of eGFP fusion tagged chimeras has been shown to be an effective method of identifying targeting signals (Levine and Monroe 2002) and in this chapter the eGFP-tagged chimeras provide a clear identification of the segment of SERCA required for ER targeting.

Foletti *et al.* (1995) and Guerini *et al.* (1998) have undertaken previous studies into the SERCA ER targeting sequence. Folletti *et al.* made a set of SERCA/PMCA chimeras and identified the first 85 residues as the region important for ER targeting. Although these chimeras were shown to insert into membranes correctly, only one was found to be able to form the phosphoenzyme intermediate, and no calcium uptake was observed. Proteins that are not active are possibly not folded properly, and mis-folded or defective proteins have been shown to be retained in the ER (Beggah *et al.* 1996, Bergeron *et al.*1994). Further elucidation of the targeting signal was carried out (Guerini *et al.* 1998), suggesting that the targeting information was limited to the cytoplasmic N-terminal region, but again no activity data was obtained.

The set of chimeras created by Black (1998) were designed to maintain activity, by joining the chimera sections at highly conserved regions of sequence. This approach was made successfully by Luckie *et al.* 1991, the SERCA/PMCA chimeras made this way were able to transport calcium and to form the phosphoenzyme intermediate. Due to the unpredictability of the antibodies however (four different anti-bodies were used), and the damaging fixation techniques - a definitive set of chimeras was difficult to obtain. By GFP tagging SERCA1b, PMCA, and John Black's SERCA/PMCA chimeras two distinct localisation patterns can be identified.

ER localisation is typified by the SERCA-eGFP construct (see figure 3.10). This strong perinuclear localisation and peripheral reticular pattern is comparable with other ER/SR targeted proteins (Treves *et al.* 2000). This localisation is also similar to SERCA expression observed using theY1F4 monoclonal antibody (Tunwell *et al.* 1991), or by using a fluorescent ER-specific dye (Bhat and Ma 2002).

Plasma membrane localisation of PMCA-eGFP is not completely typical of most PM localised proteins. Ryadine-receptor subunits tagged with GFP have been shown as a clean 'halo' around the cell's surface, with no diffuse fluorescence within the cell (Bhat and Ma 2002). The use of a more directed confocal focusing plane could be one reason for this difference in fluorescence pattern Andersson *et al.* 1999 were able to show both internal membranes and external fluorescence However, even Bhat and Ma show a slightly more diffuse pattern for a Ryanodine-receptor and ICAM chimera and PMCA overexpressed in COS-1 cells (Heim *et al.* 1992) is very similar to that obtained in this chapter (see figure 3.11).

The lack of activity data for the chimeras and PMCA-eGFP was disappointing. Only purified sarcoplasmic reticulum and microsomes transfected with SERCA1b and SERCA-eGFP provided any calcium uptake data, comparable to Maruyama and MacLennan (1988). There could be many reasons for this. The GFP fusion tagging of proteins, for example has been the cause of lack of function (Greenfield and High 1999), but this is unlikely to be the main cause because the SERCA-eGFP construct has a calcium transport rate close to (if slightly lower than) untagged SERCA1b.

Another possibility is that adding the eGFP tag to the large C-terminal loop of PMCA3 could cause a reduction in activity. Calmodulin normally interacts with this region of the protein to prevent it from binding to another part of the protein and auto-inhibiting (Grover and Khan 1992). The additional GFP sequence could interfere with this interaction and this would have a negative effect on the activity of the protein. This hypothesis however does not seem to explain the lack of activity of the constructs which have SERCA sequence at their C-termini.

The quality of the microsomes might have contributed to the unpredictability of the results – 'leaky' microsomes would not accumulate calcium as efficiently. If this

were the case then it might have been advantageous to carry out experiments to detect calcium dependent phosphoenzyme formation.

The Western blotting results provide the most likely explanation for the lack of activity (see figure 3.20), as it is clear that the expression of the constructs is highly variable. The lane containing cells transfected with SERCA-eGFP has by far the most material that is positive for GFP, and the next highest concentration is that of chimera 5 which is still considerably less. All of the others have a very feint band at about 150 kDa (the approximate size of a calcium pump linked to enhanced Green Fluorescent Protein), and chimera 3 does not have any detectable band at this molecular weight. If this were the reason why no meaningful activity data could be obtained, then trying to get results for phosphoenzyme formation would encounter the same problem.

Because of the way that the chimeras were designed, the lack of activity (and therefore the possibility of mis-folded proteins) is not as much of a problem as it could be. Black (1998) obtained activity data for the un-tagged chimeras, and the chimeras were made in pairs.

What is essentially being looked for when comparing the localisation of chimeras is a loss of targeting function. If one chimera in a pair is seen in the ER then the other will have lost that targeting information and will have escaped to the later protein export pathway and onto the plasma membrane. Although there is no guarantee that the ER targeting of one chimera is genuine, the plasma membrane localised member of the pair **must** have lost a targeting signal. Table 3.3 summarises the localisation of all of the eGFP-tagged chimeras.

Chimera 1 (consisting of the first two segments of SERCA1b, and the last segment of PMCA3) is paired with chimera 2 (first two segments of PMCA3 and the C-terminal part of SERCA1b). Chimera 1 shows a similar localisation pattern to SERCA, and Chimera 2 is targeted to the plasma membrane, like PMCA. This means that the SERCA targeting signal has been lost from Chimera 2 and is therefore is not located

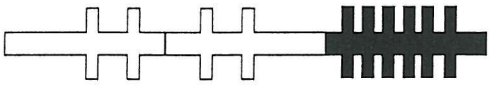
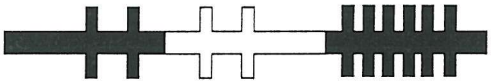
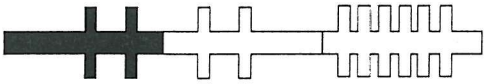
Chimera name	Chimera structure	Localisation
Chimera 1		Endoplasmic Reticulum
Chimera 2		Plasma Membrane
Chimera 3		Endoplasmic Reticulum
Chimera 4		Plasma Membrane
Chimera 5		Endoplasmic Reticulum
Chimera 6		Plasma Membrane

Table 3.3

Summarises the localisation of the eGFP-tagged chimeras. A schematic diagram is presented for each chimera to demonstrate its sequence and topology. Black indicates PMCA3 sequence and white signifies SERCA1b sequence. Vertical bars are present to show the approximate position of the ten transmembrane helices.

in the last segment of SERCA. This part of SERCA consists of the last five transmembrane helices (residues 712-994).

The localisation of the next two chimeras (3 and 4) shows that the targeting signal is not located in the middle section which consists of transmembrane helices 3 and 4 and the large cytoplasmic head. Chimera 4, which consists of PMCA sequence at the C-terminus and the N-terminus but SERCA sequence in the middle section, is targeted to the plasma membrane. Chimera three is located in the ER, and the only part of the SERCA sequence that is missing is the middle segment.

The first two pairs of chimeras showed that the ER targeting signal is not in the last two segments of the SERCA1b sequence. It could have been predicted therefore that the targeting information is contained in the first region. If a chimera which contained only the first segment of SERCA1b and the rest of PMCA3 it would still be located in the ER, and its opposite chimera would have lost that important targeting information. This is indeed what is seen with chimeras 5 and 6

The construct comprised of the N-terminal 212 residues of SERCA tagged to eGFP seems to be expressed in the cytosol (figure 3.24). This is what you would expect if the protein was not properly targeted to or inserted into the ER membrane. If this is the case, then this technique is not a viable method of identifying the targeting signal.

Another possibility is that there is a smaller construct produced that does not include an ER targeting signal. The start codon for eGFP could be responsible for the production of such a transcript, and eGFP could be produced on its own , as well as the tagged protein. This would not be a problem with any of the larger GFP-tagged constructs, as the ribosome binding site would be too far away from the start of eGFP sequence, but it might be close enough in such a truncated construct to cause cytoplasmic fluorescence. Simply mutating the AUG into another codon would eliminate the production of eGFP and allow the true targeting of the construct to be observed.

Double labelling could still be a possible technique as well, despite the impracticalities of using BFP as a fluorescent tag (see section 3.5.5). A new red fluorescent protein is now commercially available (DsRed), with better spectral qualities and more convenient excitation/emission values (for commonly used fluorescence filter sets). A PMCA or SERCA construct tagged with DsRed could be used in a double labelling experiment with GFP, as described in Zamyatnin *et al.* (2002).

To conclude: these results demonstrate that there is a strong ER targeting signal located in the first 212 residues of the SERCA1b sequence. These residues correspond to the cytoplasmic region and the first two transmembrane helices. Whether there is another, weaker signal elsewhere in the sequence, or the nature of the identified targeting sequence is not determined by these results.

The natural progression of this work would be two fold: to further narrow down the targeting signal responsible for SERCA localisation and to identify what kind of targeting signal(s) are contained within the sequence.

Chapter Four:

The mechanism of targeting SERCA to the ER

4.1 The method of ER targeting

Another aspect of SERCA targeting that could be investigated is the method by which such a large transmembrane protein is targeted to the Endoplasmic Reticulum. Although there seems to be an abundance of data on how luminal proteins and simple single spanning membrane proteins are targeted, there is little information concerning the sorting of large multi-spanning membrane proteins such as SERCA.

As described in chapter one (section 1.6), there are two potential methods by which proteins can be maintained in the ER. They can be prevented from entering anterograde vesicles (retention). Alternately, they can be recycled from a post ER compartment (retrieval). It could be argued that because SERCA is vital for one of the ER's major functions i.e. as a calcium store, the most obvious method of targeting would be retention. Indeed, SERCA is known to form dimers which are stable enough to be apparent on SDS-PAGE gels, and oligomerisation is thought to be a mechanism for preventing proteins from leaving a compartment (Gleeson 1998). Some recent work on Sec61 (a core component of the translocon), however has raised the possibility that retrieval may play a role.

Sec61 is also a large multi-spanning membrane protein, with an important role in ER function, which was widely believed to be targeted purely by retention. This was shown not to be the case by Greenfield and High (1999). They showed that a Sec61α-eGFP fusion protein was localised not only in the ER but also in a post ER compartment. Because of the apparent similarities, it would seem prudent to try and find whether the same was true of SERCA.

4.2 ER to Golgi transport: the intermediate compartment

Studies into ER to Golgi transport showed that rather than there being direct transport between the ER and the Golgi apparatus, there was a distinct post-ER compartment that displayed properties different to the ER and the Golgi. Known as the intermediate compartment, or the ER/Golgi Intermediate Compartment (ERGIC), or as Vesicular Tubular Clusters (VTC's), this structure was identified in cells infected with Semlinki Forest Virus (SFV) when the temperature was lowered to 15°C (Saraste and Svensson, 1991).

At 15°C, any transport to the Golgi is halted and the SFV proteins were observed to aggregate in vesicle-like structures in the peripheral cytoplasm. Upon returning the infected cells to 37°C, the SFV proteins continued to the Golgi, indicating that this 15°C compartment was separate from the cis-Golgi. This compartment was characterised further by Schweizer *et al.* (1991), and was recognised as a specific location, not just a specialised section of the ER. It is highly concentrated in COP-I coatomer and Erd2 (the KDEL receptor), which constantly recycle between the ER and the Golgi, emphasising the recycling and sorting nature of the ERGIC.

4.2.1 ERGIC-53

Another protein that is concentrated in the ERGIC is known as ERGIC-53 (also known as a rat homologue p58), and this is commonly used as a marker for the intermediate compartment. Structurally related to the plant lectins, ERGIC-53 contains an ER retrieval motif, which allows it to cycle between the ERGIC and the ER. It has been found to be important in the targeting of specific glycoproteins such as coagulation factors V and VIII (Nichols *et al.* 1998) and has been cross linked with

another glycoprotein; catZr (Appenzeller *et al.* 1999). It is thought act as a receptor by binding to their carbohydrate modifications in the ER and transporting them to the ERGIC to proceed further in the protein export pathway.

4.2.2 Sec61 targeting

Greenfield and High (1999) showed that Sec61 was present in the ERGIC despite being an ER resident protein, and therefore contained a retrieval signal. They established this by creating a Sec61α-eGFP fusion protein and expressing it in COS1 cells. Despite the fact the tagged construct did not retain its functionality, it was found to be co-located with Sec61β and γ (components of the Sec61 complex). Confocal microscopy studies were carried out using Sec61α-eGFP and antibodies directed against the ERGIC and the trans-Golgi. Sec61α-eGFP co-localises with ERGIC-53 but not with a marker for the trans-Golgi marker TGN-46, indicating that it leaves the ER but does not progress any further in the protein export pathway and is recycled back to the ER.

These results were consolidated by using the additional method of sub-cellular fractionation. By separating sub-cellular material on a Nycodenz gradient it was possible to separate the Endoplasmic Reticulum from the ERGIC/Cis-Golgi and fractions from these gradients were then analysed by Western blotting. The Sec61α-eGFP protein was found to be not only in the ER fraction (characterised by the ER resident protein calnexin) but also in a lighter compartment which was positive for Erd2 (the KDEL receptor).

4.3 Materials and methods

4.3.1 Antibodies

The antibody for ERGIC-53 was a gift from H.P. Hauri. The anti-TGN-46 was from Serotech. Antibodies for the KDEL receptor, green fluorescent protein, and calnexin were obtained from Stressgen.

4.3.2 Co-localisation with ER/Golgi Intermediate Compartment

COS-7 cells were seeded onto coverslips to a confluency of 50-80% and transfected with SERCA-eGFP as described in section 3.3.2. 48-hours after transfection, the COS-7 cells adhering to the coverslips were then washed once with PBS and fixed with -20°C methanol for 5 minutes and washed twice with PBS. The fixed cells were then blocked in 0.1% BSA in PBS for 15 minutes, and treated with the primary antibody, in 0.01% BSA/PBS (mouse anti-ERGIC-53) for one hour. They were then washed three times with PBS and incubated with the secondary antibody in 0.01% BSA/PBS (anti-mouse IgG) conjugated to Texas red for one hour. The coverslips were washed three times with PBS and then mounted onto microscope slides with Mowiol mountant (0.1% citifluor), and viewed using a Biorad MRC-600 confocal microscope. Selected coverslips were also placed in a 15°C incubator for one hour before fixation.

4.3.3 Co-localisation with the trans-Golgi Network

COS-7 cells were seeded onto coverslips and transfected as above. Before fixing, the cells were treated with Brefeldin A (5 μg/ml) and left for 1 hour. After fixation and blocking the coverslips were incubated with sheep anti-TGN-46 as a primary antibody (one hour), washed three times with PBS and then incubated with donkey anti-sheep IgG secondary antibody, conjugated to rhodamine (one hour). The coverslips were washed three times with PBS and then mounted and viewed as described above.

4.3.4 Preparation of Nycodenz Gradients

A solution of 27% Nycodenz was made in state 10 mM Tris (pH 7.4), 3 mM KCL, 1 mM EDTA, and from this stock solution 24%, 19.33% 14.66% and 10% Nycodenz solutions were prepared using dilution buffer (0.75% NaCl, 10 mM Tris (pH 7.4), 3 mM KCl, 1 mM EDTA). These solutions were then layered in a 13.5 ml centrifuge tube to form a discontinuous gradient. To create a continuous Nycodenz gradient this gradient was left upright overnight.

4.3.5 Sub-cellular fractionation

COS-7 cells were seeded onto 14 mm cell culture dishes and transfected with SERCA-eGFP. After 48 hours the cells were washed twice with homogenisation buffer (250 mM Sucrose, 10 mM triethanalamine, 10 mM acetic acid, 1 mM EDTA, 1 mM DTT), and harvested in 500 μl of homogenisation buffer. The cells were homogenised by passing them five times through 19 Gauge, 21 Gauge, and 25 Gauge needles consecutively, and span at 1500g for 5 minutes. The supernatants were then

loaded onto pre-made Nycodenz gradients and centrifuged at 37,000 rpm in a Beckman L7 centrifuge for 1.5 hours using a Kontron TST41.14 swing out rotor. Fractions of approximately 1 ml were then collected by making a hole in the bottom of the centrifuge tube with a 19 Gauge needle. Protein in each of the fractions was precipitated by adding an equal amount of 1M perchloric acid or 20% trichloric acid (TCA), leaving on ice for one hour and then spinning at 10,000 g. After washing once with 1 ml H_2O, the fractions were analysed by using SDS-PAGE and Western blot analysis.

4.3.6 SDS-PAGE

Protein was suspended in 40 µl SDS loading buffer containing 8 M urea and solublised by being held in a sonicating water bath for 10 minutes, and heated to 90°C for 10 minutes. The samples were then loaded onto a 10-15% polyacrylamide gel, and electrophoresed at 60mA for approximately one hour.

4.3.7 Western Blotting

After SDS-PAGE, proteins were transferred to nitro-cellulose for western blot analysis. The nitro-cellulose blot was blocked for one hour in blocking solution (5% dry milk, in PBS-Tween (0.05% Tween-20), and washed once for 15 minutes and twice for 5 minutes with PBS-Tween. The blots were the incubated with primary antibody diluted in PBS-Tween for at least one hour, washed three times with PBS-Tween (1x 15 minutes, 2x 5 minutes), and incubated with secondary antibody (conjugated to HRP) for an hour. After a final set of washes (1x 15 minutes, 2x 5

minutes) with PBS, the blots were analysed using the ECL detection system (Amersham Pharmacia).

4.4 Results

4.4.1 SERCA-GFP co-localises with ERGIC-53

Figure 4.1 shows a selection of cells expressing SERCA-GFP, also showing immunolocalisation of ERGIC-53. Figures 4.1A and 4.1D each show eGFP fluorescence. A shows the eGFP fluorescence of two cells transfected with SERCA-eGFP, and these display ER localisation (similar to that observed in chapter three) with a strong perinuclear fluorescence and a reticular structure positioned on one side of the nucleus. Both cells in 4.1A display a concentrated signal close to the nucleus and the reticular structure displayed by SERCA-eGFP fluorescence is less extensive than in cells which are not fixed but just mounted in Mowiol mountant (see section 3.3.3), this probably relates to the methanol fixation protocol. Figure 4.1D shows two more cells transfected with SERCA-eGFP, which also display a similar fluorescence pattern, although both cells contain numerous vesicles which gives the ER a different shape.

Figures 4.1B and E show the immunofluorescence staining of ERGIC-53 with a Texas Red conjugated secondary antibody. This structure can either be very concentrated (as indicated by the arrow in figure 4.1B and the top arrow in 4.1E) or as a more diffuse and tubular structure (the bottom arrow in figure 4.1E). It is always located close to the nucleus. When the fluorescent signals for SERCA-eGFP and Texas Red are overlaid, a distinct yellow signal was observed (4.1C and F). This overlap of signal can be seen in the left hand cell in figure 4.1C and in both cells in figure 4.1F, indicating that SERCA-eGFP and ERGIC-53 are located in the same intracellular compartment. No such overlap can be seen in the right hand cell in figure 4.1C due to the low Texas Red fluorescence.

4.4.2 SERCA-GFP is not present in the trans-Golgi network

Figure 4.2 shows three cells expressing SERCA-GFP, which display typical immuno-fluorescent staining for the trans-Golgi network protein TGN-43. 4.2A and 4.2D show the eGFP fluorescence for the cells and display ER localisation. 4.2B and 4.2 E show the location of the trans-Golgi, and 4.2C and 4.2F show the two signals overlaid.

Figure 4.2A shows two transfected cells treated with Brefeldin A, with different intensities of fluorescence; the left-hand cell expressing higher levels of SERCA-eGFP than the more central one. The Brefeldin A treatment required to clearly distinguish the trans-Golgi from the ER causes the SERCA-eGFP fluorescence pattern to be slightly different from that seen in untreated cells (see ERGIC-53 data- figure 4.1). There is a distinct perinuclear pattern but the reticular region of the ER does not seem to extend as much into the cytoplasm, with most fluorescence observed close to the nucleus.

4.2B and 4.2E show TGN-43 localisation (with the use of rhodamine conjugated secondary antibody) within the transfected cells shown in figures 4.2A and 4.2D. The trans-Golgi network can be observed in most cells in the field, as a central structure surrounded by a more vesicular staining pattern. 4.2C and 4.2F show the two fluorescent labels (eGFP and rhodamine) superposed over each other, without any co-localisation. Both cells in 4.2C have two distinct structures, despite the variation in SERCA-eGFP expression, and figure 4.2F is particularly clear.

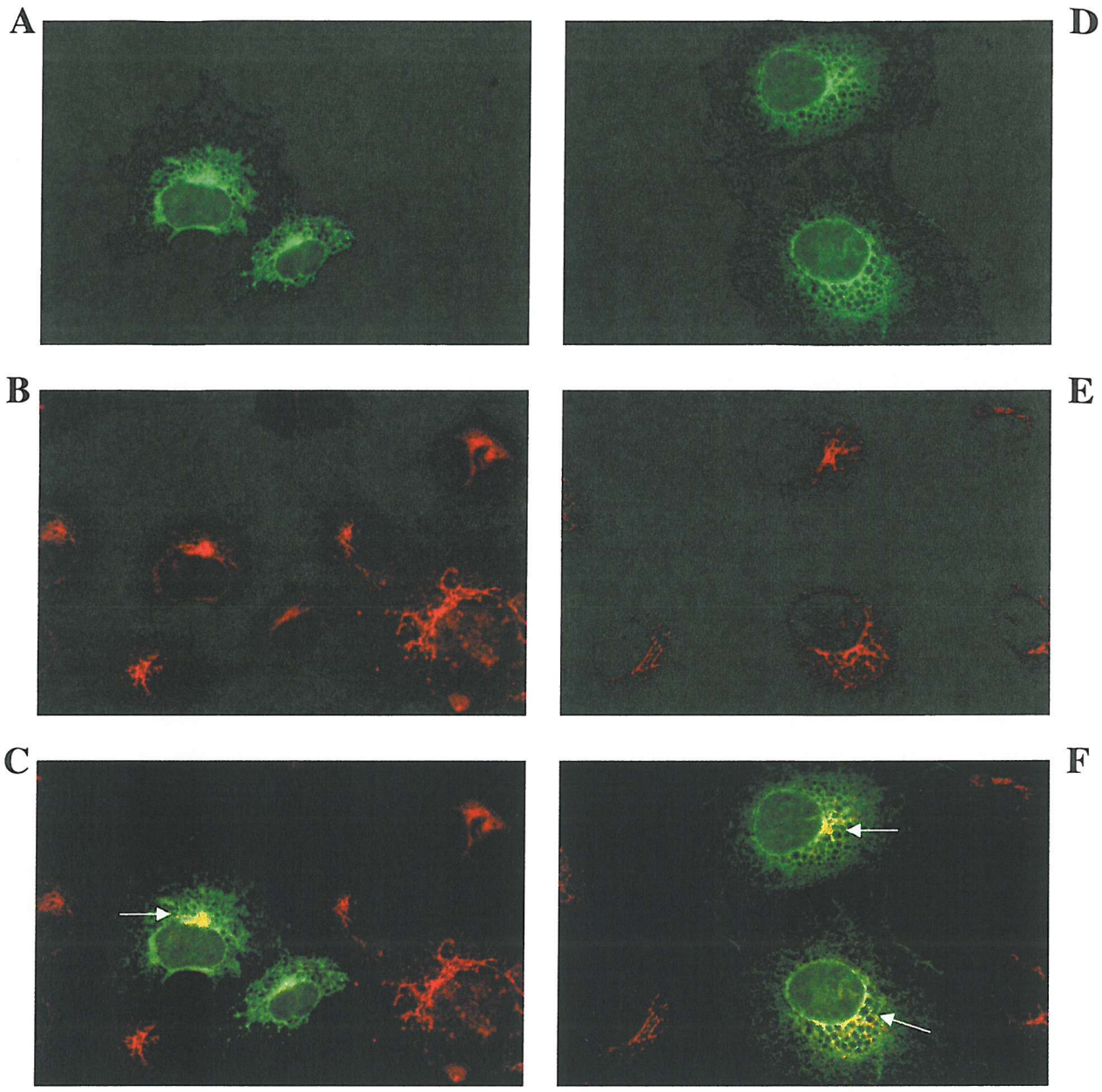


Figure 4.1: Co-localisation of ERGIC-53 and SERCA/GFP.

This shows two sets of COS-7 cells expressing SERCA/GFP; their GFP fluorescence (A and D), immunofluorescence of ERGIC-53 using a mouse anti-ERGIC-53 primary antibody and a sheep anti-mouse secondary antibody conjugated to Texas Red (B and E), and the two images superimposed (C and F). Arrows show yellow signal overlap that indicates colocalisation.

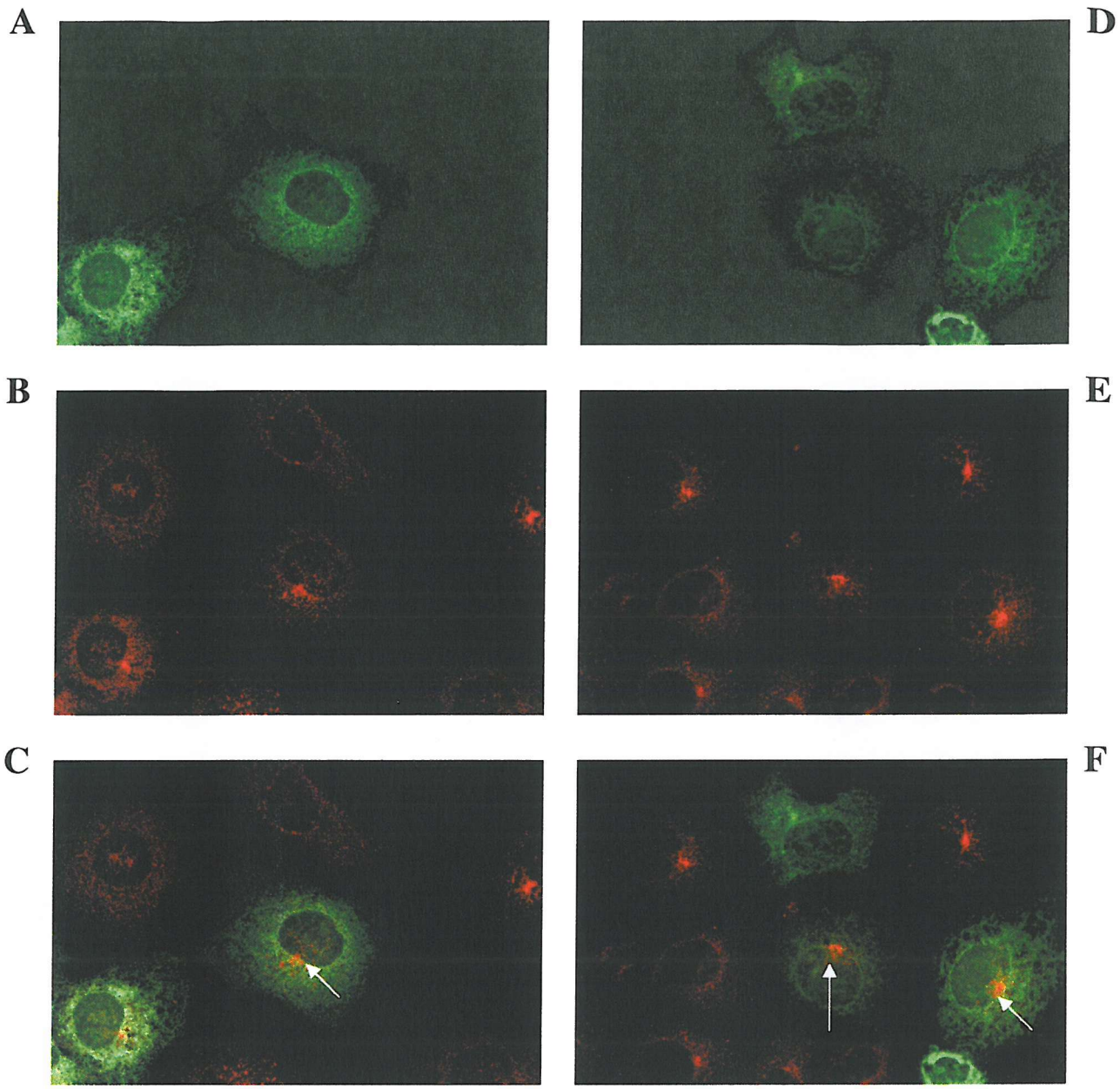


Figure 4.2: Confocal microscopy pictures showing the localisation of Trans Golgi Network and SERCA/GFP in COS-7 cells

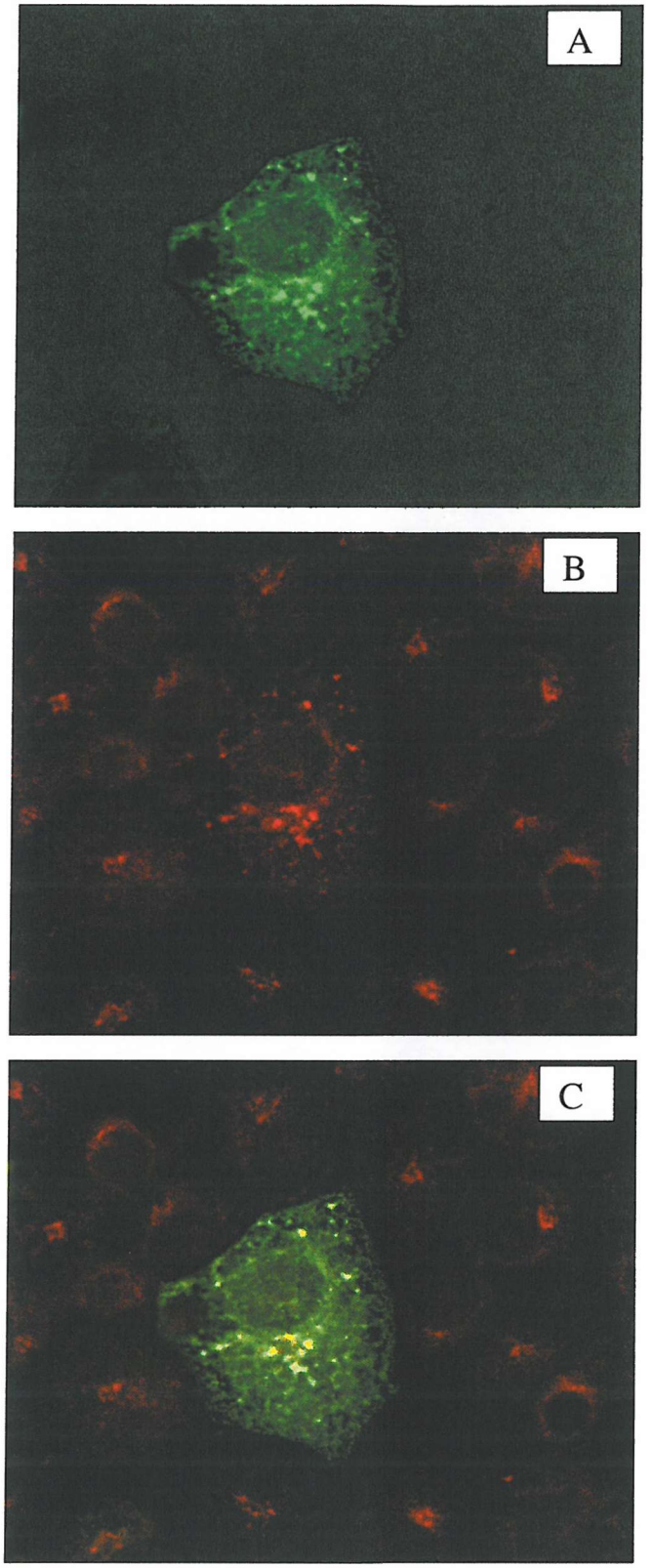
A, and D show the GFP fluorescence of two sets of cells, B and E shows the location of the TGN as determined by immunofluorescence using a sheep anti-TGN antibody and an goat anti-sheep secondary antibody conjugated to Rhodamine. C and F show the two images superimposed. Arrows show the position of the TGN in these two frames.

4.4.3 SERCA-eGFP co-localises with the tubular-vesicular structures formed after 1 hour 15°C block

When looking at localisation to the ERGIC, a technique known as 15°C block can be used. Incubating COS-7 cells for 1 hour at 15°C causes a block on ER-Golgi transport and has an effect on the nature of the Intermediate compartment. Most cells observed maintain the staining pattern shown in figure 4.3 (a single ERGIC-53 positive structure), but there is an increased vesicularisation of the ERGIC in some cells.

Figure 4.3A shows typical eGFP fluorescence in a cell transfected with SERCA-eGFP. There is a clear ER-like fluorescence pattern in this cell: clear perinuclear fluorescence is observed, as well as a fine reticular network extending into the cytoplasm. Strong fluorescence can be observed in vesicular structures in the peripheral cytoplasm, with a high concentration of these clusters just under the nucleus. A very similar pattern is seen when ERGIC-53 localisation is shown (figure 4.3B), and when Texas Red fluorescence is overlaid with the SERCA-eGFP fluorescence, yellow signal overlap was seen in all of these structures.

Figure 4.3: co-localisation after 15°C block



a) Typical eGFP fluorescence observed in a COS-7 cell transfected with SERCA-eGFP and incubated at 15°C for one hour.

b) Shows the location of vesicular structures containing ERGIC-53 using a mouse anti-ERGIC-53 antibody and a sheep anti-mouse secondary anti-body conjugated to Texas Red

c) An overlay of eGFP fluorescence and Texas Red fluorescence. Yellow signal indicates co-localisation

4.4.3 Sub-cellular Fractionation and Western blotting- Verification of antibody specificity

Figures 4.4, 4.5 and 4.6 show western blot analysis of cell suspensions containing COS-7 cells transfected with SERCA-eGFP. This was done to show that the antibodies that would be used in the cell fractionation experiments were specific to the proteins that were to be studied and that no cross-reactivity can be observed.

Figure 4.4 shows results of a Western blot in which Y1F4 was used as a primary antibody. Y1F4 is a monoclonal antibody that was raised against residues 510 and 515 in SERCA1b (characterised by Tunwell *et al.* 1991) and has been well characterised. The antibody binds strongly to a protein at approximately 150 kDa. This major band corresponds with the molecular weight of a SERCA-eGFP monomer. Other bands can also be observed at lower molecular weights but these are likely to be degradation products, which still contain the Y1F4 epitope.

Figure 4.5 shows a Western blot in which an antibody against the KDEL-receptor was used as a primary antibody. The KDEL-receptor is a 23 kDa protein, and the antibody used is cross-reactive with human and monkey Erd2 proteins. A feint band exists at the top of the gel but the main immuno-reactive band is present at approximately 23 kDa Figure 4.6 shows the reactivity of an antibody raised against calnexin. A single band can be seen at approximately 98 kDa.

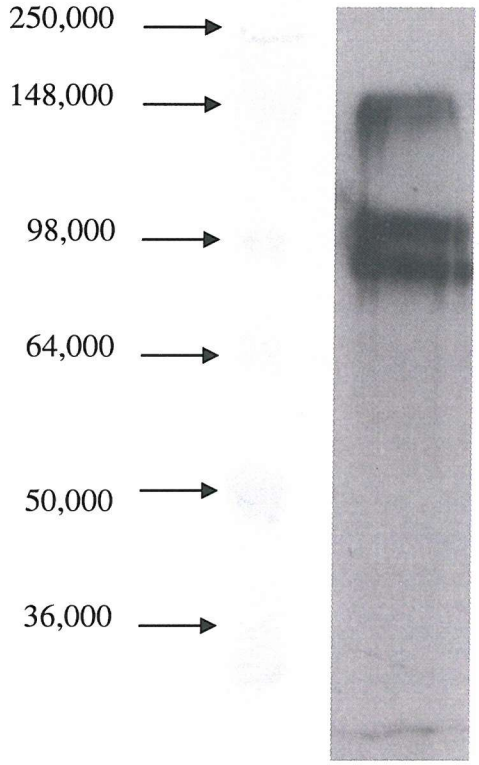


Figure 4.4: Western blot of cell suspension of COS-7 cells transfected with SERCA-eGFP.

Lane A is a pre-stained coloured marker on nitrocellulose, and lane B shows the results of ECL detection following Western blot analysis using Y1F4 monoclonal antibody (diluted 1 in 50), and sheep anti-mouse antibody conjugated to HRP (diluted 1in 10,000). Molecular Weights for the protein standards are shown on the left in Daltons.

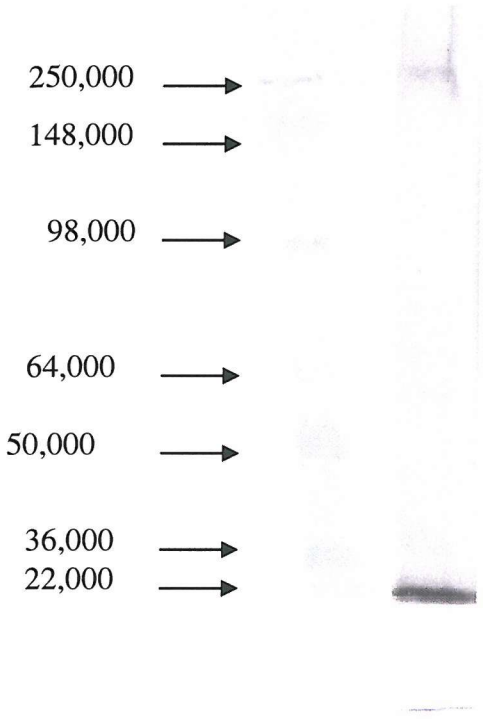


Figure 4.5: Western blot of cell suspension of COS-7 cells transfected with SERCA-eGFP.

Lane A is a pre-stained coloured marker on nitrocellulose, and lane B shows the results of ECL detection following Western blot analysis using anti-KDEL-receptor (Erd2) monoclonal antibody (diluted 1 in 200), and sheep anti-mouse antibody conjugated to HRP (diluted 1in 10,000). Molecular Weights for the protein standards are shown on the left in Daltons.

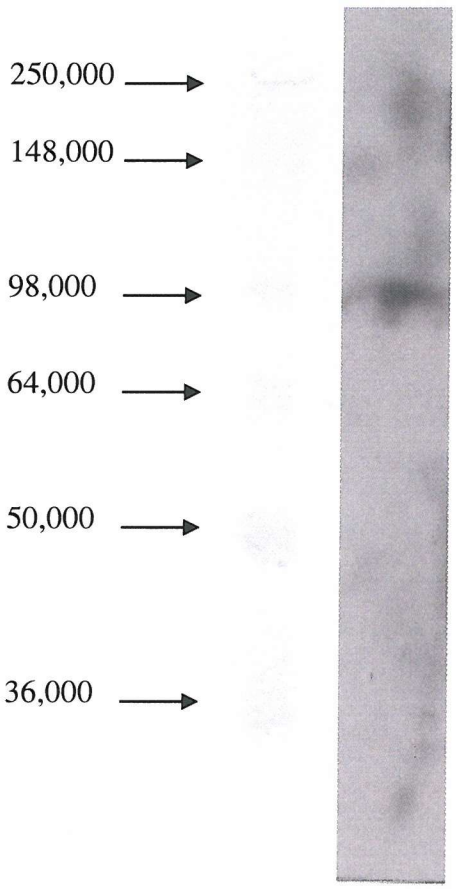


Figure 4.6: Western blot of cell suspension of COS-7 cells transfected with SERCA-eGFP.

Lane A is a pre-stained coloured marker on nitrocellulose, and lane B shows the results of ECL detection following Western blot analysis using anti-calnexin polyclonal antibody (diluted 1 in 10,000), and donkey anti-rabbit antibody congugated to HRP (diluted 1in 100,000). Molecular Weights for the protein standards are shown on the left in Daltons.

4.4.4 Western blot analysis of fractions taken from Nycodenz density gradients

Both TCA and perchloric acid were used as methods of precipitating the proteins from the gradient fractions. The most repeatable results were obtained using 0.5M perchloric acid. Figure 4.7 shows the results of western blot analysis from three sets of fractions, using the three primary antibodies (previously characterised in section 4.4.3). The fractions were taken from the bottom of the gradient, so fraction 1 contains the heaviest material and fraction 9 contains the lighter organelles.

Calnexin was used as a marker for the ER fraction, and the results using an anti-calnexin antibody are shown in 4.7c. Calnexin was observed to only be present in the heaviest fraction (fraction 1). Erd2, in contrast 4.7b was observed to be present in the lighter set of fractions (fractions 7,8 and 9), as well as being detected to a lesser extent in fraction one as well. By observing these two results, as well as comparing them with previous observations on Nycodenz fractionation (Hammond and Helenius 1994), fraction 1 can be considered to contain the ER fraction, were the lighter fractions correspond to the ERGIC fractions.

4.7a shows the result of a western blot in which Y1F4 has been used as a primary antibody. SERCA-eGFP was present in two separate sets of fractions. The first set is concentrated at fraction one, although smaller amounts of SERCA were detected in fractions two and three. A high concentration of SERCA-eGFP is also found in the lighter fractions, peaking at fraction 8 but also present in considerable amounts in 7 and 9. Sub-cellular fractionation shows that SERCA-eGFP is not restricted to the ER fractions, but is also present in fractions corresponding to the ER/Golgi Intermediate Compartment (ERGIC).

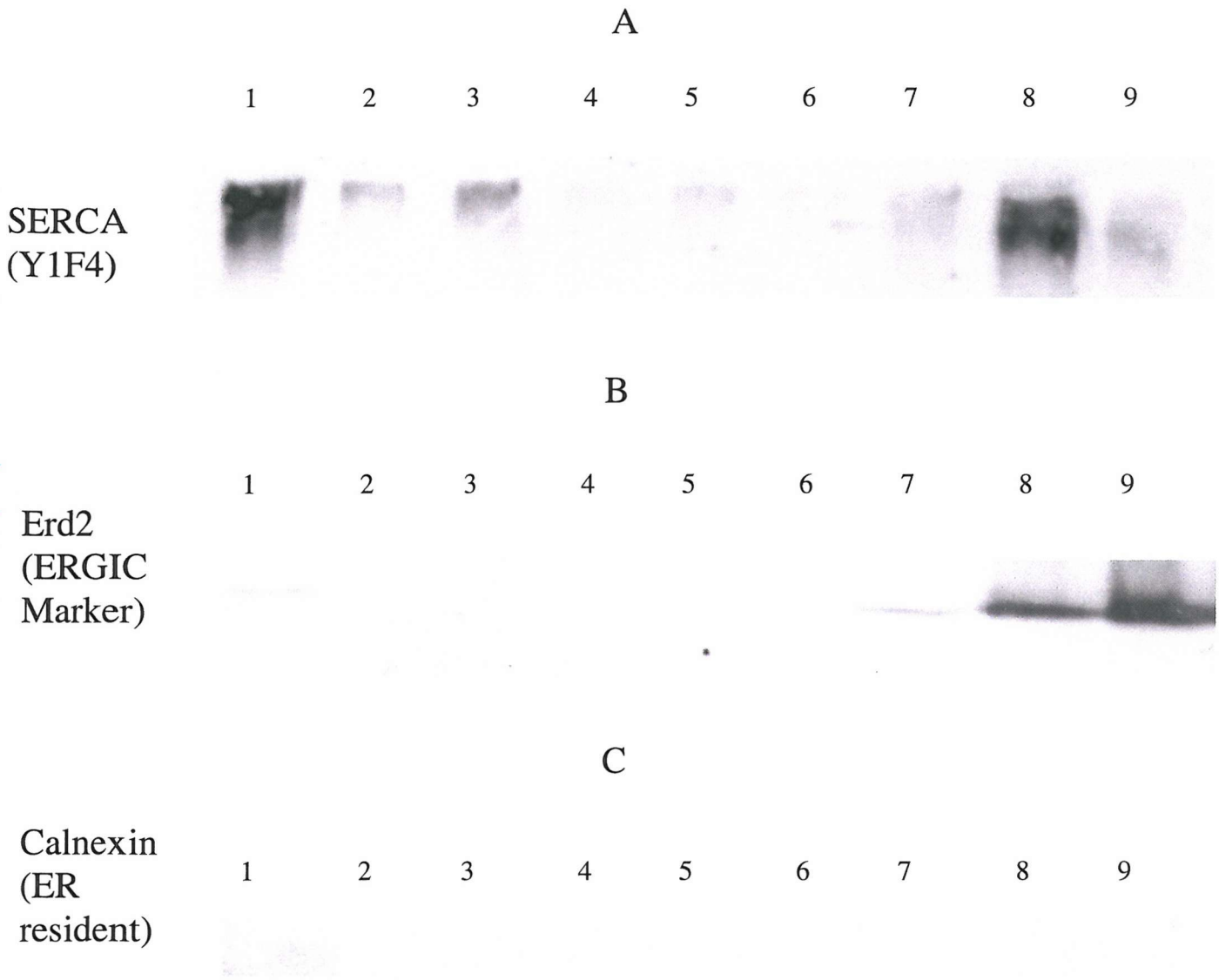


Figure 4.7: Showing the results of western blot analysis on fractions taken from Nycodenz gradients

Sections of the gels are shown with corresponding to fraction numbers (1 being the heaviest fraction, 9 the lightest. The sets of fractions shown were analysed for presence of A) SERCA B) Erd2 (the KDEL receptor), and C) Calnexin.

4.5 Discussion

This chapter set out to determine whether SERCA is targeted to the ER by a retrieval mechanism or by some other method like retention. The work presented here is similar to that of Greenfield and High (1999) where they tagged Sec61α with enhanced Green Fluorescent Protein and showed that it escaped to post-ER compartments. SERCA-eGFP has been investigated by using the same techniques such as bi-colour confocal microscopy and sub-cellular fractionation.

By using an antibody raised against ERGIC-53 (a well-established marker for the Intermediate Compartment) on cells that have been transfected with SERCA-eGFP, it was possible to establish whether SERCA co-localises with the ERGIC. The position of the ERGIC and the localisation of ERGIC-53 (see figure 4.1b and 4.1e) is consistent with previous work (Pedrazzini *et al.* 2000). The SERCA-eGFP construct does indeed co-localise with ERGIC-53, indicating that SERCA is not permanently resident in the ER. The accumulation of the tubulovesicular structures derived from the ERGIC increases at 15°C as noticed by Seraste and Svensson (1991) and proved a useful method of confirming that SERCA was truly localised to the ERGIC. These ERGIC-53 positive structures are clearly present and co-localise with SERCA-eGFP (figure 4.3), despite being separated from the main eGFP fluorescence.

Another method of checking for presence in the ERGIC that could have been used is using the GTPase inhibitor AlF_4^-. Treating cells with AlF_4^- causes transport from the ERGIC to be blocked (Kappeler *et al.* 1997). Any protein that escapes from the ER but is normally rapidly recycled, instead accumulates in the ERGIC during AlF_4^- treatment, and this technique was used successfully by Andersson *et al.* (1999) to detect escape from the ER of different types of di-lysine motifs.

Despite SERCA being present in the ERGIC, figure shows that the SERCA-eGFP fusion protein is not found in the Trans-Golgi Network (TGN). Greenfield and High (1999) found that although TGN-46 antibodies showed that Sec61α-GFP was in a

separate compartment to the TGN-46, some overlap of signals could be seen. Brefeldin A treatment was used to distinguish between the two compartments, because it causes the Golgi to somehow reintegrate into the ER, and the trans-Golgi Network to accumulate around the microtubule organising centre (Banting and Ponnambalam 1997). When used on cells transfected with SERCA-eGFP, Brefeldin A treatment showed that TGN-46 is present in a compact structure away from the ER and that this collapsed TGN does not contain any detectable amount of SERCA-eGFP.

The lack of SERCA-eGFP in any TGN-46 positive structures is important, as it shows effective recycling of any escaped SERCA back to the ER. No 'leakage' of material to the TGN is seen, and it can be concluded that the retrieval system does not get overloaded.

Sub-cellular fractionation (see figure 4.7) confirms the confocal data. Hammond and Helenus (1994) established Nycodenz gradients as an effective method of separating the ER from the ERGIC/cis-Golgi, and western blotting was used in this chapter as a method of identifying which fraction identified with which sub-cellular compartment. Previous work determined that the heavier fractions (i.e. the lower numbers) were where the ER separated to and that the lighter fractions were identified as the ERGIC/cis-Golgi fractions (Hammond and Helenus 1994, Greenfield and High 1999).

Erd2 (the ERGIC marker) was found in both the heavy (fraction 1) and light fractions (fractions 7,8 and 9). The presence of the KDEL-receptor in both the ER and ERGIC/cis-Golgi is entirely in agreement with its recycling role (Majoul *et al.* 1998) and the results described by Greenfield and High (1999). Calnexin was used as an ER marker, and was completely limited to the heavier fractions.

Like Greenfield and High (1999), quantitative analysis of the proteins in each fraction was not possible due to the estimated large amount of ER material lost during the method (particularly the low speed centrifugation step – see section 4.3.5). In this case, the loss of material has also resulted in a very low amount of calnexin being detected (see figure 4.7c).

SERCA-eGFP is found in both the calnexin-positive, ER fraction and the lighter fractions that contain the ERGIC 'resident' Erd2, demonstrating that the SERCA-eGFP does indeed escape the ER.

Both the visual confocal data and the biochemical data gained from the sub-cellular fraction results therefore suggest that SERCA-eGFP is not a permanent resident in the ER. These results have conclusively shown that SERCA does leave the endoplasmic reticulum but is retrieved by machinery in the ERGIC/cis-Golgi to maintain a high amount of SERCA in the ER.

Chapter Five:

General discussion

5 General Discussion:

The Nature of SERCA Targeting

The nature of SERCA targeting has been investigated using two different approaches. In chapter three; chimeric proteins and enhanced Green Fluorescent Protein (eGFP) were used to identify the N-terminal cytoplasmic region and the first two trans-membrane helices of SERCA as the region required for ER targeting. Chapter four used SERCA, fusion-tagged with eGFP to establish that SERCA does escape from the ER and therefore contains a retrieval signal. The first question that these two chapters help raise is where in particular the targeting signal is?

Guerini *et al.* (1997) indicate that the first 32 residues (the cytoplasmic N-terminus) is all that is required for ER targeting. This would be consistent with previous knowledge concerning the targeting of other ER membrane proteins – like those containing C-terminal KKXX. Proteins which containing KKXX have been shown to directly interact with proteins in coatamer, and to be recruited into COP-I vesicles (Harter and Wieland 1998).

Not all targeting occurs via the cytoplasmic domains of proteins however. The transmembrane helix of the ryanodine receptor monomer is vital for ER targeting (Bhat and Ma 2002), and the recently discovered retrieval 'receptor', Rer1P is known to bind directly with its cargo proteins (Sato *et al.* 2001). Transmembrane helices are also known to be important for the proper targeting of other P-type ATPase ion pumps. Work using chimeric proteins made up of $Na^+\backslash K^+$ and H^+/K^+ ATPases has established that the 4^{th} transmembrane helix of the $H^+\backslash K^+$ is responsible for targeting it to the apical of polarised cells (Dunbar *et al.* 2000).

The only conclusive way of determining which sequence is responsible for ER targeting is to make another set of eGFP-tagged chimeras. Six more chimeras could be constructed to dissect the first 212 residues of SERCA. Figure 5.1 shows the possible composition of the six new proposed chimeras. The three sections used to make the chimeras would be **1)** the N-terminal cytoplasmic region (residues 1-50) **2)** the first transmembrane helix, and **3)** the second transmembrane helix and these would be put together like the ones used in chapter three with mirror-image pairs to determine true targeting. All of the chimeric 'cassettes' would then be linked to the remainder of PMCA sequence and tagged with eGFP.

The main problem with designing more chimeras is that the original set of chimeras were joined at conserved regions between PMCA and SERCA to avoid abnormal folding and it would be difficult to design more chimeras in a similar way. There are no conserved regions in the first two loops of the pumps. The most conserved regions in this area are in the transmembrane helices of SERCA and instead of joining the chimeras in the loops (as shown in figure5.1) they could be linked in the membrane. More problems would arise however, because of predicting the position of residues relative to the membrane. It would be possible to accurately predict the position of SERCA residues because of the models produced from the crystal structure, but no such structure exists for PMCA.

Further study into the exact identity of the targeting signal could include common methods such as alanine scanning for determination of vital amino acids, and perhaps truncation of the SERCA sequence. However, with such a large stretch of amino acids, it might be difficult to obtain meaningful information from alanine scanning and truncation of the sequence might result in poorly folded proteins as SERCA spans the membrane many times.

The second aspect of SERCA targeting is the finding that, despite having an integral function within the ER, SERCA is still not a permanent ER resident. This, along with the previous results for Sec61 (Greenfield and High 1999) has implications for all ER

'residents'. It is of course possible that there are no pure retention signals and that most (if not all) ER proteins will escape the ER and therefore would need retrieval signals within them. There is evidence to suggest that proteins previously assumed to be kept in the ER purely by retention are in fact slowly recycled between the ER and the cis-Golgi.

Methods to detect proteins in post-ER compartments such as those used in chapter four, and detecting N-glycosylation of proteins by Golgi resident enzymes (Duvet *et al.* 1998) have been used to show retention of ER proteins. However, work on Cytochrome b (5) has suggested that some proteins although not detected by these techniques, are actually detectable in the cis-Golgi by looking at O-glycosylation patterns (Pedrazzini *et al.* 2000). The reason suggested for why no evidence was found of presence in the ERGIC (by 15°C incubation for example), is that the proteins are not escaping in large enough numbers for detection but are still slowly 'leaking' out of the ER.

If this is indeed the case, then a rethink of the relationship between retention and retrieval signals is required along with examination of ER proteins such as SERCA and their methods of ER targeting. Specific investigation into whether SERCA is interacting with known proteins such as Rer1P or some other unknown part of the COP-I system might be desirable, as well as determining what role retention plays in SERCA targeting. An interesting sideline of this would be to look for any specific sarcoplasmic reticulum targeting signals, and the mechanisms involved in that membrane system.

Another interesting aspect of this retieval signal is the related Pmr1 calcium/magnesium pump of yeast. This is targeted to the Golgi (Halachmi *et al* 1996), and has a role in maintaining Golgi enzyme function. As there is no SERCA homologue in yeast, does the ER pump have a similar role to play?

Generally, this work has provided an insight into the targeting of a large multi-spanning membrane protein. This is perhaps the least well investigated aspects of protein targeting and a more detailed analysis of the mechanisms involved would be beneficial.

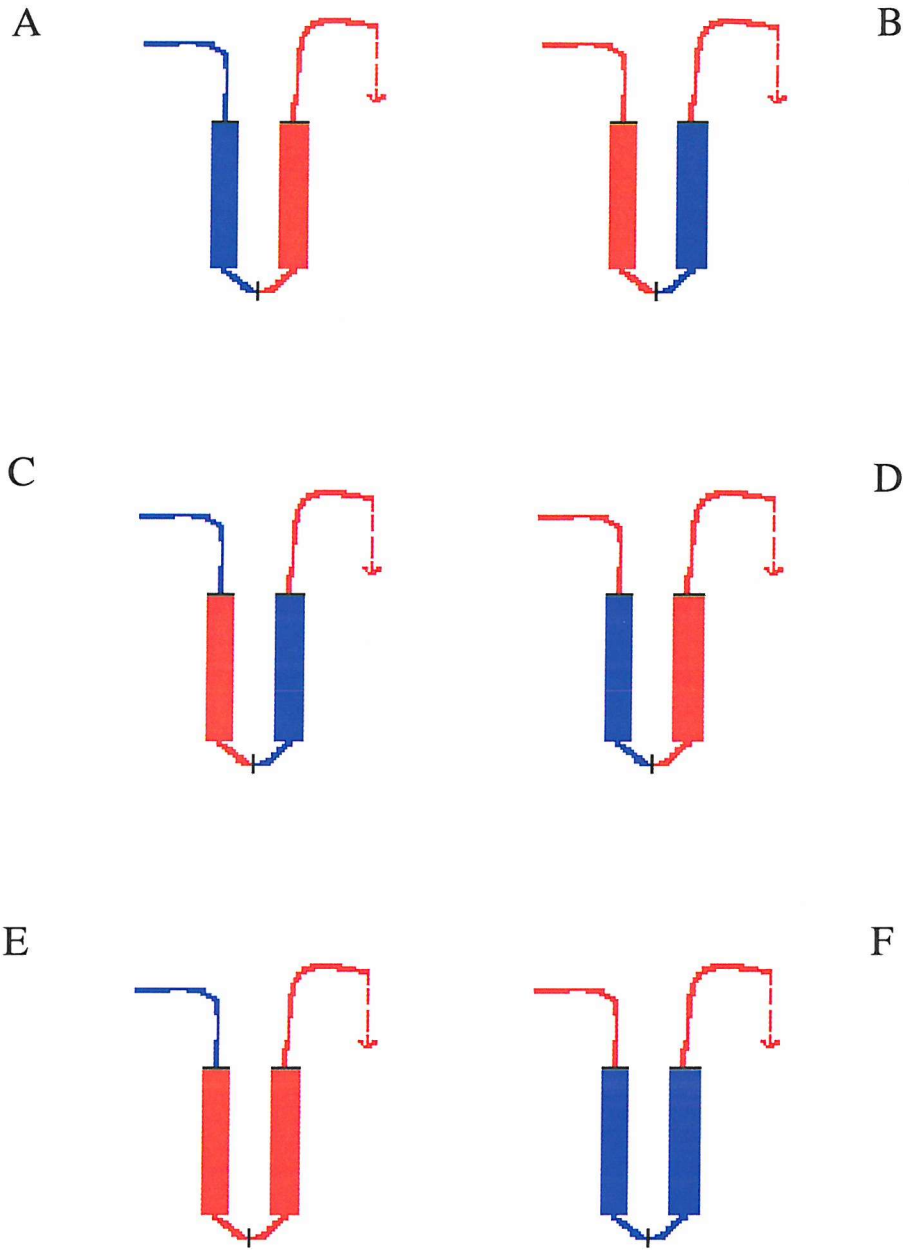


Figure 5.1: The proposed set of chimeras for identifying the targeting signal within the first 212 residues of SERCA.

Showing the set of 'cassettes' that would be introduced to replace the first segment of PMCA-GFP and create a new set of chimeras. The cassette would be split up into three segments (the N-terminus, and the two transmembrane helices). Transmembrane helices are denoted by horizontal blocks, blue showing SERCA sequence and red indicating PMCA sequence and each cassette is shown opposite its mirror-image pair.

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