

**NOVEL USE OF GLYCOSYLATION SCANNING TO MAP THE
INTRACELLULAR TRAFFICKING OF SARCO(ENDO)PLASMIC
RETICULUM CALCIUM ATPase 1A**

by

Rory J. Flinn

A thesis submitted to the Faculty of the University of Delaware in partial
fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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ABSTRACT

The sarco(endo)plasmic reticulum calcium ATPase (SERCA) family of proteins function as calcium pumps in the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) membranes. SERCA1a is found exclusively in fast-twitch muscle cells and mediates muscle relaxation by pumping calcium back into the SR after calcium has been released into the cytoplasm to elicit muscle contraction. The mechanism which allows SR biogenesis is not known, but SR membrane is believed to bud from the ER. One hypothesis is that SERCA1a proteins play a significant role in SR biogenesis in fast-twitch skeletal muscle due the protein's large size and clustering into large arrays in the SR membrane. SERCA1a arrays could recruit lipids which would allow for a large increase in membrane size that could result in the formation of the SR. Also, SERCA1a is highly expressed during the early stages of myogenesis, at the same time the first emergence of the SR is observed. It is known that SERCA1a contains ER targeting information and is synthesized in the ER membrane, however the intracellular trafficking pattern of SERCA1a before entering the SR membrane is unknown. Glycosylation events differ between glycoproteins retained in the ER and glycoproteins having traversed some or all of the Golgi apparatus compartments such that a glycoprotein can be analyzed for the particular sugar residues it contains to determine which cellular compartments it has

traversed. It was found that calsequestrin, a soluble glycoprotein found in the lumen of the SR, traveled through the Golgi apparatus and arrived in the SR lumen via clathrin-coated vesicles. These vesicles did not contain SERCA1a, and since SERCA1a and calsequestrin are synthesized at overlapping time periods in myogenesis, the hypothesis can be made that SERCA1a does not travel through the Golgi apparatus before entering the SR lumen. It is our hypothesis that SERCA1a is retained in the ER lumen and plays a significant role in SR biogenesis. In order to validate this hypothesis the intracellular trafficking of SERCA1a was investigated. Unlike calsequestrin, SERCA1a is not a glycoprotein. A novel application of a technique called glycosylation scanning was undertaken to create mutant SERCA1a glycoproteins in mouse fibroblasts and mouse myoblasts that were then analyzed for their glycosylation patterns to determine the particular cellular compartments the mutant SERCA1a proteins had traveled through. Three such mutant SERCA1a cDNA constructs were created and used in transfection experiments to generate mutant SERCA1a proteins. Only one of these three glycosylation mutants had expression levels sufficient for further analysis. However, it was concluded that the mutant SERCA1a protein was not glycosylated, and thus no conclusions could be made about the intracellular trafficking of SERCA1a.

Chapter 1

INTRODUCTION

1.1 SERCA Function, Structure, and Expression Pattern

During muscle contraction calcium released from intracellular calcium stores mediates cytoskeletal changes which physically constrict the muscle cells. A muscle relaxes when the transient calcium signal is depleted. Muscle cells have two mechanisms for accomplishing this task. The first is to pump calcium out of the cell by means of calcium ATPases located at the plasma membrane. The second mechanism involves calcium ATPases located at the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). The family of proteins that make up these latter calcium pumps is called SERCA for sarco(endo)plasmic reticulum calcium ATPase. SERCAs are multipass transmembrane proteins that usually contain ten transmembrane domains and have their catalytic domains facing the cytoplasm (Brandl et al., 1986; Karin et al., 1989). SERCAs represent up to 80% of the total protein found in the SR (MacLennan and Campbell, 1979). All SERCAs are found in either the SR and ER or just the ER depending on the cell type in which it is expressed (Karin and Settle, 1992). SERCAs are P-type ATPases, meaning that they transfer the gamma phosphate from a molecule of ATP to an aspartic acid amino acid residue (Asp 351 for SERCA1a) (Maruyama and MacLennan, 1988).

This is an intermediate step where first two calcium ions bind to SERCA in its cytoplasmic domain then the phosphate is transferred from ATP to the Asp residue. Once this occurs a conformational change (E1/E2) takes place in SERCA to allow the calcium ions to travel through the ER/SR membrane and enter the lumen of the ER/SR (de Meis and Vianna, 1979).

This family of proteins has three isoforms and splice variants for each isoform that display cell type-specific expression (Moller et al., 1996; Wu and Lytton, 1993; Wuytack et al., 1995). Table 1.1 outlines where these isoforms and splice variants are expressed (Moller et al., 1996). In fast-twitch skeletal muscle SERCA1a is the primary SERCA found, whereas in slow-twitch skeletal muscle SERCA2a is the primary SERCA found.

Table 1.1: SERCA Isoforms and Expression Profiles

Isoform	Location
SERCA1a	Fast-twitch skeletal muscle
SERCA1b	Neonatal muscle
SERCA2a	Cardiac and slow-twitch muscle
SERCA2b	Most non muscle nucleated cells
SERCA3a/b/c	Range of cells including lymphocytes, mast cells, and platelets

1.2 Theory on SR biogenesis

Skeletal, cardiac and smooth muscle cells are the only types of cells that contain SR. This organelle stores the large amounts of calcium (1.5 mM) needed to generate the large calcium signal needed for a muscle contraction (Toyoshima and Inesi, 2004). How SR is formed inside these cells still remains a mystery. In 1967, Ezerman and Ishakawa used electron microscopy to observe myogenesis in chick embryos, and were able to discern what they postulated was SR biogenesis (Ezerman and Ishikawa, 1967). Their results were visualizations of what appears to be SR budding from ER in chick embryo myogenesis. Further support for SR-ER continuities were shown with immunostaining studies showing that the ER markers glucose-regulated protein 78 (BiP) and protein disulfide isomerase (PDI), both of which function as chaperones to aid in protein folding, were contained in both the ER and SR (Fliegel et al., 1989; Villa et al., 1993; Volpe et al., 1992). The hypothesis that the SR buds from the ER is widely accepted, but the mechanism that leads to this budding remains unknown.

1.3 Expression of calsequestrin and SERCA during myogenesis

Expression analysis shows a few genes that are upregulated during myogenesis at the point when biogenesis of the SR takes place. Two of these gene products are calsequestrin, an ER/SR luminal calcium binding protein, and SERCA (isoforms SERCA1a or SERCA2a depending on muscle cell type) (Holland and MacLennan, 1976;

Zubrzycka and MacLennan, 1976). In differentiating muscle cells in culture, cell fusion events lead to the formation of myotubes which are observed to contract in culture. The expression of both calsequestrin and SERCA were observed during myogenesis *in vitro*, which mimics myogenesis *in vivo*, and it was found that prior to cell fusion calsequestrin expression levels had increased sharply (Greenway and MacLennan, 1978). It was only after cell fusion had occurred that SERCA expression was turned on (Greenway and MacLennan, 1978; Holland and MacLennan, 1976). However it was also found that SERCA expression was not dependent upon cell fusion. It was only once the earliest myotube formations were observed that SR formation was also observed (Holland and MacLennan, 1976), and therefore it can be concluded that SR is formed when pre-muscle cells (myoblasts) differentiate into myotubes at the beginning of myogenesis.

Calsequestrin was synthesized prior to myotube formation and was visualized through immunostaining to be localized to the perinuclear region (Jorgensen et al., 1977), which is part of the Golgi apparatus. It is only after the appearance of SERCA in these same cells that calsequestrin begins to spread out away from the nucleus and show a continuous, polygonal network pattern of staining typical of mature SR protein (Jorgensen et al., 1977).

1.4 Intracellular trafficking of calsequestrin and SERCA

It was determined that calsequestrin arrives at the SR via clathrin-coated vesicle transport from the Golgi apparatus after its synthesis in the lumen of the ER (Thomas et

al., 1989). The intracellular trafficking of SERCA1a or SERCA2a is not known, and neither SERCA1a nor SERCA2a was found in the same clathrin-coated vesicles that contained calsequestrin (Thomas et al., 1989). Calsequestrin seems an unlikely candidate for providing a mechanism of SR biogenesis since it arrives at the SR after traveling through the Golgi apparatus through vesicle transport, and this would not be in keeping with the current theory that SR is formed from the ER. Rather, this suggests that calsequestrin is arriving at nascent SR as the SR is forming instead of mechanistically driving the formation of an organelle.

1.5 Possible Role for SERCA in SR biogenesis

Both SERCA1a and SERCA2a are large membrane proteins that are found in densely-packed arrays within the SR membrane (Dux and Martonosi, 1984; Froemming and Ohlendieck, 1998; MacLennan et al., 1971). These arrays of SERCA1a or SERCA2a offer an interesting hypothesis about a mechanism of SR biogenesis. SERCA1a or SERCA2a (cell type dependent) would be highly expressed during SR biogenesis and its synthesis could take place at or near the site of SR budding. The large amount of SERCA protein made in the ER would be enough to drive the formation of the newly budding SR membrane. Since SERCA1a is a large transmembrane protein and is found in arrays in the SR membrane it could be able to recruit membrane lipids, thus building great amounts of membrane during its abundant synthesis to force the budding of the SR. Figure 1.1 illustrates this hypothesis.

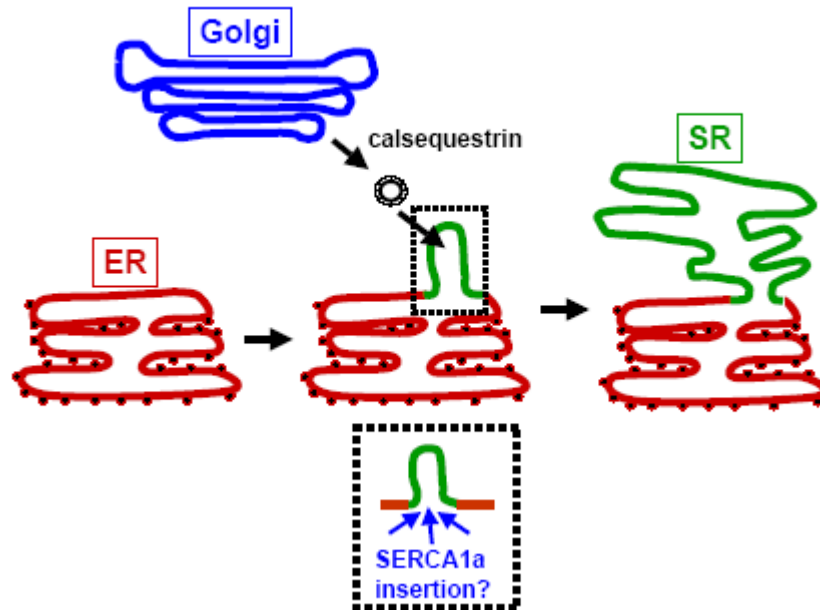


Figure 1.1: Proposed model for SR biogenesis with newly-forming SR budding from the ER. The SR is depicted in green, while the Golgi apparatus is pictured in blue and ER in red. A clathrin-coated vesicle containing synthesized calsequestrin is shown arriving at the growing SR having been transported from the Golgi apparatus. SERCA1a is shown in a close-up box enclosed by dotted lines. In this model SERCA1a would be synthesized at the ER membrane and drive the formation of the SR by being inserted into an SR growing point along the ER membrane.

1.6 Significance of mapping the intracellular trafficking of SERCA1a

In order to validate the hypothesis that SERCA is driving the biogenesis of SR from the ER, one important piece of this puzzle must first be elucidated. SERCA1a is synthesized at the ER (Chyn et al., 1979), but it is uncertain as to whether or not it travels directly to the SR via membrane continuity with the ER, or if it travels through the Golgi apparatus first, as calsequestrin does. Some preliminary evidence suggesting that SERCA does not travel through the Golgi apparatus is that no SERCA1a was found in the clathrin coated vesicles containing calsequestrin (Thomas et al., 1989). It would seem likely that if the expression of both SERCA1a and calsequestrin were highly upregulated during myogenesis and both were arriving at the SR from the Golgi apparatus at overlapping time points (Greenway and MacLennan, 1978), then they would be found together in the same clathrin coated vesicles. However, it would be possible for SERCA1a to travel through the Golgi apparatus and arrive at growing points for SR at the ER membrane without being in the same vesicles as calsequestrin. This would be one of a few possible hypotheses about the intracellular trafficking of SERCA1a. Another is that SERCA1a is synthesized in the ER and is retained there to mediate SR formation. This could occur in two ways. An unknown retrieval mechanism from an early or late Golgi compartment could allow SERCA1a to initially escape the ER only to be retrieved back as is the case with several other ER retained proteins. In this manner SERCA1a would be retrieved back to the ER membrane and then pack into arrays at an SR growing point in the ER membrane. Alternatively SERCA1a could be synthesized in the ER membrane at the site of SR biogenesis and therefore would drive SR biogenesis at its very site of synthesis.

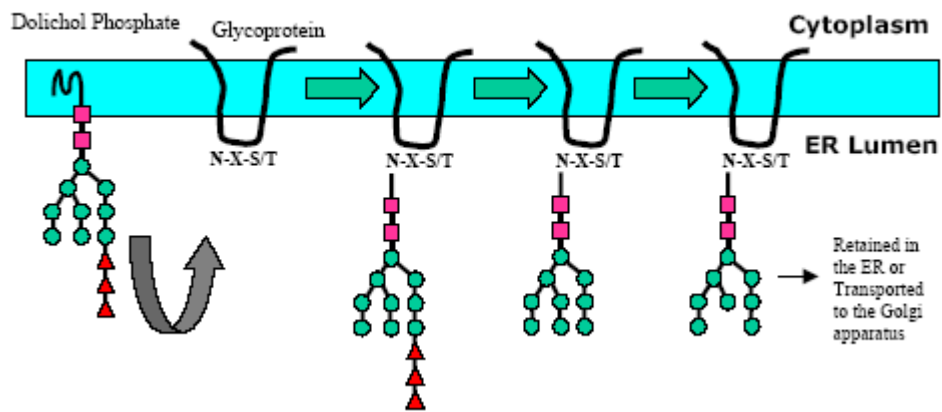
Inevitably some SERCA1a in this process would flow from the site of synthesis through all parts of the ER membrane accounting for its known presence in the ER membrane as well as in the SR membrane. Lastly, SERCA1a could be synthesized in the ER membrane at random sites, be retained in the ER, and migrate within the ER membrane to arrive at SR growing points. With all of these different possibilities of SERCA1a intracellular trafficking it would also be possible for SERCA1a not to play a significant role in SR biogenesis, and thus once synthesized, SERCA1a might just arrive at the growing SR regardless of where it first travels.

1.7 The experimental approach of glycosylation scanning to map the intracellular trafficking of SERCA1a

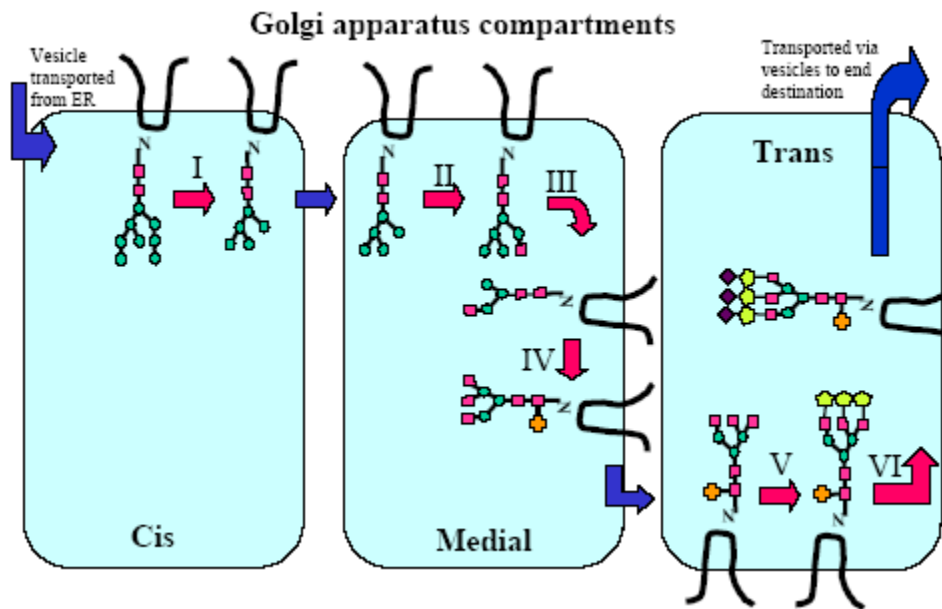
In order to study the trafficking pattern associated with SERCA1a, a novel approach was undertaken. In previous examinations of membrane topology of glycoproteins (Hamilton et al., 2001; Hresko et al., 1994; O'Brian et al., 2002; Schmidt-Rose and Jentsch, 1997), the N-linked glycosylation patterns on these proteins were analyzed to determine where transmembrane domains were located and which parts of the protein faced the lumen of the ER. This method is called glycosylation scanning. In the glycosylation scanning undertaken by these research groups (Hamilton et al., 2001; Hresko et al., 1994; Schmidt-Rose and Jentsch, 1997), novel N-linked glycosylation sites were added to the proteins through mutagenesis techniques. Since N-linked glycosylation initially occurs in the lumen of the ER and further processing occurs as a glycoprotein

travels through the Golgi apparatus, the N-linked glycosylation patterns would differ between a protein that is retained in the ER versus a glycoprotein that has been fully processed during its travels through the Golgi apparatus. An ER glycoprotein that has been retained in the ER would have a "high mannose" composition consisting of eight mannoses and two N-acetylglucosamines whereas a glycoprotein that has traveled through the Golgi apparatus and has either been retrieved back to the ER or sent for secretion or to another cellular organelle would have considerable trimming of the mannose residues, leaving the glycosylation moiety with only a core structure consisting of the two N-acetylglucosamines and three mannoses. Other sugars would be added in the Golgi apparatus to give the Golgi apparatus-processed glycoprotein a specific glycosylation moiety for further protein folding or subunit binding or to target the protein to the lysosome. Figure 1.2 shows the different glycosylation events that take place inside a cell and the organelles in which the glycosylation events take place.

A



B



■ : N-acetylglucosamine, ● : mannose, ▲ : glucose, ⊕ : fucose,
 ◆ : galactose, ◆ : N-acetylneuraminic acid

Figure 1.2: N-linked glycosylation events and cellular compartments in which they take place (Lodish, 2000). Panel (A) depicts the initial N-linked glycosylation events that occur in the ER. The green arrows show the step-wise addition and subtraction of sugars that takes place after the high mannose structure has initially been added to the glycoprotein. Panel (B) depicts the glycosylation trimming events that take place once a glycoprotein has traveled from the ER to the Golgi apparatus and in which Golgi compartment each of the trimming processes take place. A legend below panel (B) displays what each colored shape represents.

SERCA1a is not a glycoprotein, based on amino acid sequence analysis and the observation that no radiolabeled sugars are incorporated in the protein (Chyn et al., 1979). The novel application of glycosylation scanning undertaken here was designed to determine the trafficking pattern of SERCA1a through the use of mutagenesis to add N-linked glycosylation sites to chicken SERCA1a protein and then analyze its expression and glycosylation patterns in mouse fibroblast-like (Ltk-) cells and mouse myoblast (C2C12) cells. Previous studies using glycosylation scanning used cell-free systems with microsomes to synthesize and glycosylate mutant proteins to analyze protein topology, whereas the novel approach undertaken here uses transfections of live cells to study glycosylation patterns of mutant proteins to determine intracellular trafficking.

1.8 Strategy for incorporating N-linked glycosylation sites into SERCA1a

Previous studies showed that chicken SERCA1a was correctly targeted to the ER and the nuclear envelope in mouse myoblast cells (Karin et al., 1989). Also, it was shown in transfection experiments in Ltk- cells that chicken SERCA1a was properly targeted to the ER based upon these cells displaying typical ER staining and colocalization of

SERCA1a with the ER marker protein BiP (Karin and Settle, 1992). A great advantage to using Ltk- cells is that they have high transfection efficiencies.

The N-linked glycosylation sites that were added into SERCA1a protein were added as DNA consensus sequences encoding the N-linked glycosylation site to the cDNA construct of chicken SERCA1a. The amino acid sequence of an asparagine followed by any amino acid except for proline followed by either serine or threonine allows for N-linked glycosylation to occur, with the initial glycosylation moiety consisting of two N-acetylglucosamines, eight mannoses, and three glucoses to be transferred from a dolichol phosphate in the luminal-facing ER membrane leaflet to the asparagine residue. These N-linked glycosylation sites had to be added to regions of the SERCA1a protein that would face the lumen of the ER, since this is where initial N-linked glycosylation occurs. SERCA1a possesses five ER facing luminal loops (Brandl et al., 1986; Karin et al., 1989), all of which could be potential targets of mutagenesis to add N-linked glycosylation sites. Indeed, even a tri-peptide consisting of Asp-X-Thr that was targeted to the ER lumen could be glycosylated and secreted, showing that the only requirement for N-linked glycosylation to occur was the presence of the consensus sequence for N-linked glycosylation (Wieland et al., 1987).

The ER luminal loops that are part of SERCA1a vary in number of amino acids and most are small in size, ranging from four to 33 amino acids in length (Toyoshima and Inesi, 2004). The crystal structure of SERCA1a having recently been elucidated

(Toyoshima and Inesi, 2004), shows the relative size of each of these loops and their positions as part of the SERCA1a protein. Figure 1.3 depicts the size of each of these ER luminal loops belonging to SERCA1a.

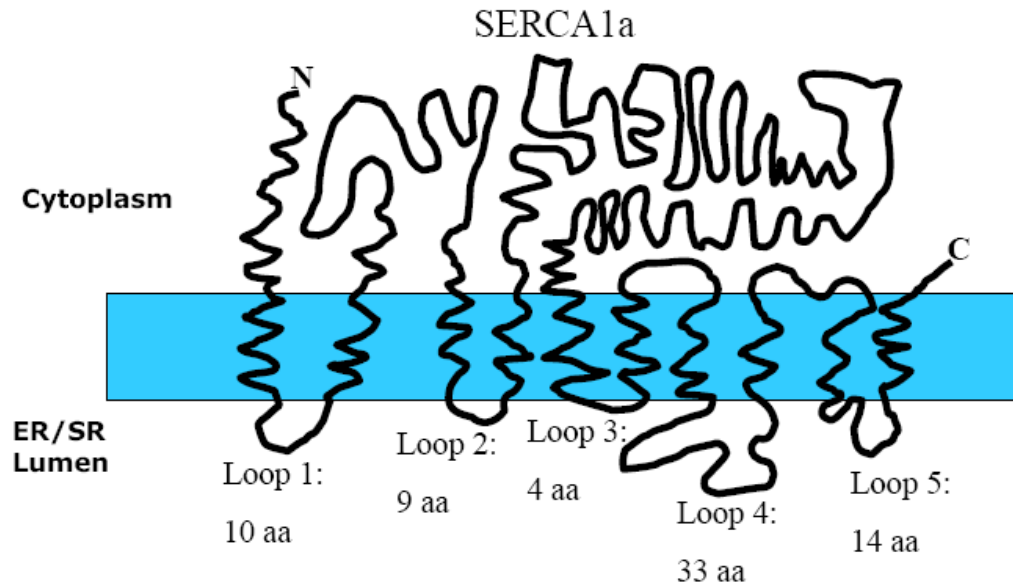


Figure 1.3: Topological map of SERCA1a based upon the elucidated crystal structure of SERCA1a (Toyoshima and Inesi, 2004). Each ER/SR luminal loop is labeled and contains information about the number of amino acids found in each loop. The blue bar represents the ER/SR membrane.

1.9 Objective

It is our hypothesis that SERCA1a is synthesized in the ER membrane and is retained there before its incorporation into the SR membrane. To investigate this hypothesis a novel application of the glycosylation scanning method was used. N-linked glycosylation sites were added to chicken SERCA1a at different ER luminal loops through mutagenesis of cDNA constructs and these mutant SERCA1a constructs were transfected into Ltk- and C2C12 cells to analyze the glycosylation status of the mutant proteins. That was done to determine which cellular compartments SERCA1a travels through before entering the SR.

Chapter 2

MATERIALS AND METHODS

2.1 Site-directed mutagenesis of chicken SERCA1a cDNA

Chicken SERCA1a cDNA contained in the pcDNA 3.1 (+) plasmid (Invitrogen), generated as previously described (Biehn et al., 2004), was used as a template to generate point mutations employing site-directed mutagenesis with overlap extension (Ho et al., 1989). Briefly, this method utilizes four primers for three separate PCR reactions that create a segment of the wild-type cDNA that encodes desired mutations. This mutant segment can be ligated back into the wild-type construct with unique restriction sites. There were two end primers, upstream and downstream, respectively, containing unique restriction enzyme cut sites. If a convenient unique restriction site was not available one was engineered into the primer. The two mutational primers are complementary and incorporate the desired mutations. These lay between the two unique restriction sites as well as the two end primers. A diagram describing the particular site-directed mutagenesis procedure employed can be seen in Figure 2.1. Specific primers used can be seen in Figure 2.2.

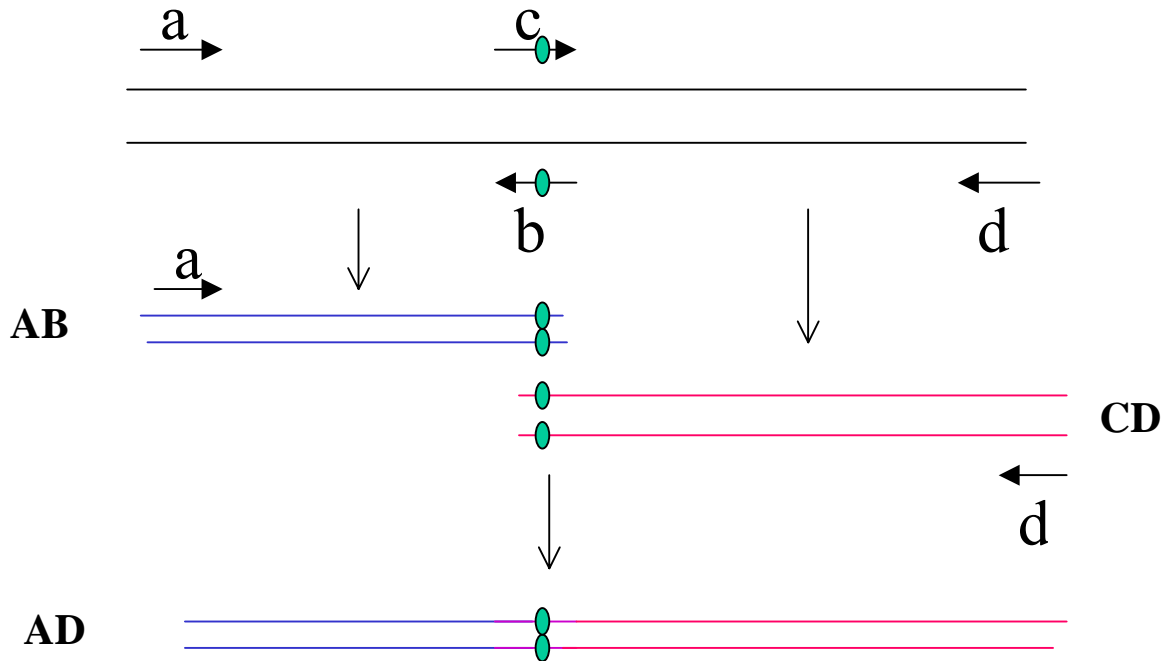
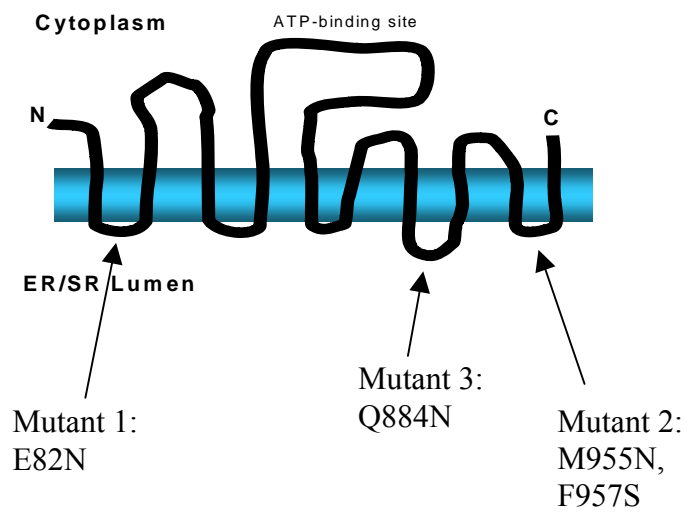


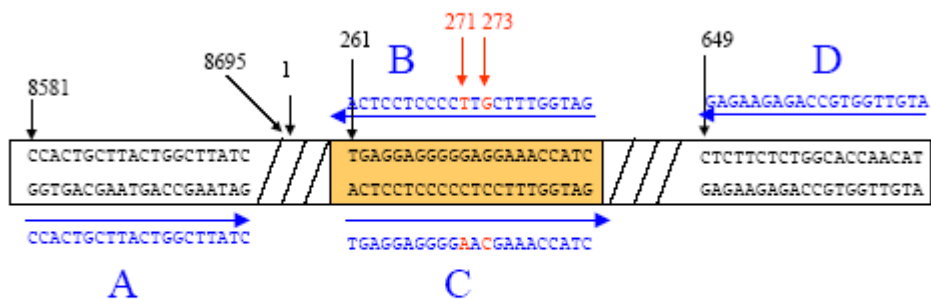
Figure 2.1: The PCR strategy employed to introduce consensus cDNA sequences encoding N-linked glycosylation sites into SERCA1a cDNA. The lowercase letters of a,b,c, and d represent the four different primers used to generate the two segments of SERCA1a cDNA containing the mutation(s) that encode the N-linked glycosylation site. The primers a and b were used to generate the AB segment of SERCA1a and the primers c and d were used to generate the CD segment of SERCA1a in the first set of PCR reactions. The two primers b and c were complementary and both encoded the same mutation(s). When a second PCR reaction is run with SERCA1a AB and CD as the templates, the two PCR products anneal and the two end primers a and d are then used to create the AD molecule which contains the desired mutation(s).

A



B

Mutant 1 Construct



Primers: 5'-3'

A: CCACTGCTTACTGGCTTATC

B: GATGGTTTCGTTCCCTCTCA

C: TGAGGAGGGGAACGAAACCATC

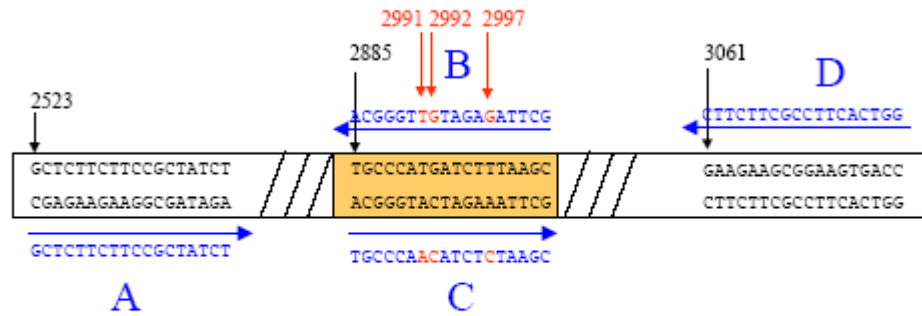
D: ATGTTGGTGCCAGAGAAGAG

Restriction Enzymes Used:

Age I : Cut site begins at base pair 568

Hind III: Cut site begins at base pair 8654

Mutant 2 Construct



Primers: 5'-3'

A: GCTCTTCTTCCGCTATCT

B: GCTTAGAGATGTTGGCA

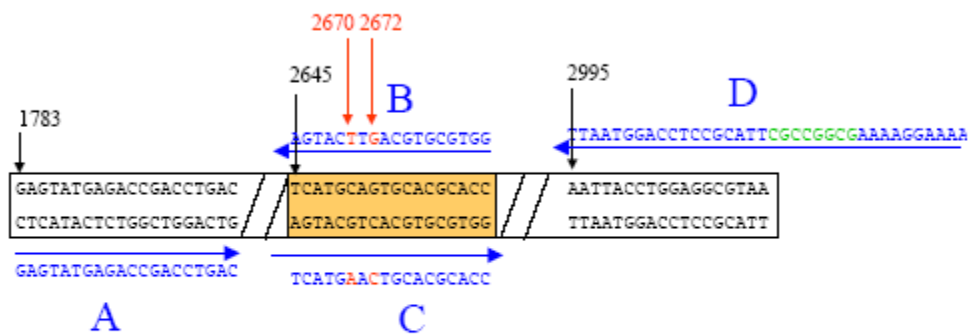
C: TGCCCAACATCTCTAAGC

D: GGTCACCTCCGCTTCTTC

Restriction Enzymes Used:

BbvCI : Cut sites at base pair 2602 and 3047

Mutant 3 Construct



Primers: 5'-3'

A: GAGTATGAGACCGACCTGAC

B: GGTGCGTGCAGTTCATGA

C: TCATGAACTGCACGCACC

D: AAAAGGAAAAGCGGCCGCTTACGCCTCCAGGTAATT

Restriction Enzymes Used:

EcoRI: Cut site begins at base pair 1996

Not I: Cut site was added in with primer D and appears green in primer sequence

C

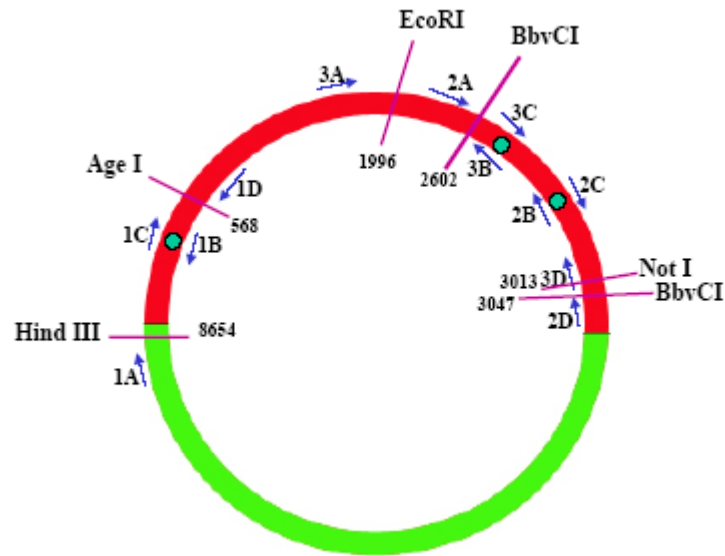


Figure 2.2: Graphical representation of the creation of SERCA1a glycosylation mutant constructs with primers and restriction enzymes used. (A) A line drawing of the chicken SERCA1a protein and where the three separate mutations were made, and what particular amino acid residues were altered to incorporate novel N-linked glycosylation sites into the SERCA1a protein. (B) DNA base changes that were made to generate mutant segments of chicken SERCA1a cDNA. (C) The complete circular SERCA1a-pcDNA 3.1 plasmid with positions of mutations and restriction enzymes. The red part of the construct represents the complete SERCA1a cDNA, while the green part represents the pcDNA 3.1 sequence. Primers for each SERCA1a mutant are represented by blue arrows and have designations of 1-3 A-D depending on the particular primer they represent. Restriction enzyme sites are depicted by purple lines and the particular restriction enzymes used are listed above each line. The cut site position for each restriction enzyme is listed below the purple line within the circle construct. The particular restriction enzymes used for the creation of each mutant can be seen in (B).

The PCR reactions conducted for mutant 1 and 2 cDNA construction were performed for 30 cycles with a 60°C annealing temperature (30 seconds/cycle), 72°C extension temperature (1 min/cycle), and a 94°C denaturing temperature (30 seconds/cycle). Hot Start Taq Polymerase (Qiagen) was used and molar amounts of primers, dNTPs, template DNA, and buffer were used according to manufacturer's specifications. Small samples (1-5µl) of each reaction were run on 1-1.5% agarose gels made with a Tris-borate-ethidium bromide solution (TBE) to confirm amplification efficiency, then larger samples (5-25µl) were run on 0.75-1% agarose gels made with a Tris-acetate-ethidium bromide solution (TAE) and desired bands extracted. In both cases a Multiimager Light Cabinet (Alpha Innotech Corporation) was used to visualize the DNA-ethidium bromide (EtBr) bands. Gel purification through either glass powder purification using glass milk (Bio 101 Inc.) or gel elution spin columns was performed to extract desired PCR products. Gel purified PCR products ('AB' and 'CD') were used as templates for mutant cDNA ('AD') production. The PCR conducted for mutant 3 cDNA used Jump Start Taq Polymerase (Sigma) and needed further optimization from manufacturer's specifications with PCR conditions. For both 'AB' and 'CD' PCR conditions (temperatures, times, and cycle numbers) were kept the same as in the generation of mutant 1 and 2 cDNAs. For the generation of complete mutant 3 cDNA ('AD') 400 nM mutant 3 A and D primers were used in the reaction. The annealing temperature was brought down to 50°C, while the extension and denaturing temperatures

remained at 72°C and 94°C, respectively. The extension time and the annealing time per cycle were increased to 1.5 and 2 minutes, respectively.

2.2 Cloning

After SERCA1a mutant cDNAs were generated they were initially ligated into the pCR[®] 2.1-TOPO vector (Invitrogen) utilizing the TOPO[®] cloning kit per manufacturer's specifications. PCR products had been generated, assayed, extracted from TAE gels and used in TOPO[®] cloning reactions within two days to minimize the degradation of the 3' A overhangs that Taq polymerase adds and which are required for ligation into the pCR[®] 2.1-TOPO vector. *E.coli* TOP10 cells, included in TOPO[®] cloning kit, were incubated with SERCA1a mutant cDNA- pCR[®] 2.1-TOPO constructs on ice for 20 minutes then transformed by a heat shock at 42°C for 45 seconds. Transformed cells were allowed to recover in S.O.C. medium (Maniatis, 1982) for 1 hour then were grown on two 100 µg/ml ampicillin (AMP) plates at 50 and 250 µl spreading volumes for at least 15 hours at 37°C. The 15-hour or greater incubation time was required in order for visible yet individual colony growth to occur. Control ligations that lacked PCR product were also used for transformations and grown on AMP plates. A positive ligation was observed when the control plates had very few (compared to test ligations) or no colonies growing on it after the incubation time period, but test ligation plates had several to hundreds of colonies growing on them. Individual colonies from test ligation plates were selected by sterile toothpicks and dropped inside of a 15 ml push cap tube containing 2 ml of 50

$\mu\text{g/ml}$ Luria broth (LB) AMP. Cultures were incubated at 37°C overnight while on a shaker. After incubation, cultures were miniprepmed to harvest the transforming plasmids they contained. Several miniprep methods were employed throughout span of this project. They included boiling minipreparations (Maniatis, 1982), alkaline lysis (Maniatis, 1982), and Qiagen spin mini prep kits. Isolated plasmids were screened by restriction digestion with EcoRI which has cut sites 3' and 5' of where PCR cDNA is inserted into cDNA- pCR[®] 2.1-TOPO. Positive clones were identified as those that possess an insert of the same size as those produced through final PCR reactions for that particular mutant construct. Positive clones had a portion of their original stock culture used for growth in 50 ml of LB AMP ($50 \mu\text{g/ml}$) overnight at 37°C on a shaker. This large volume of culture was used to perform a "medium" prep with in order to obtain a large quantity of plasmid. This method is a modified alkaline lysis method that includes two phenol chloroform extractions as well as two ethanol precipitations. Also the method makes use of a Sephadex-50 column to remove the free ribonucleotides generated by RNase A. Plasmids concentrations and quality were assessed via a Beckman DU[®] 530 spectrophotometer reading at 260 and 280 nm.

The TOPO vector and cloning kit are very efficient for initial cloning of PCR fragments, however the vector is not suitable for expression in eukaryotic cells. To this end, pcDNA 3.1 was employed. Both wild-type SERCA1a cDNA contained in pcDNA 3.1 and mutant SERCA1a inserts contained in pCR[®] 2.1-TOPO were cut with restriction enzymes to cleave the mutant insert from the pCR[®] 2.1-TOPO vector. The same restriction enzymes were used to cut the wild-type insert from wild-type SERCA1a-

pcDNA 3.1 that corresponded to the mutant segment. The mutant segment was then ligated into the wild-type construct. Figure 2.2 describes the particular restriction enzymes used for each mutant as well as the location of the cut sites. Restriction digestions of plasmids were gel purified and used in subsequent ligation reactions. Ligations were performed in a 10-20 μ l total volume containing appropriate amounts of T4 DNA ligase, T4 DNA ligase buffer, as well as varying amounts of double-cut and gel-purified mutant inserts and SERCA1a-pcDNA 3.1. Ligation reactions were incubated overnight at 14°C then used in subsequent transformations of competent bacterial cells. Competent DH5 α *E.coli* cells, either subcloning or max efficiency, were used for transformations. Transformation with these cells was performed similar to those performed with TOP10 cells except the heat shock was at 37°-42°C for 30-45 seconds. Cells were grown on LB AMP plates in the same way as with TOP10 cell transformations. Control ligations were also performed that contained water instead of mutant inserts. Colonies were picked from positive ligations and grown up in same manner as with TOP10 transformed cells. Again minipreparations were performed to determine which cultures possessed the proper mutant insert. The same restriction enzymes that were used to initially do the double restriction digestion were used for diagnostic mapping. Cultures that possessed plasmids containing desired mutant inserts were used for medium preparations and the plasmids were sequenced to confirm that the desired mutations were present as well as to make sure that no undesired mutations occurred during PCR reactions. Sequencing was performed at the University of Delaware sequencing facility under the direction of Dr. Carl Schmidt's UD genome project

(mutants 1 and 2), and Cornell University's Biotechnology Resource Center's DNA sequencing facility (mutant 3). Sequencing reactions required that both DNA templates and primers be diluted to specific concentrations that they requested in ultra pure water. To ensure that very clean DNA was sent out for sequencing, Qiagen miniprep kits were used.

2.3 Expression and Targeting Analysis

Immunostaining was utilized to evaluate the expression and targeting of SERCA1a. Two mouse cell lines were used extensively for this project. Ltk- mouse fibroblast-like cells were used due to their high transfection efficiency and their ease in cell culturing. C2C12 mouse myoblast cells were used since these are pre-muscle cells and will exhibit a differentiated phenotype in culture. This cell line however has a low transfection efficiency and must be maintained at low cell densities to prevent selective growth of cells that cannot be differentiated. Routine cell culturing involved splitting cell cultures once they were nearly confluent, every 2-5 days, with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA in Hank's Balanced Salt Solution (HBSS) without calcium, magnesium, or sodium bicarbonate) and seeding cells in new flasks, either T-25 or T-75s (Corning) at a low cell density. To split cell cultures, cells were washed with HBSS containing no calcium no magnesium (Cellgro) then trypsin was added and cells were incubated at 37°C for several minutes. Then 10% Fetal Bovine Serum (FBS) Dulbecco's Modification of Eagles Medium with 4.5 g/L glucose and with L-glutamine (DMEM),

DMEM10, was added to each culture and a small portion of this was aliquoted into new flasks. Ltk- cells were used to evaluate whether SERCA1a mutants could be expressed and whether the mutant protein was being trafficked to the ER, as is the case with wild-type SERCA1a. Both Ltk- and C2C12 cells were used to create stable lines expressing mutant SERCA1a. For transient and stable transfections LipofectAMINE 2000 (Invitrogen) was employed. Transfections were carried out with cells growing in 35 mm dishes containing UV-sterilized glass coverslips for transient transfections and 60 mm dishes for stable transfections. After stable lines were created, expression of these lines was evaluated in the same way as transient transfections were, with cells grown on coverslips in 35 mm dishes. C2C12 cells do not grow readily on glass, thus glass coverslips were coated with collagen prior to cell seeding. Amounts of DNA, LipofectAMINE, and serum-free medium (SFM) were used per manufacturer's instructions for culture vessel size, 35 mm or 60 mm. Transiently transfected cells were grown on coverslips for 3 days then fixed with methanol at -20°C for 10 minutes. Prior to fixation, the medium was changed 24 hours post transfection. Either regular DMEM10 was used or DMEM10 with 7 mM N-butyric acid added. N-butyric acid was used to amplify SERCA1a expression in transfected cells for easier evaluation of expression. This was not used with C2C12 cells. Creation of stable cell lines involved initial transfection of cells in a 60 mm dish, then 24 hours later, cells were split 1:10 into three 100 mm dishes using DMEM10 as growth medium. Twenty-four hours after this, the medium was aspirated and DMEM10 containing 400 µg/ml Geneticin was added. This was the selection medium and cells were grown in this medium for several days until

most of the cells had been killed and discrete colonies of positively transfected cells were growing. These colonies were selected using the "scratch and sniff" procedure (Karin, 1999). Briefly colonies were identified and circled on the bottom of the dish with a marker. Using a 200 μ l pipette tip attached to a pipettor, colonies were scratched and slowly aspirated into pipette tip. Next each colony was dispersed into separate wells of a 12-well plate with each well containing one drop of trypsin. Cells were very briefly incubated in trypsin then 1 ml of DMEM10 was added. Twenty-four hours later the medium was aspirated and DMEM10 containing 40 μ g/ml Geneticin was added. This selection medium was used hence forth to maintain stable cell lines. Expression was evaluated for all stable cell lines. Occasionally stable clones would continue to grow in selection medium but only sporadically express SERCA1a as determined through immunostaining. In these cases further subcloning was performed to isolate subclones from the initial expressing clone. This was done by seeding the subclones at one cell per well into a 12-well plate and characterizing each of the subclones for enriched expression.

Immunostaining was performed after cells had been fixed in methanol as previously described. Coverslips were rinsed with PBS and then placed on a rubber stopper with the cells facing up. A blocking solution containing 10% horse serum in HBSS was added to each cover slip and cells were incubated for 1 hour at room temperature. Cells were washed once with PBS, then incubated in blocking solution containing 5 μ g/ml of a monoclonal mouse anti-chicken SERCA1 antibody (5C3) for 1 hour at room temperature. Following this incubation cells were washed twice in PBS then

blocking solution containing 5 $\mu\text{g/ml}$ of polyclonal fluorescein-conjugated goat anti-mouse IgG (Molecular Probes) was added and cells were incubated for 1 hour at room temperature. Following this incubation cells were rinsed with PBS twice then placed, cells facing down, onto a glass slide containing 1 drop of Prolong Gold antifade solution (Molecular Probes) and stored overnight at 4°C prior to assaying on a Nikon Microphot-FX epi-fluorescence microscope. Positively expressing cells were photographed using a Nikon DXM1200 digital camera and imaging was analyzed for targeting compared to cells transfected with wild-type SERCA1a. Stable clones had their expression and targeting evaluated in the same way and, after identification of positively expressing clones, these cells were grown up in a T-75 flask and frozen for storage. To freeze cells, a flask containing cells was rinsed with HBSS, trypsinized as with routine splitting, but then the cells were harvested and placed into a 15 ml conical tube. Cells were pelleted at a medium speed in a clinical centrifuge for 2-3 minutes then resuspended in 3 ml of DMEM10 containing 10% DMSO. Cells were then aliquoted into 1 ml portions in 1.5 ml cryotubes (Corning) and placed in a -80°C isopropanol chamber overnight. The tubes were then transferred to a liquid nitrogen storage unit for long-term storage.

2.4 Total Protein Extraction and Immunoprecipitations

Cells were grown in 10 cm dishes to near confluence then total proteins extracted. Cells were rinsed once with PBS then 500 μl of cell lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1% SDS, and 1 mM DTT was added to the dishes. Lysates were harvested

using a cell scraper then sonicated for ~ 2 seconds until the lysate was uniform. Aliquots from each extract were used for protein quantification and the remaining extract was stored at -20°C.

For protein preparations that were going to be used for immunoprecipitations a different protein extraction method was employed. This method used non-denaturing conditions, but cell lines were grown in the same way as for the other protein extraction method. Cells grown on 15 cm dishes were washed once with PBS, then 5 ml of PBS was added and cells were harvested by scraping them and collecting them in separate 15 ml conical tubes. A 50µl sample was then taken out in order to calculate the total protein concentration. Cells were pelleted at a medium speed for 2-3 minutes in a clinical centrifuge. Cells were resuspended with 0.5 ml of ice-cold cell lysis buffer (1% BSA (w/v) in PBS with 1% (v/v) Triton X-100) and aliquoted into 5ml ultracentrifuge tubes. Tubes were kept on ice until spun. Tubes were placed into ultracentrifuge and spun at 100,000 x g for 30 minutes to clear cell lysate. Supernatants containing the solubilized proteins were transferred to 1.5 ml microfuge tubes and stored in -20°C freezer for later use.

For immunoprecipitations (IPs), agarose-bound Protein G (Kirkegaard and Perry Laboratories) was employed. For IP of SERCA1a the 5C3 monoclonal antibody (mAb) was used and for IP of Na⁺/K⁺ ATPase β-1 subunit (control glycoprotein) a rabbit antiserum against rat/mouse Na⁺/K⁺ ATPase β-1 subunit (Upstate Cell Signaling Solutions) was used. The first step in the IPs was to couple the antibodies to the Protein G beads. To do this 1.5 ml microfuge tubes were filled with 1 ml of ice-cold PBS, one tube

for each cell extract and each protein to be immunoprecipitated. To each tube 10-20 μ l of Protein G agarose bound suspension (50%) was added along with 3-5 μ g of particular antibody for that IP. Tubes were rocked end-to-end for 2 hours at 4°C to couple antibodies to Protein G agarose beads. After 2 hours the beads were spun down and washed three times with cold cell lysis buffer. An equal volume (to bead volume) of 10% BSA in PBS was added to each bead pellet. Then 10-50 μ l protein extract was added and cold cell lysis buffer was added to a final incubation volume of 250 μ l. Tubes were rocked end-to-end overnight at 4°C. After incubation beads pelleted down and the supernatants were collected to test for IP efficiency. Beads were washed four times with cold PBS over a time course of 30 minutes. Beads were then resuspended in 50 μ l of 1X protein sample loading buffer (all amounts in v/v: 2.1% stacking buffer containing 1 M Tris-HCl, pH 6.8, 0.67% SDS, 3.3% glycerol, 0.17% 2-mercaptoethanol, 86.25% water, and bromophenol blue to desired color) and incubated at 60°C for five minutes to elute proteins and antibodies from the protein G agarose beads. Beads were then spun down and supernatant containing protein and denatured antibodies were collected and stored in -20°C freezer.

2.5 Western blotting

For Western blots either poured or pre-cast SDS-polyacrylamide gels were employed. The poured gels were initially used but when better consistency between gels and better resolution was needed to discern mobility shifts the switch was made to pre-

cast gels. Protein samples in 5-30 μ l volumes were mixed with water and 6 X Sample loading buffer (all amounts in w/v: 12.5% stacking buffer containing 1 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1 % 2-mercaptoethanol, 17.5% water, and bromophenol blue to desired color) for poured gels or 2 X sample loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue, 5% 2-mercaptoethanol) for pre-cast gels. Samples in loading buffer were heated to 60°C for 5 minutes prior to loading onto SDS-PAGE gels. Five, seven-and-a-half, and nine percent polyacrylamide gels were poured for use with western blotting, or alternatively PAGER[®] Gold Precast 4-12% Tris-glycine gels (Cambrex) were used. All of the poured gels consisted of a resolving gel which varied with the differing percents of polyacrylamide and a stacking gel which was the same for all the gels. The resolving gel was always poured first and the 9% polyacrylamide gel consisted of (amounts in w/v) 30% acrylamide, 25% 1.5 M Tris-HCl pH 8.8, 0.1% SDS, 44.8% water, 0.05% APS, and 0.05% TEMED. For the 7.5% and 5% gels the acrylamide and water percent volumes were the only two to differ, and they were 25% and 16% for the acrylamide, and 49% and 57% for the water, respectively. The stacking gel consisted of (amounts in w/v) 15% acrylamide, 13.8% 1.5 M Tris-HCl, pH 8.8, 0.1% SDS, 70% water, 0.1% APS, and 0.05% TEMED.

All gels were run at constant voltage of between 200 and 300 volts in a Bio-Rad mini protean 3 system, with the current varying from 20-80 milliamps. The running buffer used for all gels consisted of 25 mM Tris, 190 mM glycine, and 0.1% SDS in water. Gels were run until tracking dye was near bottom of resolving gel or until a desired marker band was near end of gel for better separation of higher molecular weight

proteins. Gels were then placed against an equal-sized piece of nitrocellulose membrane paper (Pall Life Sciences BioTrace NT Pure Nitrocellulose transfer membrane 0.2 μ m) and placed between two sheets of 3 MM paper and two sponges to form a transfer sandwich that was placed into a cassette. Each cassette was placed into an Owl Transfer System gel transfer box. A stir bar was included to keep the transfer buffer moving over the entire cassette to maintain uniform temperature. The Owl Transfer System gel transfer box employs cold tap water to cool the transfer box. Transfer cassettes were lined up with their nitrocellulose side facing the cathode. The transfer buffer contained 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol, and was chilled to 4°C before use. Proteins were transferred to nitrocellulose membranes at 75 volts for 1.5 hours. After transfer, blots were blocked either for 1 hour at room temperature or overnight at 4°C in either 10 ml TBS-T (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.1% (v/v) Tween-20) containing 5% dry milk (w/v) or 10% BSA (w/v) in PBS containing 0.1% (v/v) Tween-20. The latter blocking buffer was used for blots that were probed with biotinylated Concanavalin A (Vector Laboratories) and Avidin coupled to horse radish Peroxidase (HRP) (Pierce), all other blocks used the milk-TBS-T blocking buffer, called BLOTTO. After blocking for one hour or overnight, blots were incubated with primary antibody or lectin. BLOTTO was used for all primary antibody incubations while buffer containing 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 0.1% (v/v) Tween-20 was used for lectin incubations. Blots were incubated with primary antibodies or lectins for 1-2 hours at room temperature or overnight at 4°C both while on shaker. Blots were then washed with TBS-T 3-4 times over 20-30 minutes to wash away

unbound primary antibody or lectin. The blots then were incubated with secondary antibody. For anti-mouse and anti-rabbit HRP antibodies (Sigma), BLOTTO was used as buffer. For Avidin HRP 3% BSA in PBS with 0.1% Tween-20 was used as the buffer. Blots were incubated with secondary antibodies for 40-60 minutes at room temperature while on a shaker. Blots were again washed as before in TBS-T, this time to wash away unbound secondary antibody. Blots were then incubated with HRP substrate, with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech) per manufacturer's instructions, placed in a clear plastic folder and transferred to a film exposing cassette. Kodak Biomax Light Film was placed against the blots inside film exposing cassette and left to be exposed for 1 second to 1 hour, depending on how strong the chemiluminescent signals were. Exposed film was then developed in a Kodak M35A X-OMAT Processor and analyzed.

Chapter 3

RESULTS

3.1 Generation of mutant SERCA1a PCR fragments

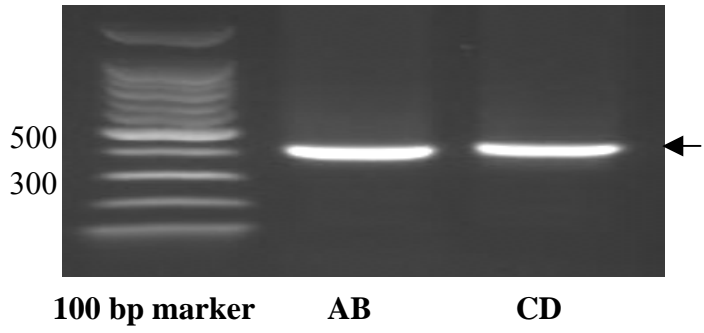
The fast-twitch muscle-specific SERCA isoform SERCA1a was investigated in this study. The intracellular trafficking of SERCA1a in mouse fibroblast-like Ltk- cells was studied using the expression of chicken SERCA1a mutant proteins containing N-linked glycosylation sites, a feature not native to the SERCA1a protein. In order to generate the SERCA1a glycosylation mutant proteins, a site-directed mutagenesis procedure was employed to incorporate specific base changes in a chicken SERCA1a cDNA construct that was placed into the expression vector pcDNA 3.1. These base changes introduced a change in the amino acid sequence of the SERCA1a protein in order to incorporate the consensus sequence for N-linked glycosylation which is asparagine-X-threonine/serine, where X is any amino acid besides proline. The particular mutagenesis strategy employed was called site-directed mutagenesis via PCR through overlap extension (Ho et al., 1989) and is outlined in figure 2.1. After generation of the segment of SERCA1a cDNA, referred to in figure 2.1 as 'AD' which encodes the desired mutation(s), the AD segment was ligated into the pCR[®] 2.1-TOPO vector as described in

methods. The purpose of this step, since the pCR[®] 2.1-TOPO vector is not an expression vector, was to use a high-efficiency cloning step and to increase the efficiency of cutting the PCR product correctly with the desired restriction enzymes. After this step the 'AD' PCR product was cut out of the TOPO vector and ligated back into the wild-type SERCA1a-pcDNA 3.1 construct used as the original template for PCR steps. This was done such that the 'AD' segment with desired mutation(s) could be ligated into the wild-type construct replacing the wild-type segment corresponding to the 'AD' cDNA fragment. This was accomplished using restriction endonucleases to cut out the wild-type segment and to give the 'AD' cDNA fragment compatible ends for ligating into the wild-type SERCA1a-pcDNA construct.

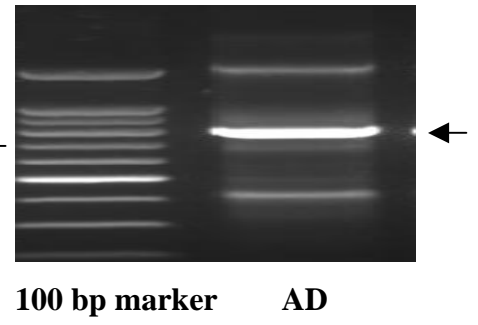
Three SERCA mutant constructs were generated and each mutant construct encoded a single N-linked glycosylation site at a different ER luminal loop. Figure 2.2(A) shows on a basic topological diagram of the SERCA1a protein where the particular mutations were generated and which amino acids were changed to create the N-linked glycosylation sites. Figure 2.2(B) displays line drawings detailing each SERCA1a mutant with details of the primers and restriction enzymes used. Figure 2.2(C) is a depiction of the SERCA1a-pcDNA 3.1 construct with the restriction enzymes used for the cloning and the validation of mutant construct orientations.

Figure 3.1 shows the results from the PCR reactions used to generate the different pieces of the mutagenesis strategy as outlined in Figure 2.1 for each mutant. In some cases non-desired PCR products were generated. However as outlined in the methods section, gel extractions were performed to isolate the desired size PCR generated products from the TAE-agarose gels.

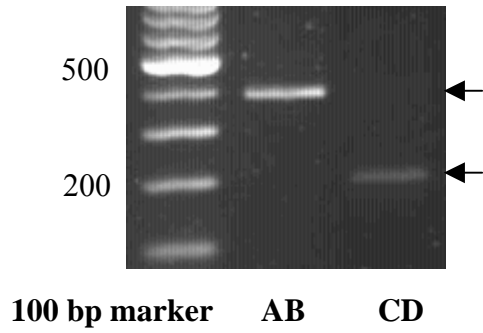
A



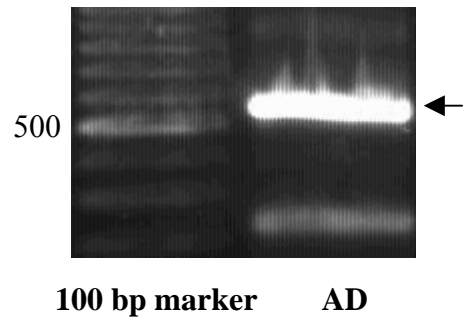
B



C



D



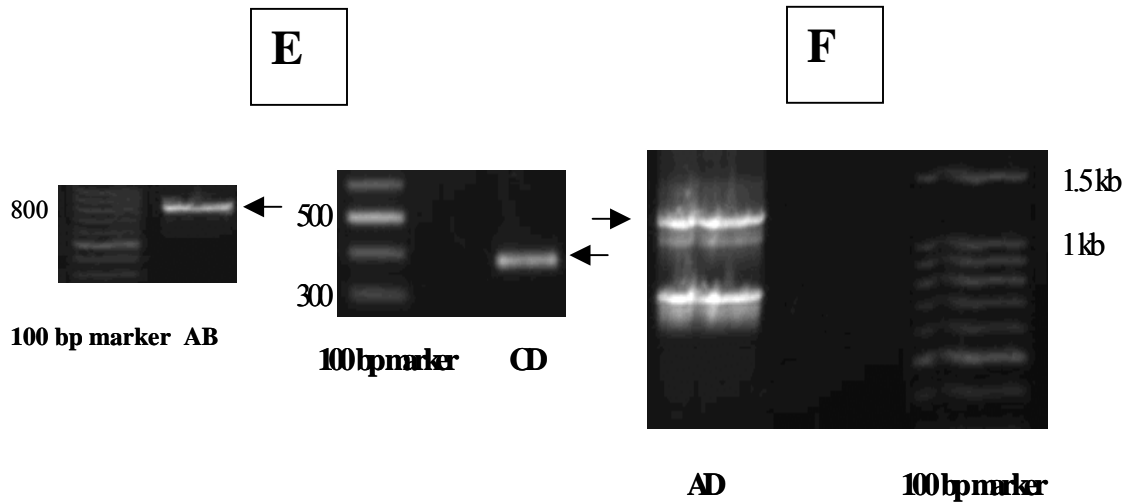
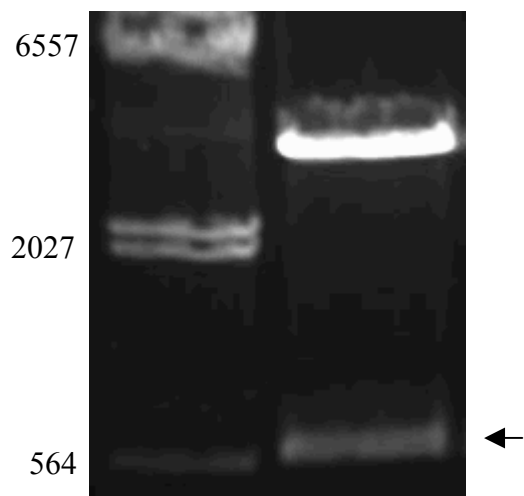


Figure 3.1: PCR-generated segments of mutant chicken SERCA1a cDNA. In each panel the arrows to the sides of the pictures point to the desired PCR product that was gel extracted. Panel (A) shows the PCR products for the generation of the mutant 1 cDNA construct with expected sizes of AB, CD, and AD at 398, 408, and 783 bp, respectively. Panel (B) shows the PCR products for the generation of the mutant 2 cDNA construct with expected sizes of AB, CD, and AD at 379, 193, and 555 bp respectively. Panel (C) shows the PCR products for the generation of the mutant 3 cDNA construct with expected sizes of AB, CD, and AD at 899, 385, and 1247 bp, respectively.

3.2 Creation of mutant SERCA1a constructs

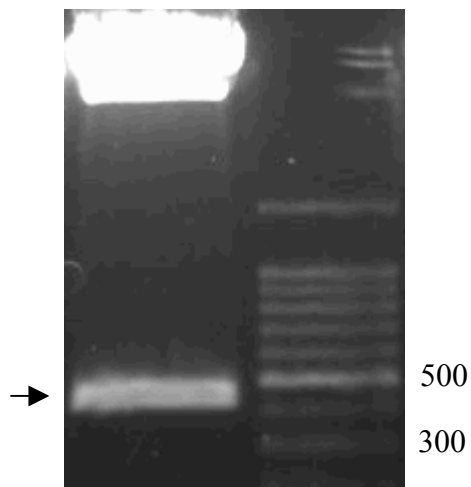
Following the production and isolation of the PCR-generated mutant cDNA segments, the PCR products were ligated into the pCR[®] 2.1-TOPO vector. Since some of the restriction sites were very close to or, in the case of mutant 3 AD, at the end of the PCR fragment, additional DNA was required for the restriction enzymes to properly cut the AD pieces for the next step of cloning. The pCR[®] 2.1-TOPO vector provided extra DNA sequences on either end of the inserted mutant AD PCR generated segments, which enabled more efficient restriction digestion to yield the mutant SERCA1a segments that were then used for subsequent ligation reactions. Figure 3.2 shows gel pictures that contain the results from the TOPO[®] cloning procedure.

A



Lambda/Hind III marker **Mutant 1 AD-pCR[®] 2.1-TOPO / AgeI + HindIII**

B



Mutant 2 AD-pCR[®] 2.1-TOPO / BbvCI **100 bp marker**

C

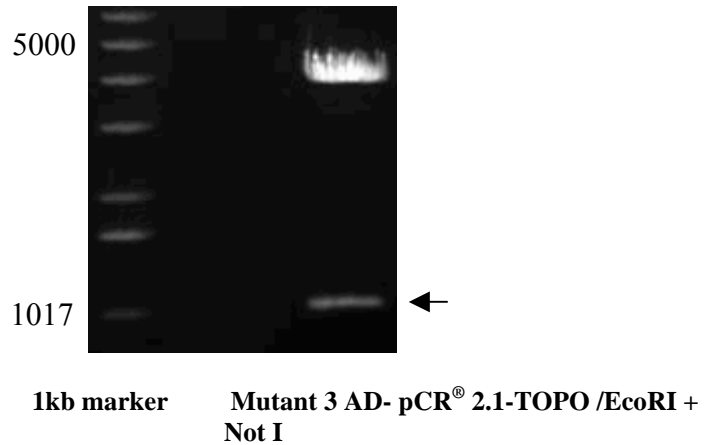
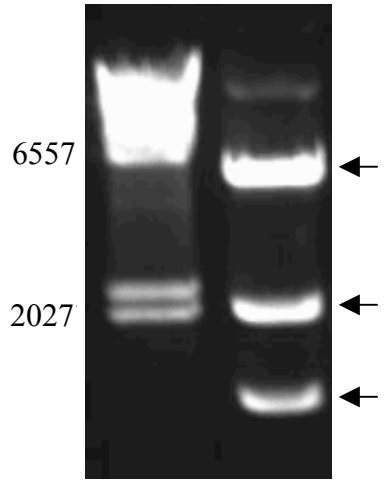


Figure 3.2: Mutant SERCA1a- pCR[®] 2.1-TOPO constructs digested with the same restriction enzymes that were used to cut out the corresponding wild-type segments in the SERCA1a-pcDNA 3.1 constructs. Mutant segments were ligated into the wild-type SERCA1a-pcDNA 3.1 construct. Each panel contains an arrow which points to the desired mutant insert. Panel (A) shows the AD segment generated by PCR for mutant 1 after having been cloned into pCR[®] 2.1-TOPO and then digested with the restriction enzymes Age I and Hind III which yields an insert size of 610 bp. Panel (B) shows the AD segment generated by PCR for mutant 2 after having been cloned into pCR[®] 2.1-TOPO and then digested with the restriction enzyme BbvCI which yields an insert size of 445 bp. Panel (C) shows the AD segment generated by PCR for mutant 3 after having been cloned into pCR[®] 2.1-TOPO and then digested with the restriction enzymes EcoRI and Not I which yields an insert size of 1025 bp.

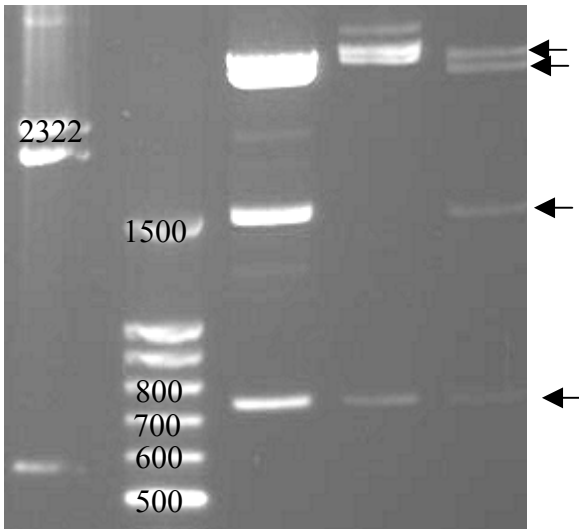
After having accomplished the TOPO[®] cloning for each mutant SERCA1a, competent bacterial cells were used in transformation experiments in order to grow up amounts of constructed plasmids for later use. These bacterial cells were plasmid prepped to harvest the replicated plasmids and individual bacterial colonies were used for this purpose in order to find particular clones that contained the correct plasmid construct. To analyze each clone, the plasmid preparations were digested with the restriction enzymes that were used to give each mutant SERCA1a segment the proper sticky ends for ligation into the wild-type SERCA1a-pcDNA 3.1. The wild-type cDNA segment corresponding to the mutant region was excised. The details of which restriction enzymes were used for each mutant cloning and where these cut sites were located are detailed in Figure 2.2 and Figure 3.2. After digestions were completed and gel electrophoresis was performed to determine which clones contained the correct plasmid construct, the restriction-digested plasmid preparations from the clones containing the correct mutant SERCA1a- pCR[®] 2.1-TOPO construct were run on TAE gels. From the TAE gels the desired mutant inserts were gel purified as outlined in the methods section. Wild-type SERCA1a-pcDNA 3.1 constructs were also restriction digested with the same enzymes as the corresponding mutant inserts and again TAE gels were run with these restriction digestions and the cut plasmid bands were gel purified. These cut plasmid segments along with the gel-purified mutant insert segments of DNA were combined in ligation reactions to yield mutant SERCA1a-pcDNA 3.1 constructs that were then used to transform competent bacterial cells. Figure 3.3 displays the results from these cloning experiments for each mutant SERCA1a-pcDNA 3.1 construct. The 3' untranslated region of SERCA1a was taken out

A



Lambda/Hind III Marker **Mutant 1-
pcDNA 3.1/EcoRI**

B



Lambda/Hind III Marker **100bp Marker** **Invalid Clones** **Mutant 2-
pcDNA 3.1/Nco I**

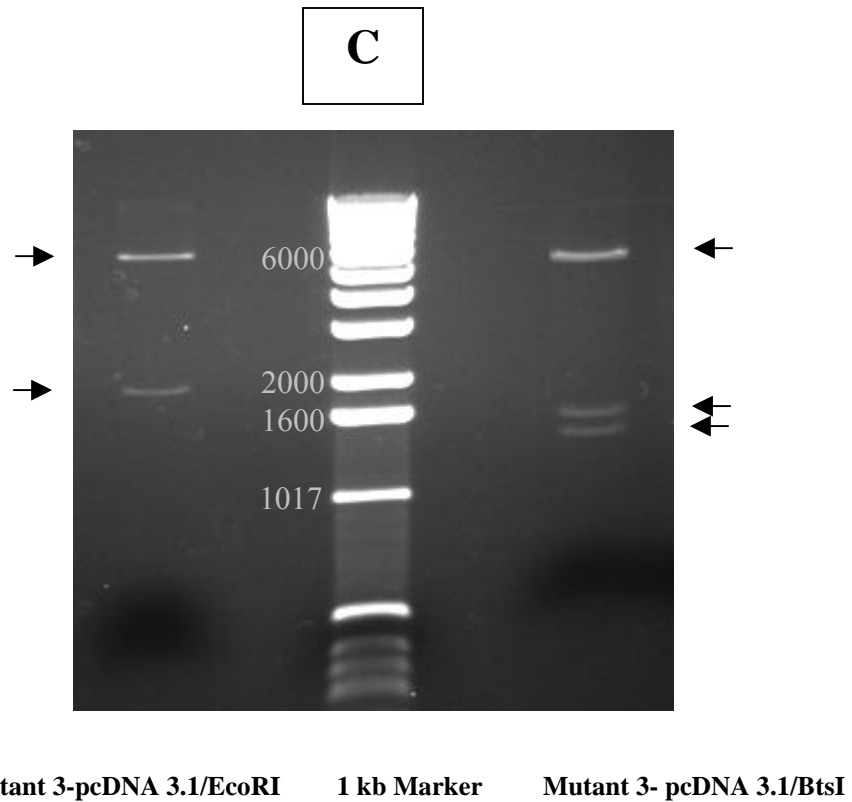


Figure 3.3: Mutant SERCA1a-pcDNA 3.1 cloning results. For each mutant SERCA1a-pcDNA 3.1 construct initial verification of clones that contained correct constructs was done through restriction digestion banding pattern analysis. Each panel contains arrows which point to the expected banding pattern for the restriction digestion of that clone with the particular restriction enzymes listed in each panel. Panel (A) shows the EcoRI digestion of mutant 1-pcDNA 3.1 which yields three bands of 5400, 2000, and 1200 bp. Panel (B) shows the NcoI digestion of mutant 2-pcDNA 3.1 which yields four expected bands of 3342, 3062, 1556, 735 bp. Panel (C) shows the EcoRI digestion of mutant 3-pcDNA 3.1 which yields two expected bands of 6000 and 2000 bp on the left part of the gel, while on the right part of the gel there is the Bts I digestion of mutant 3-pcDNA3.1 which yields four expected bands of 5900, 1500, 1300 and 220 bp.

when the mutant 3 insert was ligated back into the wild-type SERCA1a-pcDNA 3.1 construct during the addition of the Not I restriction enzyme site into the mutant 3 'D' primer.

3.3 Sequence analysis of mutant SERCA1a constructs

Following the construction of the mutant SERCA1a-pcDNA 3.1 constructs, DNA sequencing was performed to validate that the DNA sequences contained desired mutations and to determine if any non-desired mutations were incorporated. Mutant 1-pcDNA 3.1 contained the desired base changes, with only one non-desirable mutation. This non-desirable mutation changed base 505 from a guanosine to an adenosine, however this mutation is a missense mutation such that the amino acid proline is not changed. This mutation should have had no deleterious effects on the expression of this construct. Mutant 2-pcDNA 3.1 sequencing was not successful due to unknown reasons. Several attempts were made at sequencing the construct but none provided interpretable results. It was at this point in the project that it was predicted that SERCA1a mutants 1 and 2 possessed N-linked glycosylation sites that were on ER luminal loops that did not extend far enough into the lumen of the ER to be glycosylated by the oligosaccharyltransferase enzyme complex (Nilsson and Heijne, 1993). It was for this reason that SERCA1a mutant 1 and 2 constructs were no longer used in further experimentation. Mutant 1 however had already been sequenced and its expression analyzed in experiments mentioned later. However it was predicted that the mutant 3

construct encoded an N-linked glycosylation site on an ER luminal loop that did extend far enough into the lumen of the ER to be glycosylated (Nilsson and Heijne, 1993) . Sequencing of mutant 3-pcDNA 3.1 showed that the construct contained the desired mutations, but did contain one non-desirable base change at base 2673 from a guanosine to an adenosine, however this mutation is a neutral mutation such that the amino acid glutamic acid is not changed. This mutation should have had no deleterious effects on the expression of this construct.

3.4 Expression analysis of mutant SERCA1a through transfections of Ltk- and C2C12 cells

Mutants 1 and 3 in pcDNA 3.1 were used to perform transfection experiments on Ltk- (mouse fibroblast-like), and C2C12 (mouse myoblast) cell lines. Both transient and stable transfections were performed. Mutant SERCA1a-pcDNA 3.1 transfections were compared to wild-type SERCA1a-pcDNA 3.1 transient transfections as well as to the cell line LB-6 which is an Ltk- cell line stably transfected with wild-type SERCA1a (Biehn et al., 2004). Mutant 1-pcDNA 3.1 displayed only low levels of expression in the Ltk- transient transfections and no success was achieved in the C2C12 transfections or stable transfections of Ltk- cells. For the transient Ltk- transfections only a very small number of cells were seen to express the mutant SERCA1a, but the expression pattern in these cells showed typical ER staining as compared to wild-type transfections. Figure 3.4(A) shows two typical LB-6 cells expressing wild-type SERCA1a and Figure 3.4(B) shows two Ltk- cells that expressed mutant 1 protein. Many attempts were made at increasing

the transfection efficiency and further cleaning the DNA construct, but all attempts did nothing to vary the results.

Mutant 3-pcDNA 3.1 displayed a higher level of expression than mutant 1 - pcDNA 3.1. Ltk- cells were transfected with mutant 3-pcDNA 3.1 and the majority of cells expressed the mutant 3 protein. Figure 3.4 (C) shows a representative mutant 3-pcDNA 3.1 transiently-transfected cell. Figure 3.4 (D) shows a representative field of view of the stably-transfected cell line from a transfection with mutant 3-pcDNA 3.1 and will be referred to as LS1M3. SERCA1a has some characteristic traits that can be compared between mutant and wild-type to evaluate whether or not the mutations are affecting proper targeting of the protein. Wild-type SERCA1a is synthesized in the ER membrane and is targeted to the ER membrane, the nuclear envelope, and to large ER membrane protrusions the Karin lab has termed plaques (Biehn et al., 2004; Karin et al., 1989; Karin and Settle, 1992). Plaques are observed when high levels of SERCA1a protein are induced in Ltk- cells and only in the presence of the chromatin-remodeling agent N-butyric acid (Biehn et al., 2004). These plaques are seen as the dense areas of fluorescence in Figure 3.4 (A), (C), and (D). More importantly the expression of both wild-type and mutant 3 takes on a typical ER staining with mesh-like expression seen throughout the cytosol of the cell. No expression of wild-type or mutant 3 is observed in the nucleus, which is seen as the dark circular areas inside the cells. However rather dense nuclear envelope staining is observed in both wild-type and mutant 3 transfections.

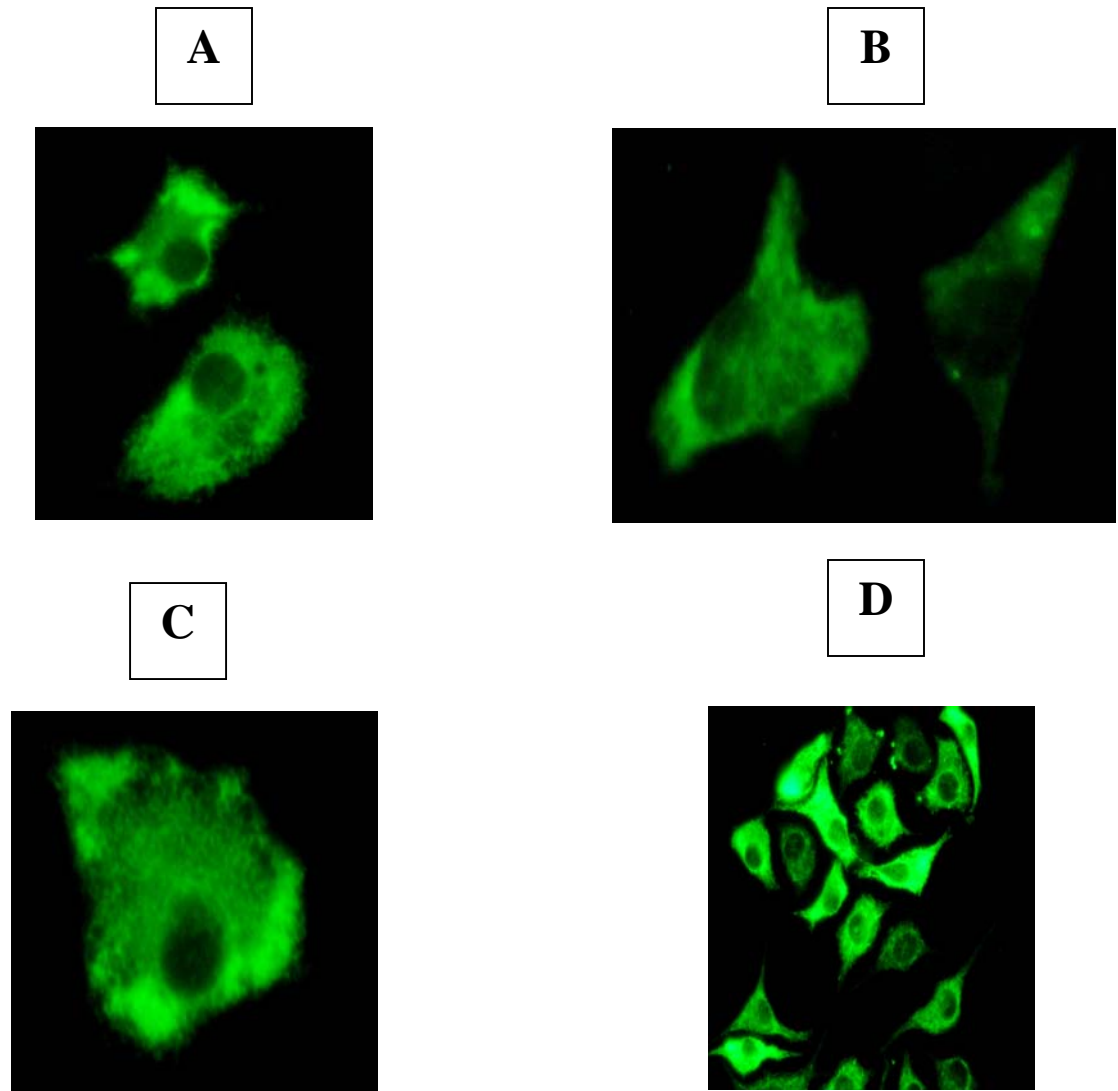


Figure 3.4: Immunostaining of SERCA1a and mutant 3 in transiently- or stably-transfected Ltk- cells. For all the transfections the monoclonal mouse anti-chicken SERCA1a antibody (5C3) was employed as the primary antibody along with the secondary antibody goat anti-mouse FITC antibody for visualizations. Cells were first fixed with methanol then blocked with 10% horse serum HBSS before staining. Panel (A) shows LB-6 cells which are a stable cell line previously made from a transfection with wild-type chicken SERCA1a in the pcDNA 3.1 vector. Panel (B) shows Ltk- cells transiently transfected with chicken mutant 1-pcDNA 3.1. Panel (C) shows Ltk- cells transiently transfected with chicken mutant 3-pcDNA 3.1. Panel (D) shows the LS1M3 cell line stably expressing mutant 3.

As a result of the very low transfection efficiency of mutant 1 in the Ltk- cells, no further analysis could be done with this protein. However mutant 3 protein expression levels in the Ltk- cells and specifically in the LS1M3 cell line were relatively at an equal level to wild-type SERCA1a from the LB-6 cell line.

3.5 Western blotting and detection of glycosylation status of mutant 3

Protein preparations were performed on both LB-6 and LS1M3 cell lines under both reducing and non-reducing conditions for both total protein Western blots and Western blots of immunoprecipitated SERCA1a. SDS-PAGE was performed followed by Western blotting to compare expression levels, but more importantly to assay mutant 3 for its glycosylation status. Initial Western blotting was performed to assay for a mobility shift that would be expected to result from the addition of an N-linked glycosylation moiety. Initial blots showed the appearance of doublets for mutant 3 as well as a mixture lane containing both wild-type and mutant SERCA1a proteins. This result was encouraging since a mobility shift was expected for the glycosylation mutant compared to the wild-type, and the appearance of two bands could mean that only a subset of mutant 3 was being glycosylated. However, the band for wild-type SERCA1a from the LB-6 cell line protein preparations was too dense to determine if it too had a doublet, which would be unexpected since it did not possess an N-linked glycosylation site. However with blot optimization it was revealed that both wild-type and mutant 3 both possessed the doublet that was seen initially. Figure 3.5 shows that wild-type SERCA1a from the LB-6 cell

line, mutant 3 from the LS1M3 cell line, and a mix lane all contain the doublet.

Therefore, the protein doublet was not due to a glycosylation of a subset of mutant 3 protein.

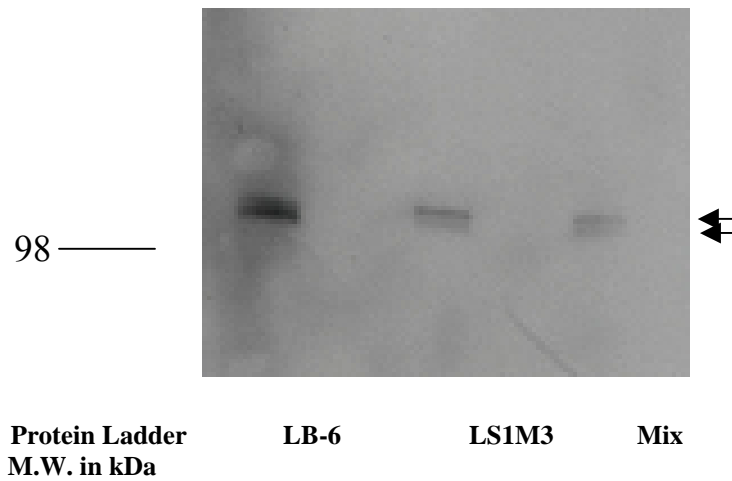


Figure 3.5: Western Blot of total protein extracted from the LB-6 and LS1M3 cell lines. The primary antibody used for this blot was 5C3 at 0.4 $\mu\text{g/ml}$ and the secondary antibody used was anti-mouse HRP at 12.5 ng/ml . The two arrows on the right part of the blot point to the doublet bands of SERCA1a. Wild-type SERCA1a has an observed molecular weight of 110 kDa. The lane marked mix is a mixture lane composed of both LB-6 and LS1M3 total protein.

Mobility shifts can be a difficult characteristic to observe, especially due to a single N-linked glycosylation site and on a large protein (110 kDa). Thus a second strategy for confirmation of N-linked glycosylation was employed. This second strategy was to use the lectin Concanavalin A (Con A) as a probe for N-linked glycosylated proteins. Con A recognizes all alpha 1-3 linked mannose residues, which are part of the core structure that does not get trimmed in any glycosylation events, and thus all N-linked glycosylated proteins contain alpha 1-3 linked mannoses and will be recognized by Con A. Immunoprecipitations were performed to isolate the chicken SERCA proteins, both wild-type and mutant 3, as well as a control protein the Na⁺/K⁺ ATPase β-1 subunit, a known N-linked glycosylated protein predicted to be expressed in Ltk- cells (Lingrel and Kuntzweiler, 1994). Before Con A could be used to determine whether or not mutant 3 was glycosylated, the immunoprecipitations were evaluated by Western blotting with the same primary antibodies used to pull down the respective proteins (5C3 for mutant and wild-type SERCA1a and rabbit anti-rat/mouse Na⁺/K⁺ ATPase β-1 subunit) (Figure 3.6).

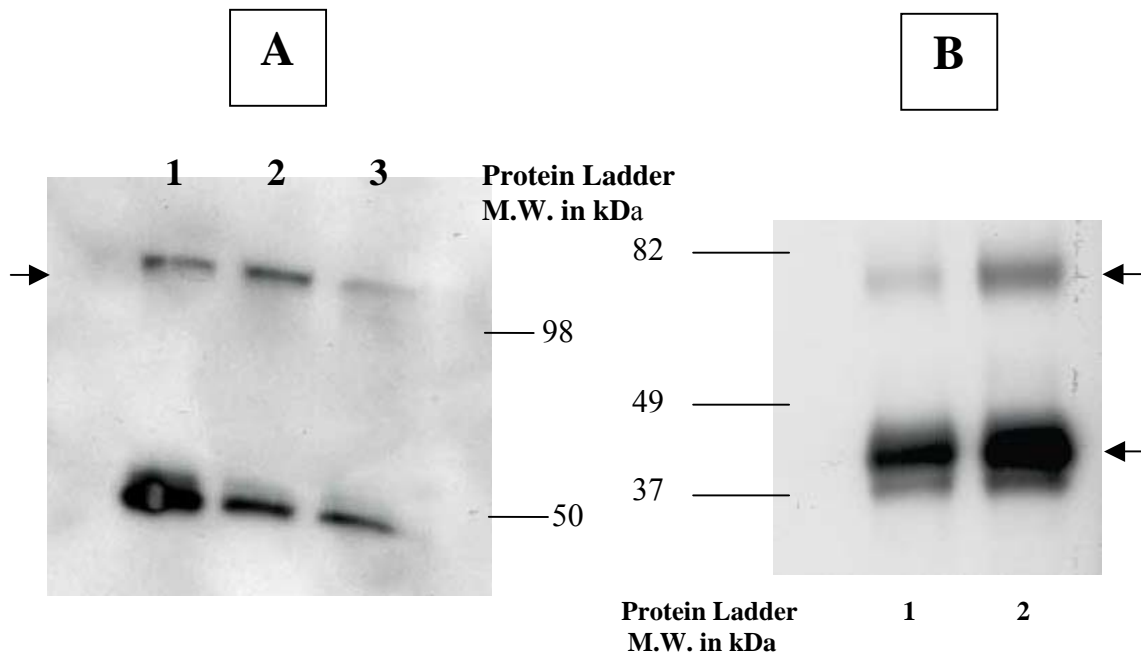


Figure 3.6: Western blots of immunoprecipitations from non-reducing protein preparations from the LB-6 and LS1M3 cell lines. In both panels the arrows indicate the bands of pulled down protein. Panel (A) shows the immunoprecipitations of SERCA1a. The dark bands below SERCA1a at 50 kDa are the mouse IgG heavy chains from the denatured 5C3 antibody used in the immunoprecipitations. Lane 1 is from the LB-6 cell line and is an immunoprecipitation of wild-type chicken SERCA1a. Lane 2 is from the LS1M3 cell line and is an immunoprecipitation of mutant 3 chicken SERCA1a. Lane 3 is from chicken breast muscle and is an immunoprecipitation of wild-type chicken SERCA1a. The primary antibody used was 5C3 at 0.4 $\mu\text{g/ml}$ and the secondary antibody used was anti-mouse HRP at 12.5 ng/ml. Panel (B) shows the immunoprecipitations of Na⁺/K⁺ ATPase β -1 subunit which is between 48 and 52 kDa. The lower indicated bands consist of the Na⁺/K⁺ ATPase β -1 subunit and the rabbit IgG heavy chains from the denatured rabbit anti- Na⁺/K⁺ ATPase β -1 subunit antibody used in the immunoprecipitations. The upper indicated bands are dimers of the Na⁺/K⁺ ATPase β -1 subunit. Lane 1 is from the LB-6 cell line. Lane 2 is from the LS1M3 cell line. The primary antibody used was the rabbit anti-Na⁺/K⁺ ATPase β -1 subunit at 0.4 $\mu\text{g/ml}$ and the secondary antibody used was the anti-rabbit HRP antibody at 12.5 ng/ml.

After the immunoprecipitations of both wild-type and mutant 3 chicken SERCA1a and the Na⁺/K⁺ ATPase β-1 subunit were confirmed to be successful, Western blots were performed using Con A as the probe to detect the glycosylation state of each of the pulled-down proteins. For each immunoprecipitation the IgG heavy chains would be detected since these are N-linked glycosylated. The internal control protein for this experiment was the Na⁺/K⁺ ATPase β-1 subunit, which is a plasma membrane glycoprotein (Lingrel and Kuntzweiler, 1994). Also varying amounts of chicken ovalbumin were used in these Western blots to assay the sensitivity level of the Con A probe. Figure 3.7 shows the results from the Western blot using Con A to detect glycosylation states of the SERCA1a proteins.

The Con A blot showed that mutant 3 was not glycosylated, or not a significant enough amount of the mutant 3 protein was getting glycosylated for detection.

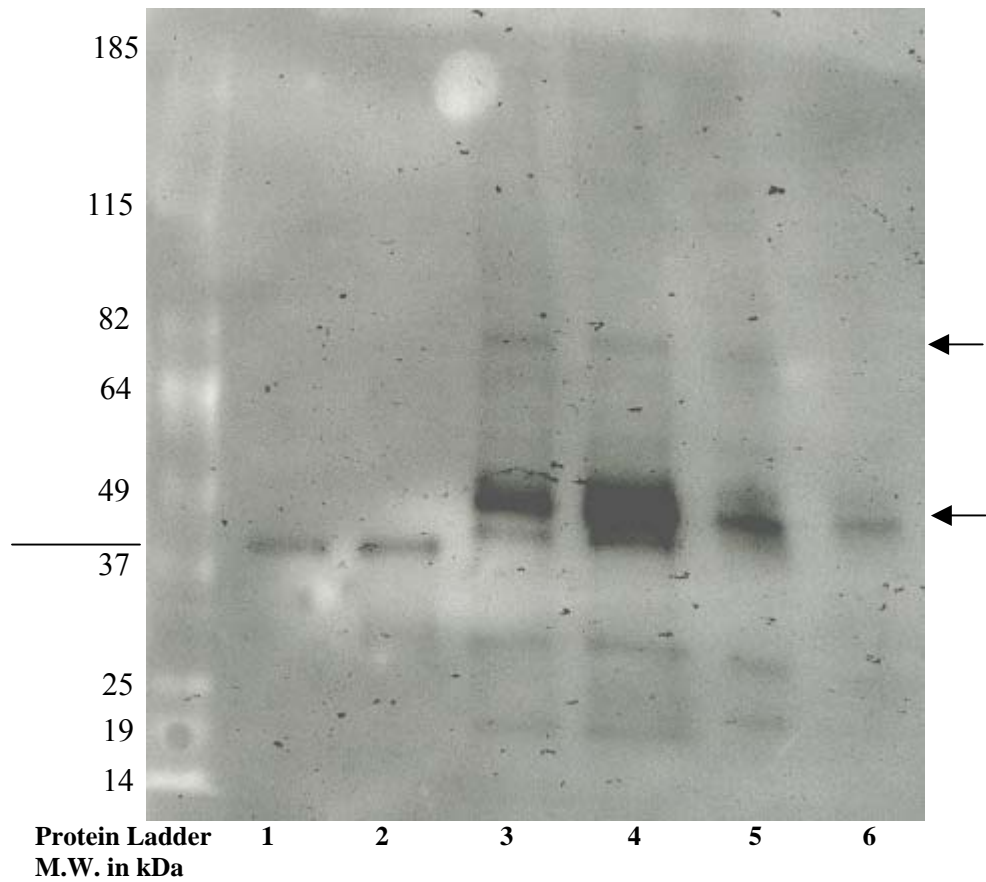


Figure 3.7: Western blots of immunoprecipitations from non-reducing protein preparations from the LB-6 and LS1M3 cell lines probed with Con A. The arrows point to the bands of pulled-down Na^+/K^+ ATPase β -1 subunit (monomer and dimer). The mouse and rabbit IgG chains from the antibodies used in the immunoprecipitations are found at 50 kDa. The line on the left part of the figure indicates where the ovalbumin of M.W. 44 kDa is found. Lanes 1 and 2 were samples of ovalbumin at 50 and 100 ng, respectively. These lanes were used as a positive control for Con A blotting. Lane 3 is from the LB-6 cell line and is an immunoprecipitation of the Na^+/K^+ ATPase β -1 subunit. Lane 4 is from the LS1M3 cell line and is an immunoprecipitation of the Na^+/K^+ ATPase β -1 subunit. Lane 5 is from the LB-6 cell line and is an immunoprecipitation of wild-type chicken SERCA1a. Lane 6 is from the LS1M3 cell line and is an immunoprecipitation of mutant 3 chicken SERCA1a. Biotinylated Con A at 0.5 $\mu\text{g}/\text{ml}$ was used as the primary probe in this blot, with Avidin HRP at 4 ng/ml as the secondary probe, which binds to the biotin part of the biotinylated Con A. The expected M.W. of the Na^+/K^+ ATPase β -1 subunit in mouse is 48 kDa while the expected M.W. of wild-type (non-glycosylated) SERCA1a is 110 kDa.

Chapter 4

DISCUSSION

Three N-linked glycosylation mutant chicken SERCA1a cDNA constructs were created to map the intracellular trafficking of the SERCA1a protein in mouse fibroblast and myoblast cells. N-linked glycosylation can be used to map intracellular trafficking of a protein since different cellular compartments, beginning with the ER and ending with the trans Golgi compartment, process glycoproteins differentially. Figure 1.2 outlines all of the N-linked glycosylation events that take place inside each of the relevant cellular compartments. Briefly, a high-mannose glycosylation moiety is added to a newly-synthesized soluble protein in the ER lumen or a transmembrane protein ER luminal loop. If the protein were retained in the ER, then this high-mannose glycosylation moiety would remain on the glycoprotein. If the glycoprotein travels to the cis Golgi compartment trimming events take place to reduce the number of mannoses on the N-linked glycosylation moiety. Further trimming events followed by varying sugar additions take place in the medial and trans Golgi compartments if the glycoprotein travels through these compartments. The particular glycosylation state a glycoprotein has can be determined to reveal through which cellular compartments the glycoprotein has traveled. The significance of mapping the intracellular trafficking of SERCA1a was to

validate the hypothesis that SERCA1a is synthesized at the ER and is retained in the ER before it travels to the SR. If this hypothesis were validated then it would support a larger hypothesis that SERCA1a plays a significant role in driving the biogenesis of the SR. SERCA1a is a large transmembrane protein that is found in densely-packed arrays in the SR membrane. SERCA1a is highly expressed at the onset of myogenesis, which also corresponds to the first observations of SR formation in muscle cells. Due to SERCA1a having high levels of expression at the onset of SR formation and SERCA1a being found in arrays once synthesized, the hypothesis can be made that SERCA1a is playing a significant role in SR biogenesis. SERCA1a could recruit lipids, and large amounts of SERCA1a found in arrays would allow for a significant increase in lipid content to drive the new membrane formation of the SR.

The particular mutagenesis strategy undertaken to create the mutant SERCA1a constructs involved PCR reactions that to generate relatively short segments of cDNA, well below the error rate for DNA Taq polymerase. This was done to minimize the chance of random mutations being incorporated into the desired segments of DNA. Also this enabled the creation of constructs known to contain the desired mutation to be placed into the wild-type construct with the corresponding mutated segment already cut out. This method of mutagenesis increased our confidence in the fact that these constructs contained a homogeneous collection of mutant constructs. This was very important since the only differences between wild-type and mutant constructs were the additions of the N-linked glycosylation sites and at the protein level this would not be able to be detected until well into the project.

Mutant 1 was shown through restriction digestion and sequence analysis to contain the correct mutations that encode the consensus sequence for the N-linked glycosylation site. This mutant construct was transfected into Ltk- and C2C12 cells but the expression levels of the protein were not adequate enough to do further analysis of the glycosylation status of the protein. There are several reasons why this could have occurred. Mutation 1 SERCA1a changed amino acid 82 from a glutamic acid to an asparagine. While this mutation does not alter this amino acid residue by a large size difference, such as would be if a glycine or alanine were incorporated, it did change a conserved amino acid and resulted in a charge change. This glutamic acid is conserved in chicken SERCAs 2a and 2b as well as in chicken SERCA3 and in mouse and rabbit SERCA1a. Unfortunately, due to a very small ER luminal loop, 10 amino acids long, that the mutation was incorporated in, no better choice of a single amino acid residue could have been mutated to generate a glycosylation site. The size of the first SERCA1a ER luminal loop was later predicted to be too small to be glycosylated since the oligosaccharyltransferase enzyme complex requires an ER luminal loop of a protein to be glycosylated to be at least 24 amino acids (Nilsson and Heijne, 1993). It is believed that changing this conserved amino acid residue altered the conformation of the SERCA1a protein such that it could not fold correctly after this point in its synthesis. The size difference between a glutamic acid and an asparagine is very slight, however it might be significant enough to sterically affect the proper folding of the first SERCA1a luminal loop, especially since this residue lies in the bend in the loop. Also, the charge change could affect amino acid interactions and result in an alteration of folding in the loop.

Alternatively, perhaps N-linked glycosylation did occur, despite the short ER luminal loop, and this created a steric hindrance great enough to affect proper protein folding. Since this mutation was incorporated at the first ER luminal loop, this corresponded to a mutation in the first part of the protein (mutation at amino acid 82 of 994 total amino acids) and if this significantly changed the folding of the protein here, it would potentially have deleterious effects for the rest of the protein. The protein could have been so misfolded that it was degraded, or it could have been misfolded such that the epitope which the 5C3 antibody recognizes could have been sterically blocked. In either case the result was undesirable since in order to investigate the intracellular trafficking of SERCA1a, the protein must have the same conformation as does the wild-type SERCA1a protein.

After the crystal structure of SERCA1a in both its E1 and E2 conformations was released in 2004 (Toyoshima and Inesi, 2004), more information about the first transmembrane domain became available. In discussing the crystal structure of SERCA1a as the protein shifts from E1 to E2, the authors describe how the first alpha helical transmembrane domain undergoes significant movement to the point of part of being pulled out of the membrane into the cytoplasm with the large conformational change that takes place in SERCA when it is pumping calcium into the lumen of the ER/SR. It can be theorized then that because this movement is taking place, altering a conserved amino acid that is part of the first ER luminal loop attached to the first transmembrane alpha helix might disturb this movement. Thus another alternative to the protein not folding correctly would be that the protein would fold relatively normally at synthesis, but as

soon as it begins calcium pumping activity the protein cannot properly change between E1 and E2 conformations and this might signal for its own degradation.

In either case the expression of mutant 1 was very minimal in Ltk- cells and not apparent in C2C12 cells at all. The reasons for this could have been one or both of the theories described above, but no conclusions can be made without further experimentation. However, because there were a small number of cells that did express mutant 1 it could be concluded that at least in some cells, the mutant 1 protein was folding correctly enough to be recognized by the 5C3 antibody.

The sequence of mutant 2 cDNA was never confirmed due to the failure of all sequencing attempts. The expression levels of mutant 2 were undetectable. Several attempts were made at cleaning up the mutant 2-pcDNA 3.1 construct for better sequencing and transfection results but no positive results were observed. Mutant 2 also was not further pursued experimentally because of a report describing the optimal distance an ER luminal loop must descend into the lumen of the ER to get glycosylated (Nilsson and Heijne, 1993). This paper concludes that the minimal distance required for the oligosaccharyltransferase enzyme complex to transfer the initial high-mannose glycosylation moiety to the asparagine residue belonging to the N-linked glycosylation consensus site of the translating protein is 12-14 amino acid residues from the luminal leaflet of the ER membrane. This means that in order for a transmembrane protein to be N-glycosylated, it would require an ER luminal loop to be at least 24-28 amino acid residues long for the oligosaccharyltransferase enzyme to catalyze the glycosylation transfer to the protein. When the mutagenesis of SERCA1a to incorporate novel N-linked

glycosylation sites was undertaken it was assumed that as long as the N-linked glycosylation site was facing the ER lumen the site would be glycosylated, based upon the study showing that a tri-peptide could be glycosylated in the ER lumen (Wieland et al., 1987). However this soluble tri-peptide would be highly mobile in the ER lumen and would inevitably end up near the oligosaccharyltransferase enzyme in such a way as it could get glycosylated. A transmembrane protein however is bound at the ER membrane and does not have the same mobility inside the ER lumen. Mutant 2 contained mutations M955N and F957S in the fifth and last ER luminal loop, which contains 14 amino acids. Neither mutants 1 and 2 contained the N-linked glycosylation sites in ER luminal loops that descended far enough into the ER lumen as to get glycosylated. As a result, the decision was made to stop pursuing these two mutant SERCA1as since the proteins needed to be N-glycosylated in order for any conclusions to be made about the intracellular trafficking of SERCA1a. As to the reasons that mutant 2-pcDNA 3.1 could not be sequenced or expressed, no firm conclusions can be made. One possible explanation for the construct not having the ability to be sequenced could be that the construct was just not clean enough to get sequenced, even after several attempts at correcting this. Restriction analysis was performed on the construct, however restriction analysis can be performed on DNA that is difficult to sequence, since restriction digestions do not require the DNA to be as clean as DNA used for sequencing. As far as the mutant 2 protein not being seen with immunostaining procedures, this could have been for several reasons. As in the sequencing problems, the mutant 2-pcDNA 3.1 construct could have contained too many inhibitory contaminants to be properly

expressed. Another possibility is that with the two amino acid changes that were made, M955N and F957S, significant folding problems with the protein could have arisen and led to the protein's degradation. The change from a methionine to an asparagine does not elicit a drastic change in size but does result in a charge change which could significantly affect protein folding. The change from a phenylalanine to a serine does result in a significant change in size at that residue, and this could lead to protein misfolding. Amino acid alignment analysis showed that the methionine at amino acid 955 is not conserved among chicken SERCAs 2a, 2b, or 3 and not conserved between mouse and rabbit SERCA1a. However, the phenylalanine at amino acid 957 is conserved between chicken SERCAs 2a, 2b, and 3 as well as in SERCA1a of mouse and rabbit. The bulky ring structure of phenylalanine could be involved with proper folding of SERCA1a, and this being changed to a serine could have deleterious effects on proper protein folding.

Mutant 3 was sequenced and shown to possess the desired mutations and was expressed in Ltk- cells very efficiently, but only very minimally in C2C12 cells. Attempts at using the C2C12 cell line were fruitless, as these cells have a very poor transfection efficiency. Initial successful stable transfections, where the C2C12 cells expressed mutant 3, showed diminished expression of mutant 3 over a short amount of time, even in selection medium. It was for this reason that the analysis of mutant 3 was then carried on solely in the Ltk- cell line, with the stably-transfected LB-6 cell line for wild-type chicken SERCA1a and with LS1M3 for mutant 3 chicken SERCA1a.

Protein preparations of both the LB-6 and LS1M3 cell lines were performed and comparisons of wild-type chicken SERCA1a from the LB-6 cell line and mutant 3 from

the LS1M3 cell line were accomplished by SDS-PAGE and Western blotting. Mobility differences between wild-type and mutant 3 proteins were not observed. This was the first indication that the mutant 3 protein had not been glycosylated. The conclusive experiments, however, were the Western blots probed with Con A. A glycoprotein with an alpha 1-3 linked mannose in its structure would be detected by Con A. Mutant 3 was not detected by the Con A probe, and thus the conclusion can be made that it was not glycosylated. Had mutant 3 been glycosylated, further analysis could have been performed to determine the precise glycosylation pattern it contained by using Endoglycosidase H (Endo H). Endo H cleaves high mannose glycosylation moieties off of glycoproteins that are retained in the ER or only travel through the cis Golgi compartment. These glycoproteins are termed Endo H sensitive. Glycoproteins that have traveled through the medial and trans Golgi compartments do not have their glycosylation moieties cleaved by Endo H and are termed Endo H resistant. Tunicamycin inhibits N-linked glycosylation from occurring and this would have been used as a control to show that the N-linked glycosylation on mutant 3 could be prevented.

The outcome of this project, although not desired, was a known possibility given the experimental design. The technique of adding N-linked glycosylation sites into transmembrane proteins and analyzing glycosylation status, called glycosylation scanning, has been performed by several other research groups (Hamilton et al., 2001; Hresko et al., 1994; Schmidt-Rose and Jentsch, 1997). In these studies N-linked glycosylation sites were added to ER luminal loops, however they were added not just as the consensus N-linked glycosylation sequence (N-X-S/T). Instead, segments of either

that particular protein that were known to get glycosylated (Hresko et al., 1994; Schmidt-Rose and Jentsch, 1997) or segments of the Glut-1 protein known to get glycosylated (Hamilton et al., 2001) were added to segments of the proteins that did not contain any N-linked glycosylation sites. In all of these cases the focus of the study was to determine the topology of the protein, not its intracellular trafficking, and thus significant mutations of the ER luminal loops were not an issue. However, potential ER retention information could possibly be encoded within any of SERCAs ER luminal loops, and for this reason the mutations that were performed in SERCA1a were kept to a minimum to preserve the intracellular trafficking that might occur if this retention signal was altered. These researchers used a cell free system utilizing microsomes and attached ribosomes to synthesize and glycosylate the proteins and then examine the glycosylation status of the incorporated N-linked glycosylation sites. In our investigation of the intracellular trafficking of SERCA1a we employed the glycosylation scanning technique to assay for which cellular compartments SERCA1a traveled, and to do this transfections of live cells were required. This was in contrast to the previous applications of the glycosylation scanning method, and makes our approach a novel use of the technique. The other research groups were able to yield efficient glycosylation of the N-linked glycosylation sites they incorporated that faced the ER lumen, however, protein folding could have been significantly altered by their mutations. Since they were using a cell free system these mutant proteins, even if folded incorrectly, would not need to be targeted correctly or even be subject to enzymes that would recognize their misfolded shape. Enzymes found in the cytoplasm of cells can recognize misfolded transmembrane proteins and

bring about their degradation. For our investigation of the intracellular trafficking of SERCA1a it was essential to keep wild-type targeting information the same in the constructed mutants. There are several potential reasons that mutant 3 was not N-glycosylated. The addition of the consensus N-linked glycosylation sequence of N-X-S/T was reported to be enough to signal the oligosaccharyltransferase enzyme complex to transfer the initial high-mannose glycosylation moiety to the SERCA1a protein (Wieland et al., 1987). However, this was found not to precisely be the case, as there is a minimum depth that the consensus sequence needs to be in the ER lumen in order for the complex to recognize it. However, also in the paper describing this minimal depth (Nilsson and Heijne, 1993) it was theorized that the N-linked glycosylation site needs to be in a parallel orientation with the ER membrane. The fourth ER luminal loop where mutant 3 had mutations performed to add the consensus sequence encoding a N-linked glycosylation site is 33 amino acids long and according to the crystal structure and deduced topological amino acid map (Toyoshima and Inesi, 2004) this loop is bent on itself and contains a cysteine-cysteine bond. Perhaps the location of the added N-linked glycosylation site is not parallel to the ER membrane enough to be N-glycosylated which would result in steric hindrance to the oligosaccharyltransferase enzyme complex such that it cannot recognize or properly transfer the high mannose glycosylation moiety to the N-linked glycosylation site. This theory seems plausible since in the studies utilizing the glycosylation scanning strategy (Hamilton et al., 2001; Hresko et al., 1994; Schmidt-Rose and Jentsch, 1997) these researchers were able to make mutant proteins that were N-glycosylated, but they did not incorporate just the consensus N-linked glycosylation

sequence. Instead these groups added a small region already containing a N-linked glycosylation sequence known to be normally N-glycosylated. These regions containing the N-linked glycosylation site known to be glycosylated in their native locations have a conformation that is recognized by the oligosaccharyltransferase enzyme complex. In our investigation it was necessary to add just the N-linked glycosylation site, which differs from these previous studies. Perhaps besides the stringent requirement for the N-glycosylation consensus sequence there is a conformational requirement of the ER luminal loop to allow access by the oligosaccharyltransferase enzyme complex to transfer the high mannose glycosylation moiety.

No conclusions about the intracellular trafficking of SERCA1a can be made from the results of this investigation. Further experimentation with mutant 3 should be performed to confirm that this protein is not N-glycosylated. One method of further testing this, which would have been performed had time allowed, would be to measure the radioactivity of tritiated glucosamine incorporation into mutant SERCA1a. If measurable amounts of radioactivity compared to positive and negative controls are observed then mutant 3 is indeed N-glycosylated. This would mean perhaps that the Con A probing of Western blots of immunoprecipitations of mutant 3 were not sensitive enough to detect N-glycosylated mutant 3. This would be of great surprise, however, since the control glycoprotein, the Na^+/K^+ ATPase β -1 subunit that was pulled down in an immunoprecipitation from both LB-6 and LS1M3 cell lines was detected by the Con A probe. It seems likely that if the mutant 3 protein were N-glycosylated it would be detected by the Con A probe more so than Na^+/K^+ ATPase β -1 subunit since the

SERCA1a mutant is being highly expressed behind the strong CMV promoter. However, one alternative could be that only a small amount of mutant 3 is N-glycosylated and that this small amount is below the sensitivity of Con A. If this were the scenario the tritiated glucosamine experiments would prove to be very beneficial since only the N-glycosylated population of mutant 3 would be detected, which might clarify the results of the glycosylation status analysis.

A completely different approach to answer the question of what cellular compartments SERCA1a travels through before arriving at the SR might be to employ Green fluorescent protein-tagged (GFP) SERCA1a constructs. A GFP-chicken SERCA1a construct has already been created, but its transfection and analysis in primary chicken myotubes was not successful. These experiments could be reexamined utilizing colocalization with known ER, Golgi apparatus, and SR fluorescently-tagged proteins to determine the intracellular trafficking pattern of SERCA1a.

Further experimentation needs to be performed to answer the main question that this investigation posed. Similar and drastically different experimental approaches have been outlined to accomplish this, and one of these should prove successful.

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