The Targeting Mechanism and Membrane Topology of Tail-Anchored Proteins.

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Membrane Targeting of Tail-Anchored Proteins

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"If those committed to the quest fail, they will be forgiven. When lost, they will find another way. The moral imperative of humanism is the endeavor alone, whether successful or not, provided the effort is honorable and failure memorable"

Edward O. Wilson *Consilience*

Abstract

Tail-anchored proteins are integral membrane proteins that are characterized by a transmembrane domain at the carboxyl-terminus of the protein. The correct targeting and localization of tail-anchored proteins is essential for cell function and viability. In this thesis, the targeting mechanism and membrane topology of tail-anchored proteins were examined using a cell-free assay system.

Initially, the targeting of tail-anchored proteins was thought to occur via spontaneous integration into the membrane bilayer. However evidence is presented in this thesis to suggest that more than one targeting mechanism exists. Unlike the spontaneous insertion of tail-anchored proteins such as cytochrome b5, one family of proteins (that includes proteins called Vamps) is shown to require both ATP and a membrane-bound receptor to mediate integration into the bilayer of the endoplasmic reticulum. The minimal region on Vamps that is necessary and sufficient to confer ATP-dependent and receptor mediated targeting was identified. Within this region are four lysine residues spaced along an amphipathic helix that was found to be necessary for binding of Vamps to endoplasmic reticulum.

While spontaneous insertion is sufficient to account for membrane integration for cytochrome b5 and Bcl-2 data was obtained suggesting that specific localization is achieved by regulating targeting. The membrane topology of one of this class of tail-anchored protein was examined using chemical modifying techniques. The membrane

topology of Bcl-2 is shown to be dynamic, as it changes in cells treated with chemotherapy drugs. This represents the first evidence of a tail-anchored protein that changes membrane topology in response to a cellular signal.

Dedication

I would like to dedicate this work to my dad and my mom, Ui-Hwi and Eun-Suk Kim whose commitment and emotional support made possible the work undertaken here. And to my brother and best friend James who never asked that dreadful question: "So…when are you going to finish?"

Acknowledgements

Firstly I wish to thank Dr. David Andrews for patiently mentoring me. I am forever grateful for his patience and guidance, and for showing me the joys of science.

I also thank Dr. Brian Leber for teaching me to smile even when all hell breaks loose. I also thank my other supervisor committee members Dr. Gerard Wright and Dr. Richard Epand for their advice and insights.

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Finally I thank Jung, Rob, Esther, Linda, and James for being the best cheering squad.

List of Abbreviations

ATP	Adenosine Triphosphate
Cb5	Cytochrome b5
CRM	crude rough microsome
CTS	Carboxyl-Terminal Sequence
Cys	cysteine
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
ER	Endoplasmic Reticulum
G-25	Sephadex G-25
HPLC	High Performance Liquid Chromatography
IASD	4-acetamido-4'-[(iodoacetyl)amino)stilbene-2,2'-disulfonic acid
KRM	potassium-treated rough microsome
LUV	Large Unilamellar Vesicles
mRNA	messenger Ribonucleic acid
MSF	Mitochondrial import Stimulation Factor
mT	middle-T antigen
MTS	Mitochondria-Targeting Signal
NTS	Amino-Terminal Sequence
PBS	Phosphate-Buffer Saline
PS	Phosphatidylserine
PM	Plasma Membrane
RNA	Ribonucleic acid
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SRP	Signal Recognition Particle
SUV	Small Unilamellar Vesicles
S.V.	Small Vesicle
TM	Transmembrane

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Chapter 1

General Introduction

1.1. Protein Targeting Mechanisms

Of the thousands of eukaryotic protein sequences in the growing genomic databases, 30%-40% of these proteins have been estimated to be integral membrane proteins (Wallin & von Heijne 1998, Boyd et al 1998). The correct targeting of these membrane proteins is essential for both the viability and function of the cell as they are involve in many cellular processes. In fact, mis-targeting of membrane proteins has been implicated in diseases such as Alzheimer's, Parkinson's, and Prion diseases (Checler & Vincent 2002, Mailman et al 2001, Di Monte et al 2002). Therefore, to further understand the various cellular processes and diseases related to integral membrane proteins intense research into targeting mechanisms has been undertaken during the last twenty years. Protein targeting studies have revealed that the targeting mechanisms to different organelles are conceptually similar, differing primarily in the specific components (Andrews 2000).

In general, the initial targeting of proteins to membranes is mediated by a cytosolic chaperone that binds a targeting sequence on the nascent or fully translated protein. The chaperone then escorts the protein to the target membrane via a specific receptor located on the membrane, and the peptide is inserted into the bilayer by a translocon complex (see figure 1.1) (Andrews 2000). Although an oversimplification, this baxic scenario is repeated for protein targeting to the endoplasmic reticulum (ER), mitochondria and peroxisomes. For targeting to both ER and mitochondria, most targeting sequences are located near the N-terminus of the protein. For ER targeting, the targeting sequence is called a signal peptide or signal sequence and is recognized by a ribonucleo-protein complex called the Signal Recognition Particle, or SRP (the chaperone). The receptor on

the ER, the SRP receptor, binds the SRP-nascent peptide-ribosome complex and transfers it to a translocon composed mainly of Sec61α, Sec61γ and Sec61β (reviewed in (Rapoport et al 1996)). Once a protein is targeted to the ER, it is sorted to its ultimate destination by the secretory pathway, which may be the ER, Golgi apparatus, plasma membrane, lysosome, or nuclear envelope. Although there is evidence that some protein sorting does occur from the between the ER and both mitochondria and peroxisomes (Vance et al 1997, Chandra et al 1998, Titorenko & Rachubinski 2001a), the majority of proteins found in mitochondria and peroxisomes are targeted directly to these organelles (Strittmatter et al 1972, Purdue & Lazarow 2001).

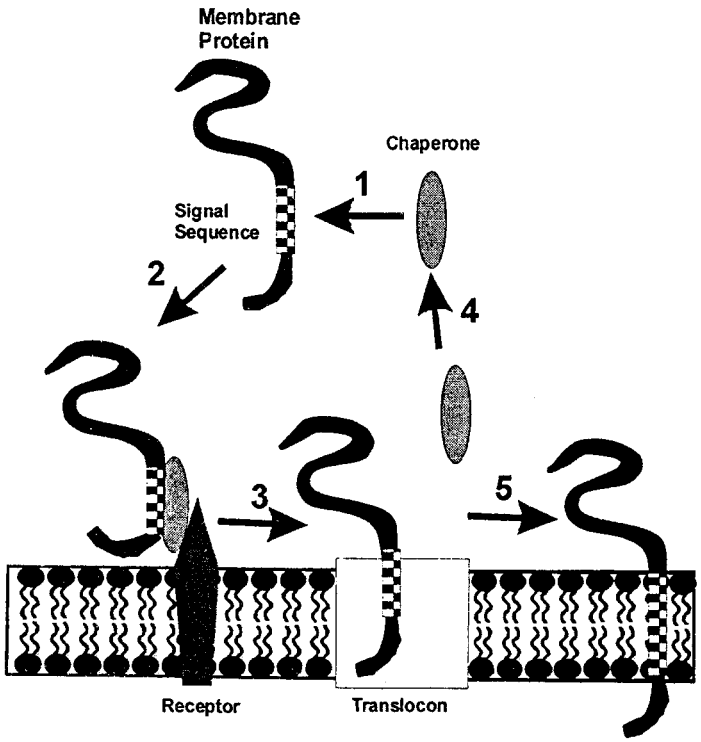

Figure 1.1. General schematic of the targeting of integral membrane protein.
1) A chaperone recognizes and binds the signal sequence of the newly synthesized or synthesizing integral membrane protein. 2) The chaperone escorts the protein to the correct membrane by binding to a specific receptor on the membrane. 3) The integral membrane protein is released by the chaperone and transferred to a translocon complex. 4) The chaperone is recycled and 5) the protein is released into the lipid bilayer.

Targeting to mitochondria is usually mediated by a sequence called the mitochondria-targeting sequence (MTS), located at the N-terminus of the protein and that is recognized by the mitochondrial import stimulation factor (MSF – the chaperone). MSF maintains the MTS in an unfolded state such that it can be recognized by the TOM 20/TOM 22 complex. The MSF/peptide complex first docks onto the TOM 70/TOM 37 complex (the receptor), before the peptide is transferred to the TOM 20/TOM 22 complex (reviewed in (Mihara 2000)). Other MTS-containing proteins are targeted to the mitochondria via an MSF-independent pathway. Instead of MSF acting as the chaperone, these peptides can also be maintained in an unfolded state by cytosolic molecular chaperones such as Hsc70. These relatives of heat shock proteins are also believed to improve recognition of the MTS, allowing it to bind to the TOM 20/TOM 22 complex directly. The TOM 40 complex acts as the translocon on the outer mitochondria membrane and translocates the protein across/into the bilayer. Besides the components involved, protein targeting to the mitochondria differs from targeting to the ER in that it occurs post-translationally, whereas the targeting to the ER is generally co-translational (reviewed in (Andrews 2000)).

Protein targeting to the peroxisome also occurs post-translationally (reviewed in (Holroyd & Erdmann 2001)). The targeting of peroxisomal integral membrane proteins to the peroxisome membrane is mediated by a mPTS (membrane peroxisome targeting signal) and cytosolic chaperone(s) (Pause et al 1997, Brosius et al 2002, Dyer et al 1996). Several cytosolic chaperones have been identified that bind newly translated membrane proteins, maintaining them in an import-competent conformation and escorting them to the

peroxisome (Pause et al 1997, James et al 1994). The import receptor on the peroxisomal membrane is made up of different combinations of two integral membrane proteins, Pex3 and Pex17, and a peripheral membrane protein, Pex19. These proteins are believed to be involved in assembling membrane proteins (Titorenko & Rachubinski 2001b).

Although the majority of integral membranes proteins are targeted to the membrane via a signal located at the N-terminal end, there is another class of integral membrane proteins, called 'tail-anchored proteins' or 'insertion sequence proteins', which bind the membrane bilayer via a hydrophobic domain at the C-terminus. These proteins lack any type of targeting signal at the N-terminal end. Instead, the C-terminal end, which includes the hydrophobic transmembrane domain and the sequences flanking it, is sufficient and necessary to target these proteins to the membrane (Kutay et al 1993, Janiak et al 1994a, Zhu et al 1998). This C-terminal signal was originally named an 'insertion sequence' to reflect what was believed to be a spontaneous mechanism for integration into the bilayer of the membrane.

Tail-anchored proteins, are a small but a growing group of integral membrane proteins that play a crucial role in many cellular functions such as apoptosis (Gross et al 1999), vesicle transport (Gerst 1999), and membrane protein targeting (Rapoport et al 1996). However, the protein targeting mechanism of tail-anchored proteins is not as well understood as the mechanism for targeting N-terminal signal sequence proteins. Although the first membrane binding study was conducted on cytochrome b5 (Cb5) some 30 years ago (Strittmatter et al 1972, Sullivan & Holloway 1973), it is only in the last ten years that substantial progress has been made in understanding the targeting mechanism of tail-

anchored proteins. In this chapter the current understanding of the targeting mechanism of tail-anchored proteins will be discussed, including some proposed models of the mechanism of targeting to specific organelles. In the following chapters my contributions to the field of tail-anchored proteins will be presented. These contributions, together with those from other members of the Andrews lab, include the first systematic evidence suggesting that more than one mechanism exists for targeting tail-anchored proteins (chapter 2); a detailed study on the targeting signal of one family of tail-anchored proteins (chapter 3); and, finally, examination of the dynamic topology of one tail-anchored protein after post-translational targeting to ER and mitochondrial membranes (chapter 4).

1.2. The Tail-anchored proteins

Tail-anchored proteins are integral membrane proteins that are characterized by a single hydrophobic transmembrane domain at the extreme C-terminus that gives them an $N_{cytosol}$-C_{lumen} topology (Figure 1.2). The C-terminal end in the organelle lumen is usually very short, with a length of 1-15 residues, and the majority of the protein is on the cytosolic side of intracellular membranes. The tail-anchor and membrane topology are the only common features shared by tail-anchored proteins, as these proteins are found performing a wide range of cellular functions on different membranes. Several membrane proteins of the SNARE complex (Vamp1, Vamp2, Vamp8 syntaxin) are tail-anchored proteins that are involved in the transport of vesicles. Others are involved in the translocation of secretory and membrane proteins (Sec61β and Sec61γ from the Sec61 complex; TOM5 and TOM6 from the TOM complex). Furthermore, many regulators of apoptosis such as Bcl-2, Bcl-xl,

and Bax are also tail-anchored proteins. Tail-anchored proteins are also involved in enzymatic and metabolic activity on the membrane.

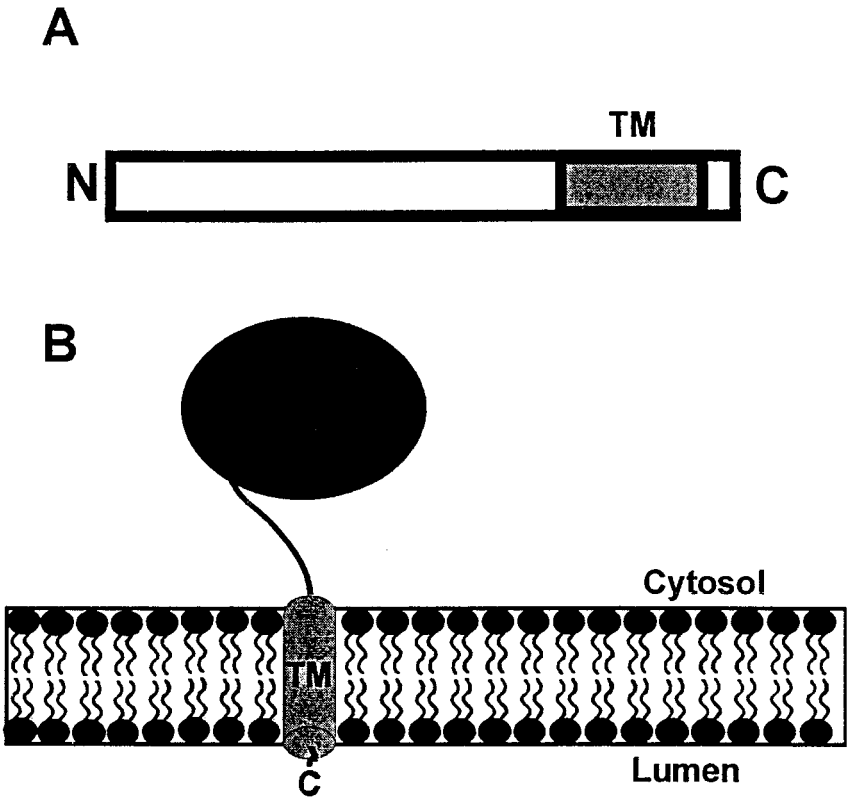

Figure 1.2. Schematic diagram of a Tail-Anchored protein.
A) Tail-anchored proteins are identified by a carboxyl-terminal hydrophobic domain (TM) of 15-23 amino acids. B) The majority of a tail-anchored protein is located on the cytosolic side of the membrane and it is anchored to the membrane via the carboxyl-terminal transmembrane domain.

The function of the cytosolic N-terminal domain is independent of the C-terminal anchoring domain, as replacement of the native insertion sequence with that of another protein does not affect the function of many of these protein (Rebrin et al 2001, Zhu et al 1996). Instead, the C-terminal insertion sequence allows the functional domain of the protein to be located on the cytosolic side of the correct organelle. Furthermore, since most

protein folding occurs co-translationally in eukaryotic cells (Netzer & Hartl 1997, Nicola et al 1999, Hardesty et al 1999), the C-terminal location of the targeting sequence allows for the N-terminal functional domain to be fully folded before the protein is targeted to the membrane. Therefore, the cytosolic portion can be properly folded without the hydrophobic domain or the membrane interrupting the folding process (Wattenberg & Lithgow 2001).

Tail-anchored proteins are found in many different organelles. However, most of these proteins are initially targeted to either the ER or the outer mitochondrial membrane. Proteins destined for organelles in the secretory pathway are first targeted to the ER and then transported to their final destination via the secretory system (Kutay et al 1995). The initial targeting information is found solely in the insertion sequence of the tail-anchored protein. The insertion sequences of several tail-anchored proteins, including the Vamps (Kim et al 1999, Isenmann et al 1998), mT (Elliott et al 1998), cytochrome b5 (Cb5) (Janiak et al 1994a, Janiak et al 1994b, De Silvestris et al 1995), and Bcl-2 (Zhu et al 1996), have all been shown to be necessary and sufficient to target these proteins to membranes. The cellular location of the cytoplasmic N-terminal domain of a tail-anchored protein can be altered by exchanging the insertion sequence. Bcl-2 is found in both the ER and the outer mitochondrial membrane (Janiak et al 1994b). However, when the insertion sequence of Bcl-2 was replaced with the insertion sequence from the ER isoform of Cb5, or with that from a mitochondrial localizing protein, Acta, the Bcl-2 chimeric proteins were no longer found in both organelles but accumulated in the one membrane specified by the insertion sequence *in vivo* (Zhu et al 1996).

1.3 Membrane targeting mechanism of Tail-anchored proteins: Post-translational & spontaneous targeting of Cytochrome b5

The precise mechanism of targeting of tail-anchored proteins is not known. The C-terminal location of the insertion sequence dictates that it associates with membranes post-translationally (Sabatini et al 1982). Both *in vitro* and *in vivo* experiments have confirmed that tail-anchored proteins bind to the membrane after the protein has been fully translated (Enoch et al 1979, Rachubinski et al 1980, Takagaki et al 1983b, Takagaki et al 1983a, Janiak et al 1994a, Janiak et al 1994b). Furthermore, the targeting of tail-anchored proteins was initially believed to be spontaneous due to the observation that purified cytochrome b5 can bind protein-free liposomes (Enoch et al 1979, Rachubinski et al 1980, Takagaki et al 1983b, Takagaki et al 1983a).

The ER isoform of cytochrome b5 (Cb5), a component of the microsomal membrane stearyl-CoA desaturase system, is the best characterized tail-anchored protein. Purified Cb5 in the absence of detergent exists in equilibrium between an octomer and a monomer (Calabro et al 1976). It is the monomeric form of Cb5 that spontaneously binds to lipid bilayers *in vitro* (Strittmatter et al 1972, Sullivan & Holloway 1973). Cb5 has two membrane binding conformations. When Cb5 first binds to the bilayer it is in the 'loose' form where the transmembrane domain forms a hairpin and dips into the lipid bilayer (Vergeres et al 1995). The hairpin structure is thought to be facilitated by a proline residue in the middle of the TM (Vergeres et al 1995). This form is designated as the 'loose' form because it can be easily transferred between lipid vesicle populations (Enoch et al 1979, Takagaki et al 1983a). The second conformation, the 'tight' form, is not transferable between lipid vesicle populations, and this is the final conformation of Cb5 in the ER membrane. Based on *in vitro* experiments

which include proteolysis (Kuroda et al 1996), cross-linking (Takagaki et al 1983b, Takagaki et al 1983a), and fluorescence (Ladokhin et al 1993) studies, the 'tight' form is postulated to be the conformation in which the TM spans the bilayer. This was confirmed *in vivo* by using Cb5 with an N-glycoslyation site at the C-terminal end. Once the chimera was transfected into CV-1 cells, the chimeric polypeptide was found to be completely glycosylated, suggesting that *in vivo* the C-terminal end is on the lumenal side of the ER (Pedrazzini et al 2000).

The 'tight' binding of Cb5 to phospholipid vesicles is dependent on the lipid composition, the curvature of the bilayer, and the cholesterol levels in the liposome (Taylor & Roseman 1995). These three factors change the conformational flexibility of the lipid in the bilayer. Cb5 tends to bind readily to more fluid bilayers (Taylor & Roseman 1995). Therefore, lipid composition with increased unsaturated acryl-chains, decreased cholesterol levels, and an increased curvature of the bilayer, all of which contribute to increasing the fluidity of the bilayer, favour Cb5 binding the bilayer in the 'tight' conformation (Taylor & Roseman 1995, Vergeres et al 1995, Vergeres & Waskell 1995).

Although the two conformations of Cb5 may explain how the protein inserts into the lipid bilayer, the mechanism of membrane-specific targeting of Cb5 to the ER is still not known. One possible answer may be the kinetics of insertion into bilayers of different lipid compositions. In this kinetic model, it is proposed that Cb5 binds to most organelles, but only in the 'loose' conformation (Enoch et al 1979, Takagaki et al 1983b, Takagaki et al 1983a). Due to the difference in the lipid composition between the organelles, the transfer to the 'tight' conformation in organelles other than the ER is slow. Therefore, in this model, the ER is the

sink for Cb5. Although Cb5 binds to other organelles, the on and off rate is faster than the time it takes Cb5 to adopt the 'tight' conformation in those organelles (Greenhut et al 1993). However, in the ER, the equilibrium favours the 'tight' conformation.

The kinetic model is supported by several *in vitro* studies on Cb5. When the binding of *in vitro* synthesized Cb5 to purified plasma membranes was compared to binding to ER microsomes, three times more Cb5 bound to ER (Remacle 1980). Similarly, Cb5 preferentially bound to ER microsomes over mitochondria when *in vitro* synthesized Cb5 was incubated with both organelles (Janiak et al 1994b). These two observations suggest that Cb5 preferentially binds the ER over other common bilayers. Bilayer specificity was also observed when the binding of Cb5 to artificial vesicles was examined. The half life of the conversion of Cb5 from the 'loose' to the 'tight' conformation in Large Unilamellar Vesicles (LUV) is much greater than in Small Unilamellar Vesicles (SUV) (24 hours compared to 1 hour respectively) (Greenhut et al 1993). Furthermore, in both lipid vesicles, the switch to the 'tight' conformation was dependent on the lipid composition (Takagaki et al 1983a). These results suggest that the different lipid compositions of the various organelles may influence the rate of Cb5 'tight' binding to the membrane.

Finally, according to the kinetic model, there should be some Cb5 in other organelles as the change to a 'tight' conformation is not prohibited in other organelles, but instead it is slow due to lipid composition and structure. Subcellular fractionation of different cell lines and tissues has led to the conclusion that Cb5 is found in other organelles, such as the Golgi (Ichikawa & Yamano 1970, Fleischer et al 1971, Borgese & Meldolesi 1980, Collot et al 1982, Elhammer et al 1983), and the plasma membrane (Jarasch et al 1979, Bruder et al 1980). The

levels of Cb5 in these organelles were much lower than the concentration found in the ER, however the levels were higher than that expected from ER contamination of the fractions (Ichikawa & Yamano 1970, Bruder et al 1980). Cb5 was also found in the mitochondria in significant amounts (Borgese & Meldolesi 1980). Although most of the Cb5 in mitochondria was found to be the mitochondrial isoform (OM-Cb5), using isoform specific antibodies, about 5% of the Cb5 found in the mitochondria was determined to be the ER isoform (D'Arrigo et al 1993). Together these experiments strongly suggest that Cb5 is indeed found in other organelles in amounts that may suggest non-specific binding via the spontaneous insertion mechanism.

Recently, Pedrazzini et al. have demonstrated that Cb5 (the ER isoform) recycles slowly between the ER and the *cis*-Golgi, thus giving another explanation for the levels of Cb5 found in the Golgi (Pedrazzini et al 2000). Using Cb5 with either an N-glycosylation or an O-glycosylation site at the C-terminus of the protein, their results suggest that some ER-Cb5 can escape the ER to the *cis*-Golgi apparatus, but it is then retrieved back to the ER (Pedrazzini et al 2000). Very little Cb5 is found in the *trans*-Golgi apparatus suggesting that the amount found in the PM cannot have originated from the ER. Ito's group has found that the length of the TM domain and C-terminal lumenal portion of Cb5 are important for ER retention, as changing either one results in distribution of Cb5 throughout the secretory pathway (Honsho et al 1998). The length of the TM has been previously found to be important in the organelle specific retention of number of membrane proteins. This phenomenon is thought to be related to the observation that different organelles differ in the thickness of their bilayers (Bretscher & Munro 1993, Munro 1995, Nilsson et al 1991, Yang et

al 1997). However, since the charged residues in the 10 amino acids at the lumenal end of Cb5 were found to be necessary for targeting to the ER (Mitoma & Ito 1992), it is possible that the lumenal mutants of Cb5 used by Ito's group are not just escaping the ER, but that the ER-specific targeting mechanism may have been disrupted. Therefore an increasing amount of Cb5 may be targeting directly to the PM and the Golgi apparatus instead of escaping the ER to these locations. To determine if the Cb5 lumenal mutants are first targeted to the ER and then distributed to other organelles via the secretory pathway, or if they target directly to other organelles in the secretory pathway, chimeric proteins containing a N-glycosylation site at the C-terminus need to be examined. By determining the extent of glycosylation of the Cb5 proteins in the PM and the Golgi, the targeting specificity of the lumenal mutants of Cb5 can be determined.

1.4. Membrane-specific targeting signals on other tail-anchored proteins

Various labs have examined the insertion sequences of different tail-anchored proteins to elucidate the membrane specificity of tail-anchored proteins. These studies identified the length of the TM, and the positively charged residues flanking the TM, to be important in membrane-specific targeting (Chapter 3) (De Silvestris et al 1995, Kim et al 1999, Isenmann et al 1998). To simplify the discussion of these various regions of the insertion sequences of tail-anchored proteins, they are divided into three sections: the N-terminal sequence (NTS) which is the 15 to 20 amino acids preceding the TM domain; the TM domain; and the C-terminal sequence (CTS) which is located C-terminal of the TM domain. In Table 1, the number of charged residues in the NTS and CTS, and the length of the TM are listed for various tail-anchored proteins, along with their cellular location. In

general, there appears to be some correlation between the positively charged residues and organelle targeting specificity. ER-localized proteins tend to have more positively charged residues within the NTS than mitochondrial-targeted tail-anchored proteins. In contrast, the CTS of mitochondrial-targeted proteins is more basic than that of ER-targeted proteins. There is some experimental evidence to suggest that these positively charged residues are involved in dictating the membrane localization of the tail-anchored proteins. Therefore, the targeting mechanism in each organelle will be discussed separately.

1.5. The targeting and insertion mechanism in the ER

In-depth studies of the membrane targeting mechanism of v-SNAREs demonstrated that the targeting mechanism of all tail-anchored proteins is not spontaneous, but instead requires at least one protein component (Chapter 3) (Kutay et al 1995, Kim et al 1999). v-SNAREs are a group of tail-anchored proteins that provide specificity in vesicle targeting and fusion. Although they are found at small vesicles, after translation they are initially targeted to the ER. The ER targeting of three mammalian v-SNARES (Vamp1, Vamp2, and Vamp8) requires ATP and a trypsin-sensitive component in canine ER microsomes (Chapter 3) (Kutay et al 1995, Kim et al 1999). The ATP is believed to be required by cytosolic chaperones, such as Hsc70/Hsp70, that prevent the aggregation of the protein due to the hydrophobic transmembrane domain (Mullen et al 1999, Lan et al 2000). The identity of the membrane receptor for v-SNAREs is not known. However, the receptor is not the Sec61 translocon complex, as ER vesicles depleted of the Sec61 complex readily bound Vamp2 (Kutay et al 1995). The Sec63 complex in *Saccharomyces cerevisiae* is also not involved in the binding of these tail-anchored proteins (Steel et al 2002). The Sec63 complex is part of a novel post-

translational pathway in *Saccharomyces cerevisiae* that binds to full-length polypeptides which are kept translocation-competent by cytosolic chaperones (Plath & Rapoport 2000). The Sec63-complex then passes the polypeptide to the Sec61 translocon in an ATP-dependent fashion (Plath et al 1998). Like the Vamps, the yeast homolog, Nyv1p was shown to bind to ER microsomes in an ATP-dependent fashion and required a trypsin-sensitive component on the membrane (Steel et al 2002). However, binding was independent of the Sec61 complex and the Sec63 complex, suggesting that a novel receptor exists that binds tail-anchors in yeast.

The ER receptor for the targeting of Vamps is still elusive. However, we have demonstrated that the ATP-dependent, receptor-mediated targeting requires an amphipathic lysine-rich domain located in the NTS of Vamps (Chapter 3 and (Kim et al 1999)). Sec61γ and aldehyde dehydrogenase also contain lysine-rich putative amphipathic helices within their NTS, which were determined to be required to bind to the ER (Chapter 3 and (Masaki et al 1994)), thus suggesting a common mechanism of binding to the ER. Interestingly, most of the proteins that contain the putative amphipathic helical domain are initially targeted to the ER (Table 1). However, not all ER-targeted proteins require a receptor or ATP. These proteins also do not have amphipathic lysine-rich NTS. This suggests that there may be two different mechanisms of targeting tail-anchored proteins to the ER.

In the first ER targeting mechanism, exemplified by Cb5, the protein is prevented from aggregating in the cytoplasm by forming hetero- or homo-oligomers. However, only as a monomer does it bind spontaneously to the correct membrane, based on the lipid composition (see above). These proteins do not have a lysine-rich amphipathic helix at the NTS. Other proteins that may belong to this group are Bcl-2, Bax and dolicolphosphate

mannose as they do not have a Vamp-like NTS signal and, in the case of Bcl-2, it does not have the ATP requirement and can bind to trypsinized membranes (Chapter 2, (Kim et al 1997)). The second mechanism requires both a cytosolic chaperone and a membrane receptor to mediate membrane-specific targeting and insertion. The lysine-rich NTS is probably recognized by the membrane receptor. A number of ER-localized proteins have the lysine-rich amphipathic NTS domain (Table 1). Of these, Sec61γ, and Sec61β were tested for ATP dependence and a receptor requirement (Chapter 3). The binding of both Sec61β and Sec61γ to ER microsomes required both ATP and a receptor (Chapter 3), suggesting a similar targeting mechanism to the Vamps. However, it is not known whether the same receptor mediates the binding of both of these two proteins to the ER. Other tail-anchored proteins containing a lysine-rich amphipathic NTS domain need to be assayed to determine if this domain is universally required for ATP-dependent and receptor-mediated binding to the ER.

1.6. The targeting and insertion mechanism in the mitochondria

The mitochondrial targeting signal consists of a shortened transmembrane domain (between 17-19 amino acids) and a basic CTS. There are several tail-anchored proteins that have isoforms that are found in different organelles, one at the ER and the other at the mitochondria. Both the ER-targeted Cb5 and Vamp1 have mitochondrial targeted isoforms. Work on the Cb5 mitochondrial isoform, OM-Cb5 (outer mitochondrial cytochrome b5), has shown that *in vivo* OM-Cb5 targets directly to mitochondria (Borgese et al 2001). *In vitro* studies on the mitochondrial v-SNARE isoform, Vamp1b, have shown that it binds to isolated mitochondria at a 15-fold greater efficiency than the ER isoform of Vamp1, again suggesting that direct targeting to mitochondria by the mitochondrial isoform is occurring (Lan et al

2000). Thus, the mitochondrial targeting signal was determined by comparing amino acid sequences of these isoforms.

Sequence comparisons of these isoforms reveal that both mitochondrial isoforms have two lysine residues in the CTS, thus making the CTS basic, while their ER counterparts do not. The two lysines in the CTS were found to be necessary for mitochondrial localization, as deleting or changing these lysines to non-charged residues resulted in the loss of mitochondrial localization when expressed in cells (Isenmann et al 1998, Kuroda et al 1998, Borgese et al 2001). The basic CTS in TOM5, a component of the yeast outer mitochondrial import machinery, was shown to be necessary for mitochondrial localization (Horie et al 2002). In fact, deletion of the lysines resulted in ER localization of the mutant (Horie et al 2002). Furthermore, the TM length was found to be important for mitochondrial localization for both TOM5 and Vamp1b. Increasing the length of the TM resulted in a promiscuous targeting of both proteins, as they were found in both the ER and the mitochondria (Isenmann et al 1998, Horie et al 2002). Therefore, these studies on the TM and the CTS have shown that a shortened TM and a basic CTS are both necessary and sufficient to constitute the mitochondrial targeting signal. Furthermore, the proximity of these two features to each other is important, as the addition of a non-charged hydrophilic residue between the TM and the basic CTS resulted in the loss of mitochondrial targeting and instead caused cytoplasmic localization (Horie et al 2002). Interestingly, both the shortened TM (17-19 amino acids) and a basic CTS appear to be conserved in most of the tail-anchored proteins found exclusively at the mitochondria (Table 1), suggesting a common mechanism of mitochondrial targeting of tail-anchored proteins.

1.7. ER vs. mitochondrial targeting: a competing pathway?

The above studies on the mitochondrial targeting of tail-anchored proteins suggest that the ER and mitochondrial targeting mechanisms are distinct, but in some cases they compete with each other. The main observation is that in all mitochondrial tail-anchored proteins examined (Vamp1b, OM-Cb5, and Tom5), the loss of either of the two distinguishing features (reduced hydrophobicity, positively charged CTS) resulted in an ER localization (Isenmann et al 1998, Borgese et al 2001, Horie et al 2002). Furthermore, Borgese et al. (2002) demonstrated that when the CTS of ER-Cb5 is made more basic by the addition of a second arginine residue and the removal of two aspartic acid residues, the mutant targeted exclusively to mitochondria (Borgese et al 2001). The significance of these results is that even though ER-Cb5 and OM-Cb5 are only approximately 60% identical, changing just the CTS of ER-Cb5 redirected the protein to the mitochondria instead of the ER.

These observations suggest two possible but opposing mechanisms of membrane-specific targeting. In the first mechanism, the mitochondrion is the default pathway and the positive charge on the CTS acts as a negative signal to the ER (Borgese et al 2001). In the second scenario, the default pathway is the ER pathway, however the positive charges on the CTS act as a mitochondria-specific signal that sequesters all these proteins to the mitochondria (Borgese et al 2001). Various *in vitro* studies support the latter mechanism. The mitochondrial signal on the Vamps does not prevent it from binding to the ER *in vitro* and, therefore, it can bind both the ER and the mitochondria, however the ER isoform of Vamp does not bind to mitochondria (Chapter 3 and (Lan et al 2000)). The ER isoform of Vamp probably does not bind to the mitochondria because it is 'shunned' by the mitochondrial

targeting machinery. On the other hand, although the mitochondrial Vamp can readily bind the ER, it is quickly shuttled away to the mitochondria after it leaves the ribosome, thereby preventing it from binding to the ER.

This mitochondria targeting mechanism suggests that there is a requirement for a cytosolic chaperone and/or a membrane-specific receptor on the mitochondria. Recently, Wattenburg's group has demonstrated that the targeting of Vamp1b requires a saturable receptor in the outer mitochondrial membrane (Lan et al 2000). Furthermore, in the presence of cytosolic chaperones, ATP is required; however in the absence of chaperones in the binding reaction, ATP is not required for binding of a chemically unfolded protein (Lan et al 2000). This result suggests that chaperones may be involved in the specific targeting of Vamp1b to the mitochondria.

1.8 The universal targeting mechanism of membrane proteins

In his review on protein targeting, David Andrews suggested a universal theme in targeting membrane proteins (Andrews 2000). In his model, a chaperone recognizes and binds to the signal on the protein and escorts it to the specific membrane via a membrane receptor. Although the model is simplified, in this thesis I will show that a similar mechanism is also involved in targeting some tail-anchored proteins. In this model, both a chaperone and a membrane receptor are required for membrane-specific targeting. Using this general model, I suggest that the membrane specificity is determined by the signal on the C-terminus of the tail-anchored protein, which is probably recognized by both the cytosolic chaperone and the membrane receptor specific to the target membrane.

Although the general theme may be similar in the targeting of all tail-anchored proteins, there are some distinct differences in their precise mechanism, even within the targeting to the same organelle. The difference between the targeting of various tail-anchored proteins may be due to the role and identity of the receptor. In some cases, the receptor just binds to the protein, which by maintaining proximity allows spontaneous insertion of the protein into the lipid bilayer (ex. Cb5, Bcl-2). However, for other tail-anchored proteins the receptor itself may be required to translocate the TM (Vamps).

1.9 Project Goals and Strategies.

Proper function of tail-anchored proteins requires the correct membrane localization. For example, Bcl-2, an anti-apoptotic protein, has been shown to regulate apoptosis through different pathways depending on its cellular location (Annis et al 2001). For Vamps it is essential that they are localized to their specific vesicle in order for the vesicle to be targeted to the correct membrane. Furthermore not only the localization of the of the protein but also its topology may be important for function (Gerst 1999, Muchmore et al 1996).

In other to gain a better understanding of tail-anchor proteins, a series of studies were carried out to examine the initial targeting mechanism of newly synthesized protein and the membrane topology of tail-anchored proteins. Using a cell free system, the targeting mechanisms of four tail-anchored proteins, mT, Vamp1, Cb5 and Bcl-2 were examined (Chapter 2). These studies revealed differences between the targeting mechanisms of these tail-anchored proteins to ER microsomes. Two models for targeting of tail-anchored proteins were devised based on this work (Chapter 5). Next, the targeting mechanism of the Vamp family of proteins was further examined using Vamp2 fusion proteins (Chapter 3). The aim of

this work was to further characterize the targeting mechanism of Vamp in order to identify the Vamp receptor. Finally the membrane topology of one tail-anchored protein, Bcl-2 was examined in order to determine if the topology of Bcl-2 is related to its function. Using chemical modifying techniques, the membrane topology of Bcl-2 was shown to be dynamic, changing from one topology to another in response to the induction of apoptosis in cells by treatment with chemotherapy drugs. This represents the first evidence of that a tail-anchored protein can change membrane topology in response to a cellular signal. In the last chapter (Chapter 5), the biological implication of these findings and future directions are discussed.

	NTS		TM	CTS		CTS			
	+ve	-ve	Length	+ve	-ve	Length	(+)-(+) *	Amphipathic Helix?	
OMF25	1	2	19	2	0	6	**-1**	N	MITO
Bax	2	3	19	3	0	9	**-1**	N	MITO/ER?
MAO-A	2	1	16	3	0	8	**-1**	N	MITO
MAO-B	2	1	18	3	0	8	**-1**	N	MITO
APX	3	3	19	4	1	7	**-1**	Y	Peroxisome
Cb5 ER	1	2	19	1	2	7	**0**	N	ER
Inp54p	1	1	14	1	0	3	**0**	N	ER
Sec61B	2	2	20	2	0	5	**0**	Y	ER
UL56	1	1	17	1	0	2	**0**	N	ER->Golgi
Bcl-2	2	1	19	1	0	1	**1**	N	ER/MITO
PTN1	2	2	19	1	0	7	**1**	Y	ER
Cb5 Mito	3	3	18	2	1	10	**1**	N	MITO
CPT	3	2	16	2	0	2	**1**	Y	MITO
Tom5	4	5	18	3	0	5	**1**	N	MITO
FIS1	5	2	16	4	0	5	**1**	N	MITO
Cb5 Reductase	2	2	19	1	2	13	**1**	N	ER
Bcl-XL	4	2	19	2	0	2	**2**	Y	MITO/ER?
mT	5	0	22	3	0	6	**2**	Y	?
DPMS	3	1	14	0	0	16	**3**	Y	ER
UBC6	4	4	17	1	0	1	**3**	Y	ER
Ramp4	4	2	19	1	0	10	**3**	Y	ER
HEME-OXYGENASE	4	0	19	0	0	0	**4**	Y	ER
ALDH	5	0	17	1	1	4	**4**	Y	ER
SNC1_yeast	5	1	20	1	0	4	**4**	Y	ER->S.V
Sssp1	6	2	16	2	0	12	**4**	Y	ER
Vamp1B	6	0	17	2	1	3	**4**	Y	MITO
Vamp8	5	0	22	0	0	2	**5**	Y	ER->ENDO
giantin	5	0	22	0	0	2	**5**	Y	ER-Golgi
Vamp1	6	0	20	0	0	0	**6**	Y	ER->S.V
Vamp2	6	0	20	0	0	0	**6**	Y	ER->S.V
Sec61γ	7	2	19	1	0	14	**6**	Y	ER
Syntaxin	8	0	21	0	0	0	**8**	Y	ER->PM
Ufe1p	8	1	19	0	1	4	**8**	Y	ER->PM
US9	9	0	25	1	1	6	**8**	N	?

Table 1: TM length and charged residues flanking the TM of Tail-Anchored Proteins

The number of positively and negatively charged residues in the NTS and CTS of the tail-anchored proteins is listed above along with the length of the TM. NTS is defined as the 15 amino acids preceding the TM. Whether the NTS can form amphipathic helix and the cellular location is indicated. *(+N)-(+C) the total number of positively charged residue at the N-terminus of the TM was subtracted from the total number of positively charged residues at the C-terminus. S.V, small vesicle.

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Chapter 2

Evidence for Multiple Mechanisms for Membrane Binding and Integration of Tail-Anchored Proteins.

Preface.

Part of the material in this chapter has been reproduced with full permission from the journal Biochemistry in which it was first published by Peter K. Kim, Janiak-Spens F, William S. Trimble, Brian Leber, David W. Andrews in 1997 under the title "Evidence for multiple mechanisms for membrane binding and integration via carboxyl-terminal insertion sequences". It was published in Biochemistry Vol. 36(29):8873-82. Copyright © 1997 by American Chemical Society.

All the data was produced by Peter K. Kim. William Trimble provided the full length Vamp1. Fabiola Janiak-Spens generated some of the DNA constructs used and with Brian Leber contributed to the conceptual ideas of the manuscript. The manuscript was prepared by Peter Kim and David Andrews.

2.0 Introduction

The ER specific isoform of rat liver cytochrome b5 (Cb5) is the best characterized tail-anchored protein. Experiments *in vitro* and *in vivo* have confirmed that the hydrophobic carboxyl terminus of Cb5 spontaneously inserts into the bilayer of the target membrane (Enoch et al 1979, Rachubinski et al 1980, Takagaki et al 1983b). *In vitro*, Cb5 binds to a variety of physiological membranes and phospholipid vesicles (Enoch et al 1979, Takagaki et al 1983b, Takagaki et al 1983a). However, in transfected cells (Mitoma & Ito 1992) and in cell free competition assays containing both ER and mitochondria (Janiak et al 1994b), Cb5 is preferentially localized to ER.

The protein targeting of several other tail-anchored proteins have been examined. These include: middle-T antigen (mT), the transforming protein of polyoma virus (Treisman et al 1981, Rassoulzadegan et al 1982); the proto-oncogene product Bcl-2 (Vaux et al 1988, Chen-Levy et al 1989, Liu et al 1996); and v-snares such as the synaptic vesicle membrane protein, Vamp2 (synaptobrevin) (Elferink et al 1989, Sollner et al 1993). Although it is widely believed that other molecules with insertion sequences are targeted to and integrated into organelles by mechanisms similar to that of Cb5, there is some evidence to suggest that not all tail-anchored protein share the same mechanism of Cb5. However the current published data results are difficult to interpret and compare because membrane assembly of these molecules has not been directly compared using the same assay system. Therefore, the mechanisms of membrane integration for four different tail-anchored proteins for which there is published evidence for insertion into endoplasmic reticulum (ER) have been examined.

To determine if different targeting and/or integration mechanisms exist for tail-anchored proteins, the mechanism of membrane binding of Cb5 was directly compared to those of mT, Bcl-2, and Vamp1 using a cell free system. Furthermore, the role of the putative insertion sequences from these proteins in membrane assembly was determined by fusing them to common passenger proteins. The results demonstrate that mT and fusion proteins, containing the mT insertion sequence, bind electrostatically but do not insert into either ER microsomes or liposomes. In contrast, the insertion sequence of Bcl-2 is sufficient to integrate both the native protein and fusion proteins into the bilayer of ER membranes but not to lipid vesicles. Furthermore, although Cb5, Bcl-2, and Vamp1 synthesized in reticulocyte lysate all insert into ER membranes, ATP is required only for membrane assembly of Vamp1. Finally, quantitative measurements for the binding of Vamp1 to ER microsomes revealed that although binding of Vamp1 to ER microsomes is saturable, with 20 fmole of Vamp1 binding sites per 100 fmole of SRP receptor. Thus these results suggest that multiple mechanisms of membrane targeting and integrating the insertion sequences of tail-anchored proteins to ER membranes.

2.1 Methods

2.1.1. Plasmids and Membranes.

All of the plasmids used were constructed in the vector pSPUTK, which contains an SP6 promoter and a high-efficiency 5' untranslated region for efficient translation in reticulocyte lysate (Falcone & Andrews 1991). The plasmids containing the coding regions for Cb5, mT, Bcl-2, and Vamp-1 were described previously (Enoch et al 1979,

Andrews et al 1989, Elferink et al 1989, Janiak et al 1994b). A polypeptide called gPA was used as a passenger domain because the unmodified sequence behaves as a cytosolic protein yet gPA has been demonstrated to be passive to translocation across membranes. Thus, gPA can be targeted to a variety of subcellular organelles by adding the appropriate targeting information to either the amino or the carboxyl terminus (Janiak et al 1994a). The construction of the plasmid encoding gPA by fusing coding regions for the first 27 amino acids of chimpanzee α-globin to the N-terminus of the IgG binding domains of *Staphylococcus aureus* protein A (amino acids 23-271) was described previously (Janiak et al 1994a). To fuse the putative insertion sequences from Bcl-2, Cb5, and mT to gPA the corresponding coding sequences were added to the plasmid encoding gPA using a unique *Bam*H1 site at the 3' end of the coding region of gPA and an *Xho*1 site 3' of the gPA termination codon. In the resulting fusion proteins gPABcl-2, pPACb5, and gPAmT, the coding sequences for putative insertion sequences (nucleotides 643-717 from the coding region of Bcl-2, nucleotides 323-427 of the coding region of Cb5, and nucleotides 1158-1277 of the coding region of mT) replace the coding sequences for the last 14 amino acids of gPA. Thus as shown in Figure 2.1, the junction between gPA and the putative insertion sequences is identical in all of the fusion proteins. The Bcl fusion proteins were constructed similarly, except using a unique *Afl*II site within the coding region for Bcl-2 instead of *Bam*H1. The putative insertion sequences of Cb5 and Bcl-2 were placed behind nucleotide 1157 of mT using *Bam*H1 in order to construct the mT

fusion proteins. All of the fusion junctions and coding regions for the putative insertion sequences were confirmed by DNA sequencing.

Salt-extracted canine pancreatic ER microsomal membranes (microsomes) were prepared as described by Walter and Blobel (Walter & Blobel 1983). Each batch of microsomes was tested for cotranslational translocation of preprolactin before use (Andrews et al 1989). In addition, batches of microsomes were standardized by measuring the amount of signal recognition particle receptor α-subunit by western blotting. One equivalent of microsomes is defined as containing 100 fmole of signal recognition particle α-subunit and typically processes greater than 50% of the preprolactin synthesized in a 20 μL reticulocyte lysate translation reaction (Andrews et al 1989). Phospholipid vesicles (7:8:1:4 phosphatidylcholine/ phosphatidylethanolamine/ phosphatidylserine/cholesterol) were prepared by extrusion in 10 mM Tris-HCl buffer, pH 7.5, or in buffer containing 1 M NaCl. Transcription of the plasmids using SP6 polymerase was as described by Gurevich et al. (Gurevich et al 1991).

2.1.2. Translation and Membrane Binding.

Transcription-linked translation reactions were performed as described previously using rabbit reticulocyte lysate (Andrews et al 1989). After incubation at 24 °C for 60 min, cycloheximide was added to 20 μg/mL to inhibit further translation and the ribosomes were removed by centrifugation at 20 psi (100,000*g*) for 15 min in a 30 ° A-100 airfuge rotor (Beckman). ATP was removed from translation reactions after the addition of

cycloheximide by incubation with 5 units of Apyrase (Sigma) at 24 °C for 20 min. Nucleotides and other small molecules were removed by passing the translation reaction twice through a 600 µL Sephadex G-25 (Sigma) spin column.

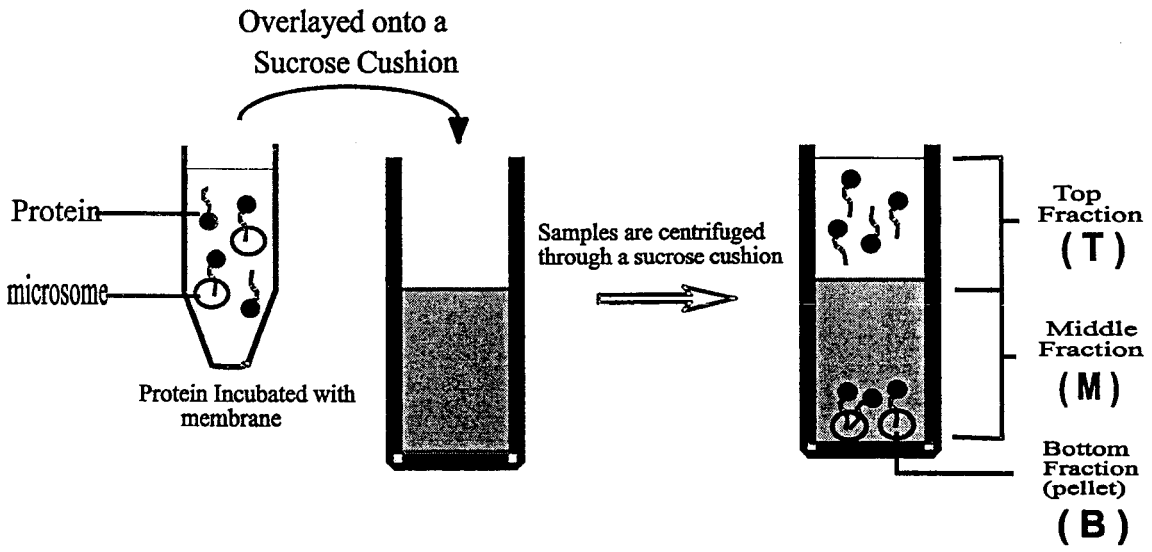

Figure 2.0. Membrane pelleting assay.

[^{35}S]-Met labelled protein synthesized in rabbit reticulocyte lysate is incubated with canine ER microsomes. The microsomes are removed from the reaction by centrifugation over a sucrose cushion. The supernatant is divided into two fractions, top (T) which is the reaction fraction, and middle (M), which is the sucrose cushion fraction. The pellet resuspended in 1% SDS/ 10 mM Tris-HCl pH 9.5 is named bottom fraction (B).

Microsomes were added to terminated translation reactions, and the samples were incubated at 24 ° C for the times indicated (also see figure 2.0). Following the incubation, the reactions were mixed with translation buffer (50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 1 mM DTT) and layered over a 0.5 M sucrose cushion in polyallomer tubes. Microsomes were pelleted by centrifugation for 10 min at 20 psi (110000*g*) at 4 °C in an airfuge. Gradients were divided into two aliquots [top (T) and middle (M) fractions]. The bottom (B) fraction containing the microsomes was obtained by solubilizing the pellets in 75 µL of 1% SDS and 0.1 M Tris, pH 9.0, at 70 °C for 10 min. Amounts of each fraction corresponding to equivalent amounts of the original translation reaction were separated by SDS-PAGE using a Tris-tricine buffer system (Schaegger & von Jagow, 1987), and radioactive proteins were visualized and quantified using a phosphorimager (Molecular Dynamics).

To assay membrane binding quantitatively, 1 or 2 equiv of microsomes was added to translation reactions containing different amounts of the molecules being assayed. To set up binding reactions with different amounts of *in vitro* translated molecules, a single large translation reaction for the molecule of interest was incubated at 24 °C for 1 h. Translation was then terminated by adding cycloheximide and aliquots of different volumes were removed from this reaction and added to sufficient mock translation (translation reaction mix containing cycloheximide but without mRNA) to adjust the final volume of each aliquot to 60 µL. Microsomes were added to these reactions and the samples were incubated at 24 °C for 1 or 2 hr as indicated. Two 1 µL calibration aliquots

were removed for later analysis. The remaining 58 μL of the translation reaction was diluted to 90 μL with translation buffer and layered on top of a 110-μL 0.5 M sucrose cushion in a polyallomer airfuge tube. The membranes were pelleted as above and gradient fractions were analyzed by SDS-PAGE. To determine the total amount of radioactive protein synthesized, the radioactivity in one of the 1 μL calibration aliquots was measured by scintillation counting after precipitation with 10% trichloroacetic acid on GF/C filters (Whatman). To calibrate the storage phosphor screen used to analyze the gradient fractions, the other 1 μL calibration aliquot was analyzed on the same SDS-PAGE gel as the gradient fractions. A correction factor used to convert phosphorimager units into counts per minute was obtained by dividing the phosphorimager units for the calibration aliquot by the counts per minute measured for the other calibration aliquot. Thus quantification of the radioactivity recorded on the storage phosphor screen from the calibration samples permits quantification of the protein in the other lanes. To convert these values to femtomoles of protein, the total radioactivity in counts per minute was divided by the number of methionines in the molecule and the specific activity of the isotope was used to convert the radioactivity [after conversion of counts per minute to disintegrations per minute using the measured counting efficiency of the scintillation counter (88%)] into femtomoles. The unlabeled methionine in the reaction (22 μM) was measured by HPLC. The radiochemical purity of the labelled methionine was greater than 98%; thus the total unlabeled methionine in the reaction is negligible. To determine the amount of protein specifically bound to membranes, the amount of protein pelleted in

a duplicate tube without added membranes was subtracted from the total amount of protein in the bottom fraction. The number of binding sites was determined by Scatchard analysis (Scatchard 1949).

2.1.3. Liposome Binding.

Samples containing liposomes were analyzed as described previously (Andrews et al 1989, Janiak et al 1994a), except that the gradient steps were composed of 70 μL of 0.86 M sucrose in translation buffer, 110 μL of 0.34 M sucrose in translation buffer, and 40 μL of translation buffer. Each samples were subjected to 30 psi (170,000 xg) for 2 hr in a 30 ° A-100 airfuge rotor (sigma). The gradient was fractionated into five fractions (55 μL each), with the pellet, solubilized as above, as the bottom fraction.

2.1.4. Gel-Shift Assay.

Cysteine residues in the polypeptides were modified by incubation with IASD [4-acetamido-4'-[(iodoacetyl)amino)stilbene-2,2'-disulfonic acid] purchased from Molecular Probes. The gel-shift assay was adapted from that described by Krishnasastry et al. (Krishnasastry et al 1994). Following termination of translation, 10 μL of the translation reaction was diluted to 40 μL with 0.3 M Tris-HCl (pH 8.5) and 1 mM DTT or with 0.3 M Tris-HCl (pH 8.5), 1 mM DTT and 8 M urea buffer. IASD was added to a final concentration of 10.5 mM from a 100 mM stock solution in 18 MΩ resistance deionized filtered water. As a control, 6 μL of the diluted reaction was removed before adding IASD. Aliquots containing equivalent amounts of the original starting material were

quenched with DTT at the indicated time points and then separated by SDS-PAGE on either 16% or 12-18% gradient polyacrylamide gels (Laemmli 1970). The radioactive proteins were visualized and quantified as above.

2.2. RESULTS

2.2.1 Binding of mT, Bcl2, Cb5 and Vamp to microsomal membranes

The four tail-anchored proteins, mT, Bcl-2, Cb5 and Vamp1 share no sequence homology and also are diverse in their function. However, all four proteins contain a putative transmembrane domain at the carboxyl-terminus (Figure 2.1A) and are membrane bound *in vivo*. To examine the post-translational binding to membranes the four proteins, synthesized *in vitro* using rabbit reticulocyte lysate in the presence of ^{35}S-Met, were incubated with cycloheximide and the ribosomes were removed by centrifugation. After incubating the supernatant of each reaction with canine ER microsomes, the microsomes were separated from the reaction by pelleting them through over a sucrose cushion. Membrane bound proteins are found with the membrane pellet. Of the four proteins examined, only mT was found not to pellet with microsomes in this assay (Figure 2.1B, lane 1-3). The small amount of mT detected in the bottom fraction (Figure 2.1, lane 3) represents only 4% of the total protein and results primarily from electrostatic interaction

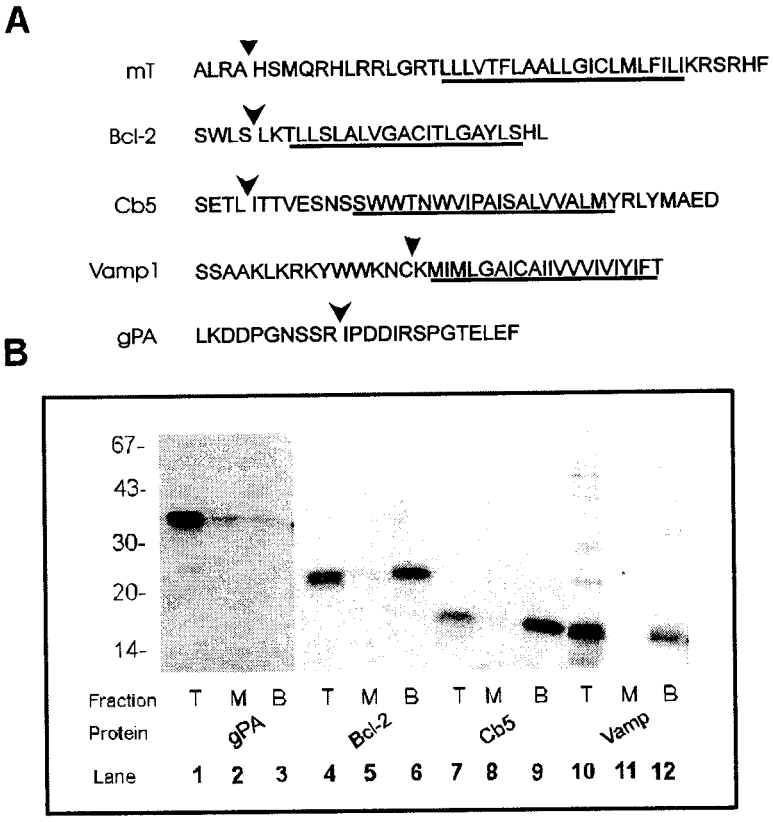

Figure 2.1. Amino acid sequence of tail-anchored proteins and post-translational binding to ER microsomes.

A) Amino acid sequences of the putative insertion sequences are given in one-letter code. mT, polyomavirus virus middle-T antigen; Cb5, the endoplasmic reticulum-specific form of rat liver cytochrome b_5. The hydrophobic core of each sequence is underlined. The junction for the fusion proteins is indicated by an arrowhead. The sequence of the carboxyl terminus of the control molecule gPA is also indicated. For gPA the arrowhead indicates the fusion point at which the insertion sequences were added, thereby replacing the last 14 amino acids of gPA. (B) Post-translational binding of mT, Bcl-2, Cb5, and Vamp1 to microsomes. Rabbit reticulocyte lysate translation reactions (10 μL) were incubated with 2 equiv of canine pancreatic microsomes for 20 min at 24 °C. The reactions were then layered on top of a 0.5 M sucrose cushion (100 μL) and the microsomes were pelleted by centrifugation. The gradients were divided into top (T), middle (M), and bottom (B) fractions. The top fraction contains soluble proteins and the bottom fraction contains microsomes and microsome-bound proteins. An aliquot of each fraction equivalent to 1.0 μL of the translation reaction was analyzed by SDS-PAGE. Lanes 10-12 are from a longer exposure of the same gel. Migration positions of molecular mass markers (in kilo Daltons) are indicated at the left of the figure.

with the ER membrane (see below). As expected, Cb5 bound readily to microsomes, as did Bcl-2. Vamp did not bind to membranes as efficiently as Cb5 or Bcl-2. About 20% of the Vamp1 protein pelleted with ER microsomes compared to 82 % for Cb5 and 61% for Bcl-2. To determine if the pelleted Vamp1 is bound to membranes instead of forming aggregates, the pellet was subjected to extraction with either sodium carbonate (pH 11.5) or high salt (1 M NaCl). In both assays Vamp1 pelleted with the membrane fraction, suggesting that Vamp1 in integrated into the lipid bilayer of the ER microsome (data not shown).

Although both carbonate and high salt extractions are standard assays for membrane integration, Young et al (1996) demonstrated that some peripheral membrane proteins are resistant to both procedures. Chemical labelling was used to provide independent verification of the insertion into the lipid bilayer of the putative transmembrane domain of Bcl-2 and Vamp1. Since both Bcl-2 and Vamp1 has two cysteines, one in the transmembrane domain and another in the cytoplasmic part of the protein, a cysteine modifying reagent, IASD was used to determine if the TM has integrated into membranes. IASD is a hydrophilic iodoacetamide that covalently modifies cysteines and causes a change in the electrophoric mobility of the labelled protein in SDS-PAGE (figure 2.2). Due to the high polarity of IASD it does not label cysteines that are in the lipid bilayer (Krishnasastry et al 1994). Therefore, a cysteine in the transmembrane domain should be protected by modification by IASD if it is inserted into the lipid bilayer. We also predicted that if cysteine in a transmembrane sequence is

labelled before targeting the protein to membranes, the label should prevent the insertion of a transmembrane domain into the lipid bilayer. Thus a transmembrane domain of a tail-anchored protein labelled with IASD should prevent binding of the protein to membranes because the two negative charges added to the sequence would not be stable in the lipid bilayer. To verify this, the transmembrane domain of Bcl-2 and Vamp1 was fused behind gPA, to generate the fusion protein gPABcl-2 and gPAVamp1 respectively (the fusion points are indicated in figure 2.1A). The gPA domain was selected as a fusion partner because it does not contain cysteine and was previously shown to have no intrinsic targeting information (Janiak et al 1994a). The only cysteine residue present in these fusion proteins, gPABcl-2 and gPAVamp1 are in the transmembrane domains of Bcl-2 and Vamp1. gPABcl-2 bound to ER microsomes at similar levels as its full length version (Figure 2.2, Lane 7-9). However gPAVamp1 did not bind to membranes (data not shown). Only the putative transmembrane domain of Vamp1 (residue 96-118) was used in the gPAVamp1 fusion since the residue 95 is a cysteine which will give the gPAVamp1 fusion two cysteines. As discussed further in the next chapter, the putative transmembrane domain alone of Vamp1 was not sufficient for binding to membranes, but it also requires at least 16 residues N-terminus of the TM is required for wild type levels of binding (chapter 3). Since a Vamp1 fusion to gPA with a extended N-terminus would result in two cysteines in the new fusion gPA-Vamp1 fusion protein, Vamp2, which is 76 % identical to Vamp1 was used as a substitute for Vamp1. Vamp2 has only one cysteine which is located at its transmembrane domain. The new gPA fusion, gPAVMB contain

residues 80 to 116 (Chapter 3 figure 3.5). This region is highly conserved between Vamp1 and Vamp2 as they only differs in eight of the 39 residues, and most of these differences are conserved changes between the two proteins. Furthermore, the binding of Vamp1 to membrane is similar to Vamp2 (this will be further described in Chapter 3). Therefore, gPAVMB was used as a substitute for gPAVamp1.

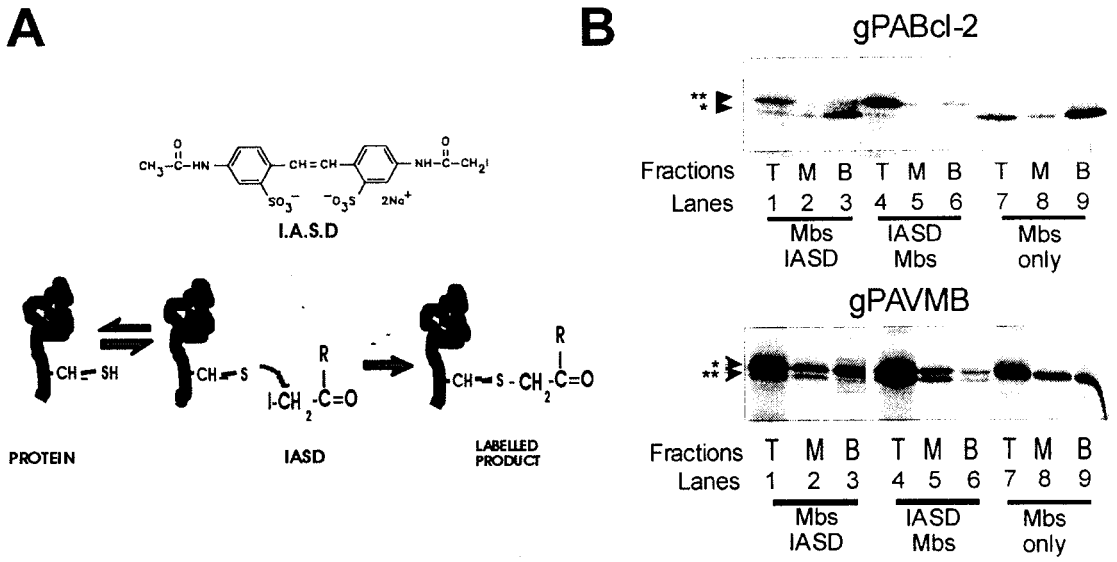

Figure 2.2. IASD labelling of gPABcl-2 and gPAVMB

A) A structural diagram of IASD and diagram of the IASD labelling reaction.
B) IASD labelling of gPABcl-2 and gPAVMB before and after membrane targeting reveals that the cysteine in the Bcl-2 insertion sequence integrates in the membrane.
Reticulocyte lysate translation reactions (10 µl) for gPABcl-2 or gPAVMB were mixed with an equal volume of translation buffer. This mixture was labelled with IASD before or after incubation with 2 equivalents of microsomes (Mbs) for 30 min. at 24°C. The cysteine in the insertion sequence of integrated molecules was protected from labelling by the lipid bilayer (lanes 1-3). Labelling with IASD before incubation with membranes prevented membrane integration (lanes 4-6). As a control, a sham reaction was performed without IASD (lanes 7-9). Microsomes were then isolated by centrifugation over sucrose steps and fractionated as above. The migration position of unlabelled (dot) and labelled gPAbcl-2 or gPAVMB (*) are indicated to the left of the panel.

gPAVMB bound to membranes at similar levels to Vamp2 and Vamp1 (chapter 3 figure 2.4). Both gPABcl-2 and gPAVMB proteins when bound to ER microsomes were protected from modification by IASD (Figure 2.2, lane 3) while the unbound fraction (lane 1) was modified. A cysteine modification by IASD in gPABcl-2 resulted in an upward shift on a SDS-PAGE while a downward shift was observed for gPAVMB. Also gPABcl-2 was more readily modified than gPAVMB. This may be due to misfolding of gPAVMB as only about 20% of the full length Vamp1 synthesized in rabbit reticulocyte lysate bound to membrane (figure 2.1). Similarly about 20 % of gPAVMB pelleted with ER microsomes (figure 2.2 lanes 1-3 and lane 7-9) suggesting that the other 80 % may be misfolded. Most of the 20% that pelleted with membranes was not modified by IASD (figure 2.2, lane 3) suggesting that the cysteine in the transmembrane domain of Vamp2 is in the lipid bilayer. This result is consistent with results of carbonate extractions suggesting that gPAVMB is integrated in the bilayer. When the proteins were labelled before incubating them with ER microsomes, binding is reduced for both proteins (figure 2.2, lane 4-6). Therefore, taken together with the carbonate and high salt extraction, these results indicate that similar to Cb5, both Bcl-2 and Vamp1 are integrated into the lipid bilayer via the putative transmembrane domain.

2.2.2. Effect of ATP on membrane targeting

Previously Vamp2, an isoform of Vamp1, was reported to require ATP and a trypsin sensitive membrane component for it to bind to ER microsomes (Kutay et al 1995). The possible ATP dependence of Bcl-2 and Cb5 binding to membranes has not been examined. Therefore, we investigated whether ATP is required for membrane binding by Vamp 1, Cb5 or Bcl2. To remove ATP from the *in vitro* translation reaction, 5 units of Apyrase were added. After incubation with Apyrase, membrane binding was impaired for Vamp1 as only 7% bound compared to 30% for control (figure 2.3 compare lanes 1-3 with lanes 4-6). Furthermore, when all nucleotide triphosphates were first removed by passing the reaction mix though a G-25 column, binding was reduced to background (3% bound) (figure 2.3, lane10-12). However, the binding to membranes of Bcl-2 and Cb5 was not affected by either hydrolyzing ATP by Apyrase or by the removal of all small molecules by gel permeation chromatography on a G25 column (figure 2.3, compare lanes 1-3 with lanes 4-6 and lanes 10-12). Binding of Vamp1 to ER microsomes was also abolished when the membranes were pre-incubated with 5 μg/mL trypsin (figure 2.3, lanes 7-9). These data are consistent with a requirement for both ATP and an ER membrane protein in targeting of Vamp2 to membranes (Kutay et al 1995). In contrast both Cb_5 and Bcl-2 bound to trypsin digested microsomes. These results indicate that the targeting to ER microsomes of Vamp1 is fundamentally different than that of Bcl-2 or Cb5.

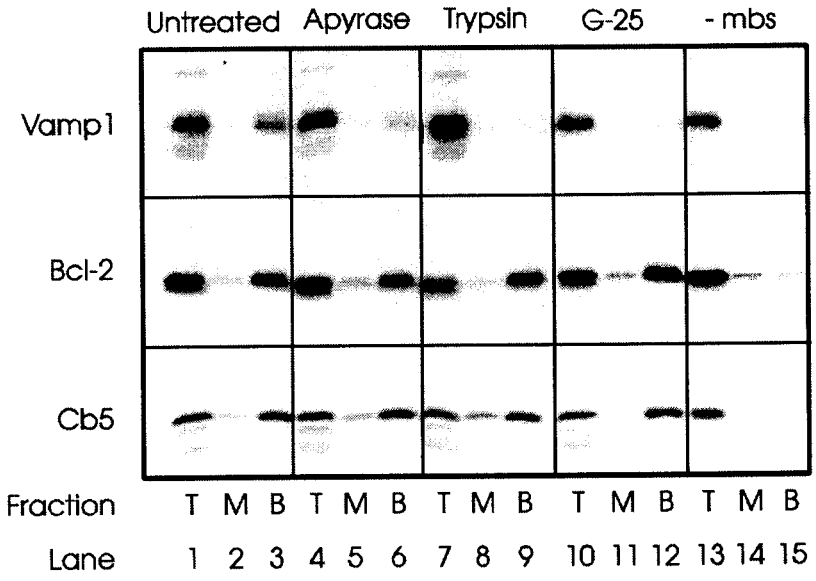

Figure 2.3. Vamp1, Bcl-2 and Cb5 ATP and trypsin dependence pelleting assay. Only Vamp1 membrane binding requires ATP and a trypsin-sensitive membrane component. Reticulocyte lysate translation reactions (10 μL) for Vamp1, Bcl-2, and Cb5 were incubated with 2 equiv of ER microsomes for 20 min at 24 °C (untreated) or treated as follows. (a) Apyrase: after translation for 1 h, 5 units of Apyrase were added to the translation reactions. After 30 min incubation, 2 equiv of microsomes was added. (b) Trypsin: microsomes were digested with 5μg/mL sequencing grade trypsin for 1 h on ice. Then trypsin was inactivated with PMSF and the microsomes were washed with high salt before 2 equiv was added to the translation reactions. (c) G-25: after translation was completed, small molecules were removed from the translation reactions by centrifugation through a column of Sephadex G-25. (d) -mbs: microsomes were not added to the translation reaction. Microsomes were isolated from the incubations by centrifugation on sucrose gradients and the gradients were divided into top (T), middle (M), and bottom (B) fractions.

The requirement for a trypsin sensitive membrane component on the ER for the efficient targeting of Vamp1 suggests that Vamp1 membrane binding may be receptor mediated. If Vamp1 stays bound to the receptor, or if the receptor binding is the rate limiting step in the targeting and inserting of Vamp1 into the bilayer then the binding

should be saturable. To determine whether the binding of Vamp1 to canine ER microsomes is saturable, increasing amounts of *in vitro* translated proteins were incubated with two equivalents of KRM (1 equivalent of KRM represents approximately 100 fmole of SRP receptor). Preliminary control experiments demonstrated that after 2 hours incubation, binding had reached equilibrium, no further increase in membrane binding occurred. To permit direct comparison of the binding characteristics of different molecules, the radioactivity recorded on the phosphorimager plate was converted to femtomoles of protein (see material and methods). Figure 2.4 shows that the binding of Vamp1 to membranes is saturable.

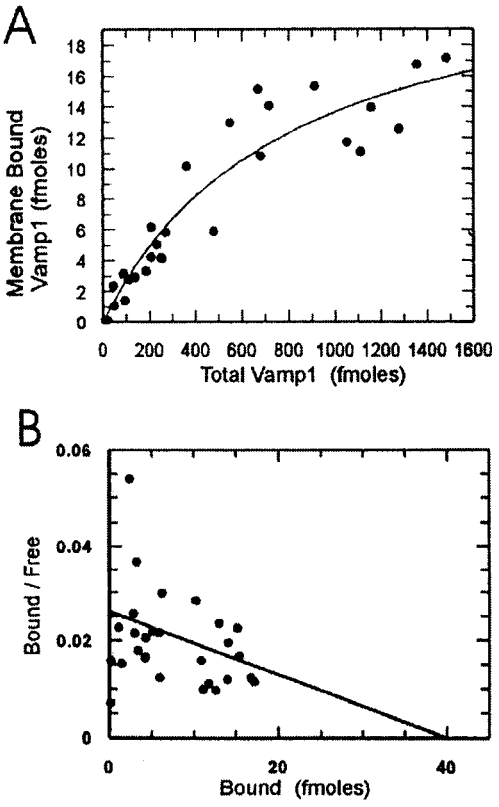

Figure 2.4. Binding of Vamp1 to microsomes.

(A) Increasing amounts of the individual proteins in translation reactions were incubated with 2 equiv of microsomes for 2 h at 24 °C. Translation buffer (30 μL) was added to the reaction mixtures and they were layered on top of a 0.5 M sucrose cushion (110 μL). Microsomes were separated from the reactions by centrifugation at 20 psi (110000*g*) for 10 min at 4 °C in an A10-300 rotor in an airfuge (Beckman Instruments). After the different factions were separated by SDS-PAGE, the total amount of the specific protein loaded and the amount recovered with the pelleted microsomes was measured using a phosphorimager and converted to counts per minute by scintillation counting of duplicate samples after trichloroacetic acid precipitation of the proteins in the translation reaction. (B) Scatchard analysis of the binding data. One of two independent experiments is shown. The number of binding site was calculated to be 20 ± 13 fmole/equiv of microsomes.

The reticulocyte lysate used to synthesize the protein was depleted of endogenous methionine by gel-filtration chromatography. Thus the amount of unlabelled methionine in the lysate is negligible. Therefore by measuring the amount the ^{35}S-methionine incorporated into the protein, the approximate number of binding sites for Vamp in microsomes can be calculated. The number of Vamp1 binding sites in one equivalent of canine ER microsomes was determined to be approximately 20 ±13 fmole using a Scatchard analysis of two independent data sets. The high error is mainly due to the limited synthetic capacity of the *in vitro* system that prohibited us from synthesizing enough Vamp1 to extend the Scatchard plot. Nevertheless, the ATP dependence, the requirement for a trypsin-sensitive component and the saturable binding of Vamp1 demonstrate that binding to ER microsomes by Vamp1 is clearly different from that of Bcl-2 or Cb5.

2.2.3. Comparison of membrane binding properties of the putative insertion sequences

The binding of mT to membranes may be similar to Vamp1. A specific membrane receptor may be present in the authentic target membrane that is not in canine ER microsomes. To test this hypothesis, mT was incubated with several different purified membranes. These included plasma membrane (P1), rat liver mitochondria, rat liver lysosome, and rat brain synaptic vesicles. mT did not bind to any of these organelles, whereas Cb5 bound readily to all of them (data not shown). These results were not surprising as it was demonstrated that when expressed in cultured cells mT is

first found in a brefeldin A resistant perinuclear compartment (Zhu et al 1998). Therefore the organelles tested here may not be the authentic organelle for the initial targeting of mT to membranes. Furthermore, mT has been shown to bind to elements of the membrane skeleton that may not be present in our cell free system (Andrews et al 1993).

Alternatively, the *in vitro* synthesized protein may be misfolded such that its insertion sequence is inaccessible to bind and integrate into membranes. To determine if the folding of mT in rabbit reticulocyte prevented the putative transmembrane domain from binding to membranes, it was replaced with the insertion sequence of either Cb5 or Bcl-2. When these proteins were targeted to ER microsomes, they readily pelleted with the microsomes, thus suggesting that the lack of binding to membrane of the wild type mT is not due to the inaccessibility of the mT insertion sequence (figure 2.5).

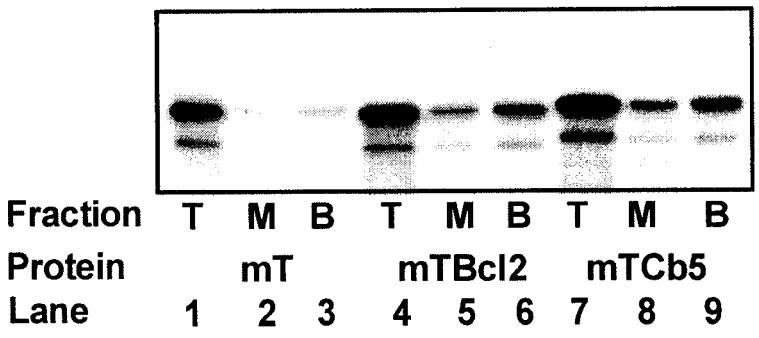

Figure 2.5. Membrane binding of mT fusion proteins
Membrane binding assays for mT and mT fusion proteins. The cytoplasmic domain of mT was fused to the insertion sequence from Bcl-2 (mTBcl-2) or Cb5 (mTCb5). Translation reactions for these molecules were analyzed for membrane binding as above.

To examine this possibility further, gPA fusion proteins containing the insertion sequences of mT, Bcl-2, and Cb5, were constructed. A scheme of the various fusion proteins is shown in figure 2.1A. As previously described (Janiak et al.) without a tail-anchor, gPA alone did not bind to KRMs (figure 2.6A). The addition of the C-terminus of either Bcl-2 or Cb5 to the C-terminus of gPA resulted in efficient binding of the fusion proteins to membranes, with 73% and 61% of the molecules pelleted with the KRMs, respectively (figure 2.6A, lanes 7-9 and lanes 10-12). In contrast, the fusion proteins with mT insertion sequence bound to KRMs less efficiently, (17%) (figure 2.6A, lane 4-6). Therefore, these results suggest that the insertion sequence of Bcl-2 and Cb5 are sufficient for membrane targeting, whereas the insertion sequence of mT is not sufficient.

The small amount of gPAmT that pelleted with membranes appears to be bound via electrostatic interaction rather than integration into the bilayer since it was prevented by increasing the ionic strength of the translation reaction (data not shown). This electrostatic interaction of gPAmT with membranes is also consistent with our supposition that the fusion protein folds such that the putative insertion sequence of mT is exposed and available for membrane binding. However, it is still possible that the insertion sequence of mT is partially masked in mT and when attached to gPA thus prevented from inserting into the membrane.

The mT insertion sequence was also examined within the context of a molecule that is efficiently targeted *in vitro* to microsomes by its own insertion sequence. The rationale for this experiment is that by exchanging the insertion sequence on Bcl-2 for

that of mT, the mT insertion sequence will be in a context in which a very similar sequence is available for membrane binding. However, even in this context the mT insertion sequence bound poorly to membranes (figure 2.6B). In figure 2.6B, only 19% of Bcl-mT pelleted with membranes (lane 10-12). The cytoplasmic domain of Bcl-2, Bcl, can fold in a conformation that binds membrane when fused to an exogenous insertion sequence as Bcl-Cb5 bound readily to membranes (41% compared to 44% bounding of Bcl-2; figure 2.6B, lanes 1-3 and lanes 7-9). The small amount of BclmT and gPAmT that bound to membranes did not increase with increased amount of membranes added whereas the binding of Vamp1, Bcl-2 and Cb5 all increased with increased membranes (see below). Taken together, these data suggest that the binding of BclmT and gPAmT to membranes is due to relatively non-specific electrostatic interactions. Therefore, these membrane binding studies indicate that binding of mT to membranes is not a result of the spontaneous insertion mechanism observed for Cb5 and Bcl-2.

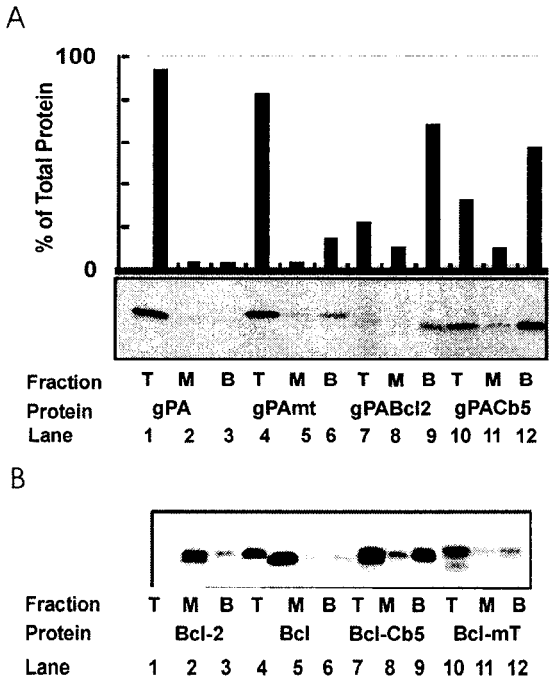

Figure 2.6. Pelleting assay of gPA and Bcl-2 fusion proteins.
The carboxyl-terminal hydrophobic sequence of mT will not bind fusion proteins to microsomes. (A) Membrane binding assays for gPA and the gPA fusion proteins. As a convenient nomenclature for the fusion proteins, the first part of the name represents the fusion partner and the second part of the name represents the protein from which the insertion sequence was derived. Thus, gPACb5 consists of the passenger protein gPA fused to the insertion sequence from cytochrome b_5. Translation reactions (10 μL) were incubated with microsomes (2 equiv) and analyzed by sedimentation in sucrose step gradients as above. The percentage of the total protein synthesized in the translation reaction that was recovered from each fraction is shown on the histogram above the autoradiogram. (B) Membrane binding assays for Bcl-2 and Bcl-2 fusion proteins. The cytoplasmic domain of Bcl-2 (amino acids 1-213, Bcl) was fused to the insertion sequence from Cb5 (BclCb5) or mT (BclmT). Translation reactions for these molecules were analyzed for membrane binding as above.

2.2.4. Binding of mT to phospholipid vesicles

Experiments with ER microsomes demonstrate that the binding mechanism of mT is different than that of Cb5. The binding of mT to ER microsomes is largely electrostatic whereas Cb5 is integrated into the bilayer. One possible reason for the inability of the mT transmembrane domain to insert into the lipid bilayer of ER microsomes is that proteins and other molecules on the ER surface may block insertion. We considered it unlikely that the mT insertion sequence would be blocked in mT, and the gPAmT and Bcl-mT fusion proteins particularly, since the other three proteins studied

inserts readily into the membrane. Nevertheless to assay binding of the mT insertion sequence to lipid bilayer, liposomes were used. The composition of the liposomes used mimicked the ER lipid bilayer. Liposomes were added to the rabbit reticulocyte lysate translation reaction of mT, or gPAmT and the vesicles were separated from the incubation mixture by equilibrium sucrose density centrifugation. The gradient was divided into five fractions, including a pellet fraction labelled 'B'. Equivalent portions of each fraction were analyzed by SDS-PAGE (figure 2.7). Proteins bound to the phospholipid are detected at the top two fractions of the gradient.

The wild type mT floated upwards with lipid vesicles (Figure 2.7). However, when the wild type protein was incubated with lipid vesicles in 1 M KOAc (Figure 2.7A), very little mT associated with lipid vesicles, whereas the Cb_5 migrated with vesicles in high salt concentration (data not shown). Similar to the full length mT, the insertion sequence of mT was sufficient to bind to liposomes in the absence of high salt as it also co-migrated with the vesicles (Figure 2.7B). The binding of gPAmT to liposome was due to the mT insertion sequence as gPA was not found in the top fractions with liposomes. When 1 M KOAc was added to the reaction, the binding was greatly diminished. These experiments further support the hypothesis that the binding of mT to membranes in these *in vitro* experiments is solely due to the electrostatic interactions between the insertion sequence of mT, and the phospholipids of the membrane. Thus the lack of insertion of gPAmT into the lipid bilayer of membranes is unlikely to be due to the masking of its insertion sequence thereby preventing it from interacting with the lipid bilayer.

Therefore these results confirm that the binding of mT to membranes is distinct from that of Cb5 and Bcl-2.

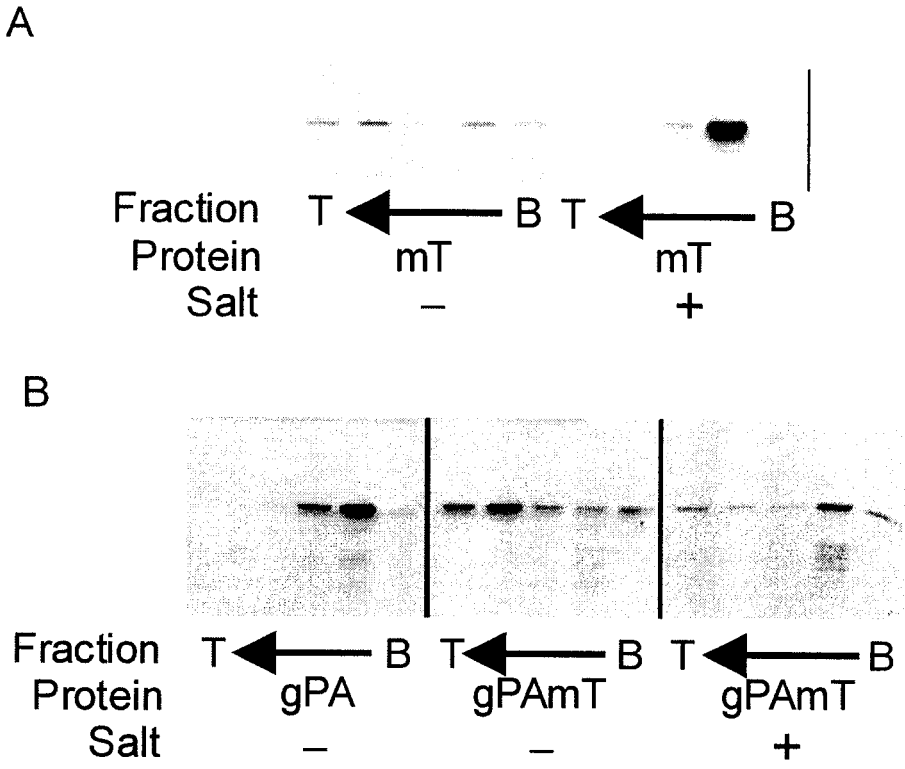

Figure 2.7. Analysis of binding to phospholipid vesicles by floatation in sucrose gradients.

A) Binding of mT to phospholipid vesicles is abolished by incubation in 1M KOAc. Phospholipid vesicles were added to translation reactions and incubated at 24 °C for 15 min. Sucrose was added to a final concentration of 0.86M and the samples (70 µl) were transferred to airfuge tubes. 110 µl of 0.34M sucrose in translation buffer and 40 µl translation buffer were layered on top. For binding assays in high salt the phospholipid vesicles were made in the presence of 1 M KOAc and the translation reactions and gradient steps were adjusted to 1 M KOAc prior to addition of phospholipid vesicles. After centrifugation in an A100-30 rotor for 2 hrs at 30 psi (170,000 xg) in an airfuge (Beckman Instruments) gradients were fractionated from the top (T) into 5 fractions (55 µl each), with the solubilized pellet as the bottom (B) fraction. Translation reactions for the wild-type proteins and fusion proteins are identified below the relevant panels. Migration positions of molecular weight markers (in kDa) are indicated at the left of the panels.
B) Binding of gPA and gPAmT fusion proteins to phospholipid vesicles.

2.2.5 The mechanism of membrane integration of Bcl-2 is similar but distinct from Cb5

The binding of purified Cb5 to microsomal membranes has been previously shown to be not saturable (Strittmatter et al 1972) and spontaneous (Enoch et al 1979, Takagaki et al 1983b, Takagaki et al 1983a). The binding of Bcl-2 to ER microsomes (figure 2.3) appears similar to that of Cb5 (Enoch et al 1979, Takagaki et al 1983b, Janiak et al 1994a). However, it is not known if the binding of Bcl-2 to ER microsomes is saturable. To examine the saturability of the binding of the proteins Cb5 and Bcl-2 to ER microsomes, proteins synthesized in rabbit reticulocyte lysate were used to determine a binding curve in the same way as for Vamp1. In figure 2.8 the binding curves for Cb5 and Bcl-2 to one and two equivalents of ER microsomes are shown. The binding of both proteins appears not to be saturable in the *in vitro* assay as the binding increased linearly to the maximum protein added. Lack of saturation may be due to the limited synthetic capacity of reticulocyte lysate to synthesize sufficient amount of proteins to reach the saturation point of Bcl-2. Only 1400 fmoles and 550 fmoles of Cb5 and Bcl-2 were synthesized respectively. Binding of Bcl-2 increased linearly when added to one equivalent of membranes (from 4 to 55 fmoles of Bcl-2 bound), whereas binding of Cb5 increased linearly from 9 to 330 fmoles. This result suggests that similar to Cb5, the binding of Bcl-2 to microsomes is not saturable. However, since one equivalent of ER membranes represents 100 fmoles of SRP receptors, it is possible that the binding site for Bcl-2 may be as abundant using the reticulocyte system. It is not possible to determine a

saturation curve for a protein with a moderate number of binding sites. Therefore, it is possible that the binding site of Bcl-2 may exceed 55 fmoles or like Cb5 its binding is not saturable. It is also possible that the binding of Bcl-2 to a membrane receptor is not its rate limiting step.

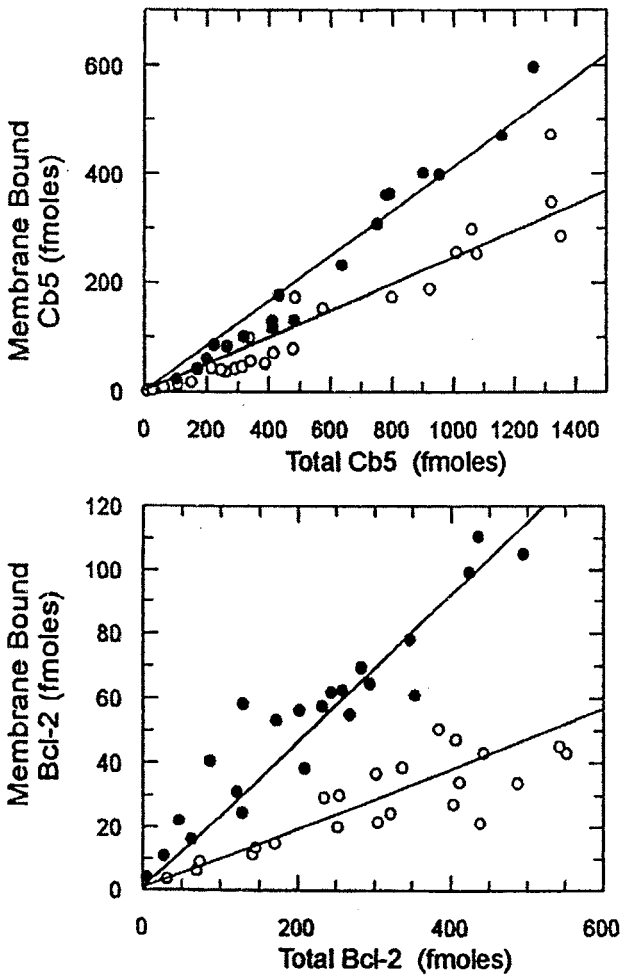

Figure 2.8. Cb5 and Bcl-2microsome binding curve.
Translation reactions (60 µL) containing increasing amounts of the individual proteins were incubated with either 1 equiv (○) or 2 equiv (●) of microsomes for 2 h at 24 °C. Membrane binding was assayed as described above (Figure 2.4).

However, these binding curves reveal some insight in the possible mechanism of membrane targeting of these proteins. Increasing the amount the membranes from 1 equivalence to 2 equivalence resulted in an increase in the slope of the binding curve of both Bcl-2 and Cb5 (figure 2.8). There are two possible explanations for these data. Firstly the data suggest that the binding of these proteins is a bimolecular event, where the concentration of both the protein and the binding site (membrane) affect binding rate. Therefore in this case neither the protein nor the binding sites are in sufficient excess to drive the reaction to completion, which results in the increase in the slope when the amount of membrane is increased. The second possibility is the existence of equilibrium between two or more forms of the protein, where one conformation is competent to bind to membranes. Therefore, increasing the amount of membranes (thus increase the number of binding sites for the protein) will affect the equilibrium between the binding competent and non-competent by favouring the binding competent form. This will result in an increase in the slope of the binding curve. Gel-filtration chromographic data add support for this latter model as both monomeric and oligomeric forms of Cb5 and Bcl-2 were detected (data not shown). Cb5 has been previously reported to exist in an equilibrium between monomeric and octameric forms (Calabro et al 1976), while Bcl-2 appears to exist as monomers and pentamers (data not shown). However, it is also possible that both processes may be contributing to the binding curve in figure 2.8.

Cb5 has been previously proposed to bind spontaneously to membranes based on the observation that it binds to liposomes (Enoch et al 1979, Takagaki et al 1983b,

Takagaki et al 1983a). In other words, Cb5 does not need any other factors (membrane or cytosolic based) to bind to membranes since purified Cb5 bound to liposomes. To determine if Bcl-2 binds to lipids membranes, the binding of Bcl-2 and Cb5, synthesized in rabbit reticulocyte lysate, to liposomes was compared. As above, vesicles and vesicle-bound proteins were recovered from the translation reaction by flotation in a sucrose gradient. In both physiologic ionic strength (figure 2.9) or in buffer containing 1 M NaCl (data not shown) Bcl-2 did not float up with the liposomes, while Cb5 did float upward with the liposomes. When the lipid composition of liposomes was changed to include an increased amount of PS (7:6:3:4 from 7:8:1:4 PC/PE/PS/cholesterol) Bcl-2 was found in the top fraction (data not shown). However, the binding was diminished in the presence of 1 M NaCl suggesting an electrostatic interaction between the protein and the liposome rather than integration of the insertion sequence into the bilayer. This result suggests that mechanism of the insertion of the tail-anchor of Bcl-2 into membranes differs from that of Cb5.

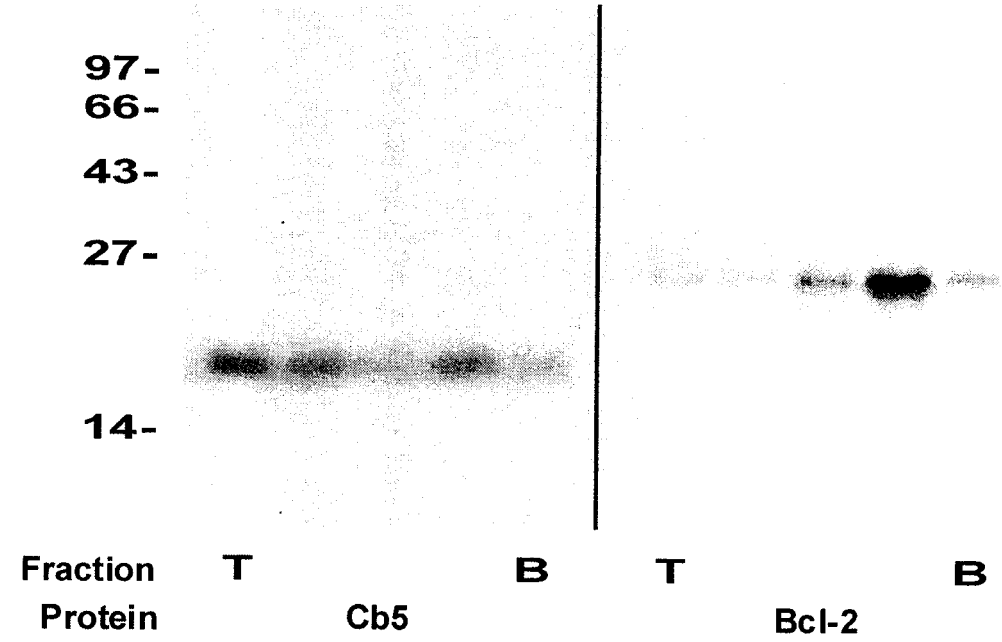

Figure 2.9. Binding of Cb5 and Bcl-2 to LUV.
Cb5 but not Bcl-2 binds to phospholipid vesicles. Phospholipid vesicles were added to translation reactions and incubated at 24 °C for 15 min. Sucrose was added to a final concentration of 0.86 M, the samples (70 μL) were transferred to airfuge tubes, and 110 μL of 0.34 M sucrose in translation buffer and 40 μL translation buffer were sequentially layered on top. After centrifugation in an A100-30 rotor for 2 h at 30 psi (170000x*g*) in an airfuge (Beckman Instruments), gradients were fractionated from the top (T) into five fractions (55 μL each), with the solubilized pellet as the bottom (B) fraction. Translation reactions for the proteins are identified below the relevant panels.

2.3. Discussion

The membrane targeting and integration of tail-anchored proteins does not require the SRP/Sec61 complex (Rapoport et al 1996). Instead the targeting and integration of these proteins was thought to occur spontaneously without specific signal or sequence but it was thought to be based on the hydrophobicity of their carboxyl transmembrane domain (Whitley et al 1996). Here we presented evidence which contradicts this common notion about the targeting mechanism of tail-anchored proteins.

The proteins examined here have been subject of membrane targeting studies (Janiak et al 1994b, Vergeres et al 1995, Kutay et al 1995, Hofer et al 1995). However, we present the first study which examines and directly compares the mechanisms of membrane targeting of all four of these proteins within the same context. Using various cell-free assays, we have shown that multiple mechanisms exist for insertion of the tail-anchored proteins into membranes. In fact, our *in vitro* assays demonstrate that the mechanisms of membrane targeting and insertion are strikingly different for each protein studied.

The carboxyl terminal end of Cb5 enables spontaneous binding to both biological and artificial membranes. Initially, Bcl-2 also appeared to share a similar spontaneous binding mechanism (figure 2.1, 2.4). Like Cb5, Bcl-2 binds to ER membranes independent of ATP and binding is not reduced by trypsinization of membrane proteins (figure 2.3). Furthermore carbonate extraction and high salt extraction indicated that Bcl-

2 was not peripherally bound to canine ER microsomes but was integrated into the lipid bilayer. Insertion of the transmembrane domain into the bilayer was verified by IASD labelling (figure 2.2). However, unlike Cb5, Bcl-2 does not bind efficiently to artificial liposomes. The inability of Bcl-2 to integrate into the bilayer of liposomes suggests that binding of Bcl-2 to membranes is not spontaneous, but some other component may be necessary for its insertion. I suggest two possible mechanisms of Bcl-2 binding to ER microsomes. First, the binding of Bcl-2 may require a factor on the ER membrane that causes a conformational change in the carboxyl terminal region to a conformation which is more favourable to insertion into the bilayer. It is also possible that the binding of Bcl-2 to an ER component increases the efficiency of membrane insertion without effecting its conformation.

Similar to Cb5, Vamp1 bound readily to ER microsomes and it was resistant to both high salt and carbonate extraction suggesting that it is integrated into the lipid bilayer. Membrane integration was further demonstrated by the protection of the cysteine residue in the transmembrane domain from modification by IASD. However, unlike Cb5 and Bcl-2, Vamp1 binding required both a cytosolic component (ATP) and a trypsin sensitive membrane component. At the trypsin concentration used here of 0.5 μg/mL at 0 °C, relatively few ER membrane proteins are degraded (Andrews et al 1989), however this concentration of trypsin abolished the binding of Vamp1. This suggests that the putative Vamp1 receptor is highly sensitive to trypsin. The analysis of the binding curve using a Scatchard plot indicated that there is about 20 fmoles of Vamp binding receptor

per 1 equivalent of the ER microsomes. Therefore, these data suggest that Vamp1 does not insert spontaneous into the lipid bilayer of the membranes. Instead, it requires the aid of membrane bound component(s) probably to change the conformation of the insertion sequence to allow membrane insertion.

In contrast to the other proteins, only electrostatic binding to membranes was observed for mT. The ability of the insertion sequence to mediate electrostatic binding to ER microsomes and phospholipids vesicles suggest that the lack of integration is not due to the inaccessibility of its hydrophobic domain to interact with the lipid bilayer. In addition, two other experimental results suggest that the C-terminus of mT is accessible to the aqueous environment. First, fusion proteins of the cytoplasmic domain of mT with insertion sequence of other tail-anchored proteins (mTBcl-2 and mTCb$_5$) binds to microsomal membranes. This suggests that the (mis)folding of the cytoplasmic domain of mT does not mask the insertion sequence. Secondly, when the insertion sequence of mT was fused to gPA or Bcl-2, these fusion proteins did not bind to microsomes. However the insertion sequences of Bcl-2 and Cb5 readily mediated the binding of the cytoplasmic portion of gPA, Bcl-2 and mT to membranes. Therefore the lack of binding of gPA-mT and Bcl-mT is unlikely due to the rest of the molecule interfering with the insertion sequence of mT from mediating the membrane binding. Taken together, these results suggest that unlike Bcl-2 and Cb5, the hydrophobic carboxyl terminus of mT does not directly insert into membranes.

The membrane insertion of mT may be similar to that of Vamp1. The ER is the *bona fide* site for membrane insertion for Cb5, Bcl-2 and Vamp1 (Zhu et al 1996, De Silvestris et al 1995, Kutay et al 1995), however the initial target membrane for mT remains controversial (Zhu et al 1998). These results suggest that ER is not the authentic target of mT. Both the full length and fusion proteins of mT did not integrate into ER membrane. Nor did it bind to other organelles like mitochondria, plasma membrane, synaptic vesicles, and lysosome. It is possible that like Vamp1, mT requires a membrane receptor which is not found in canine ER microsomes or in the other membranes examined. There is some evidence that mT targets to as yet unidentified perinuclear membrane (Zhu et al 1998). However to determine if mT binds to these membranes in a cell-free assay such as that used here, the appropriate target membrane must be first isolated.

2.4. Conclusion.

By comparing directly the mechanisms of membrane integration of mT, Vamp1, Bcl-2 with that of Cb5, I have clearly demonstrated multiple mechanisms for membrane integration of these tail-anchored proteins. At one extreme, exemplified by Cb5 and to a lesser extent Bcl-2, membrane integration occurs spontaneously and non-saturably. Therefore correct localization of these proteins must be determined primarily by targeting, for if Cb5 were to freely diffuse in the cytoplasm, it would be undoubtedly integrate equally into many subcellular locations. At the other extreme are the Vamp molecules which could diffuse freely in the cytoplasm because membrane integration is regulated. Although the targeting of these proteins can be regulated to increase the efficiency but it is not essential for the correct localization of these proteins. The integration of mT into membrane is suspected to fall into the latter category; however the ER microsomes are not an authentic target for mT molecules.

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Chapter 3

Identification of the endoplasmic reticulum targeting signal in vesicle-associated membrane proteins.

Preface.

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All the data was produced by Peter K. Kim. William Trimble provided the full length Vamp2 and Vamp8. Catherine Hollerbach contributed in the cloning of the N-terminal deletion mutants of Vamp2. The manuscript was prepared by Peter Kim, Brian Leber, and David Andrews.

3.0 Introduction

Vamp1 is a member of the v-snare family (or Vamp family) of proteins that currently includes eight members (Bock & Scheller 1997). Initially found in synaptic vesicles, the Vamps target vesicles to their target membrane by forming a tight coil-coil interaction with two t-snare proteins, SNAP 25 and syntaxin, located on the target membrane (Bock & Scheller 1997). This coil-coil complex is called the snare complex (reviewed in Bock & Scheller 1997). The snare complex has been proposed to be involved in the vesicle targeting specificity, docking of the vesicle, and the fusion of the vesicle to the target membrane (Sollner et al 1993, Ungermann et al 1998, Weber et al 1998).

Vamps 1 and 2 require ATP and a membrane-bound receptor to target to the ER (Chapter 2) (Kutay et al 1995) by a process in which targeting and integration appear to occur simultaneously. Once integrated into the ER membrane, Vamps 1 and 2 are sorted to secretory vesicles and the plasma membrane. To elucidate the mechanism of targeting of Vamp proteins to the ER membrane, I have compared the membrane binding characteristics of distantly related members of the Vamp family (Vamp1 and Vamp2 compared with Vamp8) and identified the sequence that specifies localization to the endoplasmic reticulum. Surprisingly the sequence necessary for targeting in all three Vamps includes four positively charged amino acids spaced along one side of an amphipathic sequence that is amino-terminal to the hydrophobic region. The results also indicate that there is a common mechanism and probably a single receptor used to target different Vamps to the ER.

3.1. Methods

3.1.1. Plasmid construction.

Construction of a plasmid expressing Vamp1 (GenBank accession# AAA42322) was described previously (Elferink et al 1989b). Vamp2 (GenBank accession# AAA42321) and Vamp8 (GenBank accession# T63214) cDNA sequences were amplified by polymerase chain reaction (PCR) from rat and Human cDNA respectively, and inserted into pBluescript KS-. For *in vitro* transcription/translation, the protein coding sequences were cloned into pSPUTK, a plasmid that contains both a SP6 promoter and a high-efficiency 5' untranslated region for translation in reticulocyte lysate (Falcone & Andrews 1991). Vamp2 and Vamp8 were subcloned into pSPUTK by PCR.

In vitro and *in vivo* gPA behaves as a cytosolic protein but can be targeted to different intracellular membranes when fused to appropriate targeting sequences (Janiak et al 1994). Gene fusions for gPA and either Vamp2 or Vamp8 were constructed using a unique *Bam*H1 site near the 3' end of the coding region of gPA and a *Xho*1 site 3' of the gPA termination codon. The required sequences were added to the coding regions of Vamp2 and Vamp8 by PCR amplification. N-terminal deletions of the Vamp2 coding sequence in gPAVamp2 were constructed using a whole plasmid PCR method as described (Hughes & Andrews 1996) except that PCR was performed using a 40:1 ratio of Taq (MBI, Fermentas) and Vent (New England Biolabs) DNA polymerases. To construct a plasmid encoding gPAVamp2 (96-116), the plasmid encoding gPAVamp2 was prepared from GM48 cells and cut with *Bcl*1 (within the Vamp2 coding region) and *Bam*H1 (near the carboxyl-end of gPA) restriction endonucleases and then the plasmid was re-closed by ligation. Plasmids encoding point mutations were generated by cassette mutagenesis using internal *Bcl*1 and *Eco*R1 sites. For random mutants the oligonucleotides were synthesized with multiple nucleotides added at the appropriate

steps in the synthesis reaction (Mobix central facility, McMaster University). The relevant regions of all of the plasmids were sequenced to ensure that additional mutations were not introduced during amplification or cloning.

3.1.2. Preparation of membranes

Canine pancreatic ER membranes (microsomes) were prepared as described by Walter and Blobel (Walter & Blobel 1983). Each batch of microsomes was tested for cotranslational translocation of preprolactin (Andrews et al 1989). One equivalent of microsomes is defined as 100 femtomoles of signal recognition particle receptor α-subunit as measured by western blotting (Andrews et al 1989). Mitochondria and lysosome were isolated from rat liver as described by Greenawalt (Greenawalt 1974), and Symons and Jonas (Symons & Jonas 1987) respectively. Phospholipid vesicles (7:8:1:4 phosphatidylcholine/ phosphatidylethanolamine/ phosphatidylserine/ cholesterol) were prepared by extrusion in 10 mM Tris-HCl buffer, pH 7.5.

3.1.3. In vitro transcription, translation and membrane binding

In vitro transcription and translation were performed as previously described (Andrews et al 1989). After 60 minutes incubation at 24 °C, the translation reaction was stopped by adding cycloheximide to a final concentration of 20 μg/mL. ATP was then removed from the translation reaction by incubation with 5 units of Apyrase (Sigma) at 24°C for 30 min. Nucleotides and other small molecules were removed by passing the reaction though a 600 μl Sephadex G-25 (Sigma) spin column. ATP was added to the G-25 treated translation reaction by adding a solution containing 100 mM ATP, 10 mM Tris-HCl, pH 7.5 to a final concentration of 0.50 mM ATP.

The membrane binding assay has been described previously (chapter 2). Immunoblotting with calreticulin of a parallel reaction fractionated similarly demonstrated that the microsomes remained sealed throughout the procedure.

Mitochondria and lysosome binding assays were performed similarly with the following modifications. Mitochondria were pelleted at 17,000 xg in a microcentrifuge for 10 min at 4 °C and the pellet was washed once with translation buffer (50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 1 mM DTT). Lysosomes were pelleted through a 0.5 M sucrose cushion by centrifugation at 39,000 xg in a TLA100 rotor (Beckman).

Volumes of each fraction corresponding to equivalent amounts of the original translation reaction were analyzed by SDS-PAGE using a Tris-tricine buffer system (Schagger & von Jagow 1987). Radioactive proteins were visualized and quantified using a phosphorimager (Molecular Dynamics). Quantitative membrane binding assays were performed as described previously (chapter 2).

3.2. Results

3.2.1. Common mechanism of targeting Vamps to ER membranes.

Vamp proteins are expressed in all tissue types and are highly conserved in different organisms (Elferink et al 1989b, Trimble et al 1991, Rossetto et al 1996). Out of the eight known members, Vamp1, Vamp2 and Vamp3 share the highest level of similarity (>71% identical). The other Vamps are 25% to 29% identical to Vamp1 and to each other (Bock & Scheller 1997). In addition, several spliced-isoforms of Vamp1 were recently cloned, one of which targets specifically to mitochondria (Isenmann et al 1998, Berglund et al 1999). All of the Vamps possess a hydrophobic domain at the C-terminus which is believed to be the membrane anchor domain. Studies on Vamp1 and Vamp2 have shown that both ATP and an ER receptor are required for the efficient binding of these proteins to ER membranes (Chapter 2) (Kutay et al 1995) However, the targeting mechanism of the other members of the Vamp family is not known. To answer this question and to identify potential targeting motifs, the targeting mechanism of *in vitro* synthesized Vamp8, which is only 29 % and 33 % identical to Vamp1 and Vamp2 respectively, was compared with that of Vamp2.

Both Vamp2 and Vamp8 bound efficiently to canine ER microsomes (figure 3.1, lane 1-3). After subtracting the percentage of proteins pelleted in the absence of membranes (figure 3.1, lane3 13-15), 20% and 21 % of Vamp2 and Vamp8 (respectively) was found to pellet with microsomes. This is far less than the 67% of Cb5 that pelleted with membranes (chapter 2). However, more than 80% of the membrane-bound material was resistant to both carbonate and high salt extraction, suggesting that the proteins are

efficiently integrated into the lipid bilayer of the membrane (data not shown). ATP was depleted from the translation reaction by either removing small molecules by gel filtration chromatography, or by treating the reaction with Apyrase prior to the addition of membranes. In both cases protein binding to microsomes was greatly diminished (figure 3.1, lanes 4-6 and lanes 7-9). When 0.5 mM ATP was added to the translation reaction that was previously subjected to gel filtration as above, Vamp binding was restored (figure 3.1, lane 10-12). Other nucleotides were ineffective, confirming the specificity for ATP (data not shown).

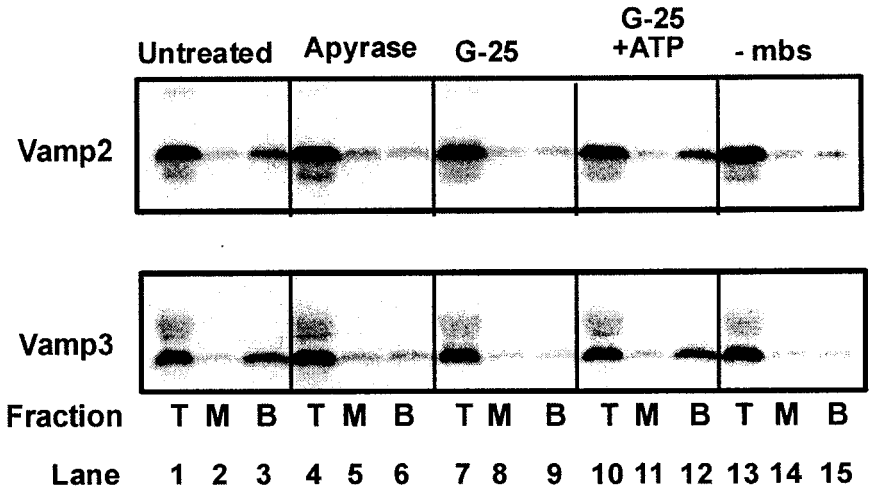

Figure 3.1. Vamp2 and Vamp8 binding to ER is ATP dependent.

Reticulocyte lysate translation reactions of Vamp2 and Vamp8 were incubated with microsomes (untreated) or treated as follows: Apyrase; after translation for one hour, 5 units of Apyrase was added to the translation reactions and incubation was allowed to continue for another 30 minutes and microsomes were added. G-25; after translation for one hour small molecules were removed from the translation reactions by centrifugation through a column of Sephadex G-25. G-25 +ATP; after G-25 column separation, a final concentration of 0.5 mM of ATP was added to the translation reaction before the microsomes. – mbs; microsomes were not added to the translation reaction. After in vitro translation of [^{35}S]-Met labelled proteins, 1 equivalent of microsomal membranes was added to 10 µl of the translation reaction and incubated for one hour at 24 °C. Membrane bound proteins were separated by pelleting through a sucrose cushion. Gradients were divided into three fractions (T) top, (M) middle and (B) bottom or pelleted fraction. Equivalent amounts of each fraction were analyzed by SDS-PAGE.

In the previous chapter, the binding of Vamp1 to membranes stripped of peripheral proteins by high salt treatment was found to be saturable. Furthermore binding of Vamp1 was abolished when the membranes were pre-incubated with a low concentration of trypsin. When compared directly, the binding of all three Vamps to non salt-treated ER microsomes observed to be very similar. In figure 3.2, the binding curves of all three Vamps are superimposed. For 1 equivalent of ER membranes, saturation occurs at approximately 45 fmole of protein (determine by Scatchard analysis). Furthermore, none of the three proteins bound to trypsinized microsomes (Chapter 2: figure 2.2, Kutay et al 1995). Taken together, these results suggest that all three Vamps bind to ER microsomes via an interaction with a membrane protein. To elucidate whether a common receptor mediates the binding of these Vamps, binding competition experiments are required. However, the amount of protein required for such experiments is difficult to produce using an *in vitro* translation reaction.

To further confirm that Vamp membrane binding requires a membrane receptor on the target membrane, the binding of Vamp1 to liposomes was assayed with a lipid composition similar to that of the ER membrane (Andrews et al 1989). Both *in vitro*-synthesized Vamp1 and Cb5 (which binds spontaneously to most lipid bilayers) were incubated with liposomes. When the binding reaction was subjected to equilibrium density centrifugation, the liposome-bound protein was found in the top two fractions (figure 3.3A, lanes 1 & 2), while the unbound protein remains in the bottom fraction (figure 3.3A, lane 4). The majority of Cb5 was recovered from the first two top fractions (figure 3.3A, lane 1 & 2), whereas Vamp1 (figure 3.3A, compare lane 1 with 4) and

Vamp8 (data not shown) remained unbound to liposomes. These results are consistent with the requirement for a Vamp receptor in the ER membrane.

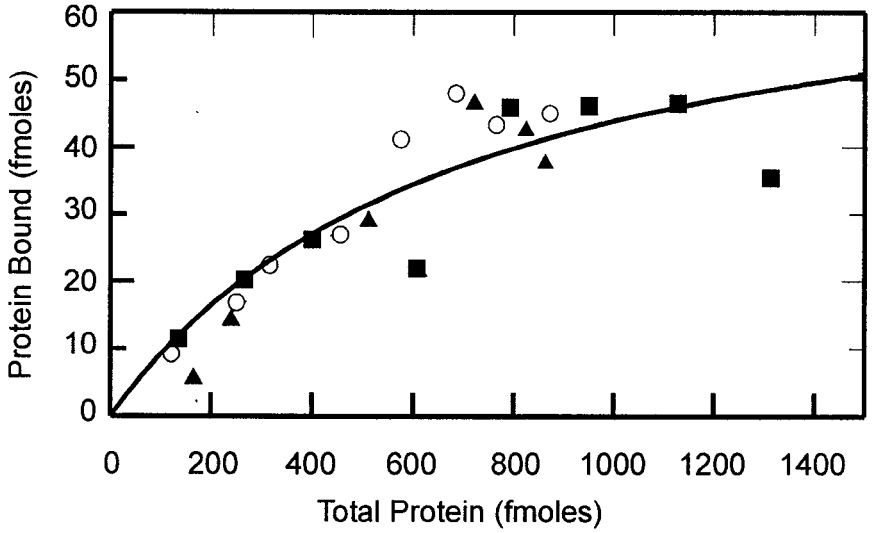

Figure 3.2. Binding of Vamps to microsomes is saturable.
Increasing amounts of individual translation reactions containing Vamp1, Vamp2, or Vamp8 were incubated with 1 equivalent of microsomes for 2 hours at 24 °C. Translation buffer (30 µl) was added to the reaction mixtures and then the reaction was layered on top of a 0.5 M sucrose cushion (110 µl). Microsomes were separated from the reactions by centrifugation at 20 psi (110,000 xg) for 10 min at 4 °C in an A100/30 rotor in an airfuge (Beckman Instruments). After SDS-PAGE, radioactivity was measured using a phosphorimager, and converted to protein concentration. A best-fit curve was plotted for all three sets of data as there was no significant difference between the three individual curves. Open circles - Vamp1. Solid squares - Vamp2. Solid triangles - Vamp8.

3.2.2. The Vamp receptor is not found in the mitochondria or lysosome.

Vamp1 and Vamp2 are known to cycle between the PM and synaptic vesicles (Bock & Scheller 1997, Jahn & Sudhof 1999). Vamp8 has recently been demonstrated to be involved in vesicle transport within endosomes, and also between endosomes and lysosome (Wong et al 1998, Mullock et al 2000, Paumet et al 2000). Therefore, it is possible that Vamp proteins may insert directly into organelles other than the ER. However, *in vitro*-synthesized Vamp1 and Vamp8 did not bind to lysosomes (figure 3.3B,

lanes 1-3 and lanes 4-6). The positive control for membrane binding, Cb5, bound readily to lysosomes, suggesting that the lysosomes are intact. In cells, Vamp1 is found in small synaptic vesicles near the PM. However, when Vamp1 was incubated with rat brain synaptic vesicles, it did not bind to them (data not shown). Together, these results further suggest that both Vamp1 and Vamp8 are first targeted to the ER before being sorted to their final destination.

Recently, Vamp1b, a mitochondrial isoform of Vamp1, was cloned (Isenmann et al 1998). The mitochondrial targeting signal on Vamp1b has been shown to be the shortened hydrophobic transmembrane domain and three positive residues at the carboxyl-terminus of the protein (Isenmann et al 1998). Vamp2 and Vamp8 were tested to determine if they have an intrinsic targeting signal for mitochondria. When *in vitro*-synthesized Vamp1, Vamp2 and Vamp8, were incubated with rat liver mitochondria, none of them pelleted with the mitochondria (Figure 3.3C), suggesting that, like Vamp1, neither Vamp2 (figure 3.3C) nor Vamp8 (data not shown) contains a mitochondrial signal. However, it is not known whether the mitochondrial signal can prevent the binding of the protein to the ER. To answer this question, the mitochondrial signal sequence from Vamp1b was placed on Vamp2. This mutant, named Vamp2mito, bound readily to ER (figure 3.4) and to mitochondria (data not shown). Furthermore, like the wild type, its binding was ATP-dependent and sensitive to trypsin treatment of the membrane (data not shown). These results suggest that the ER-specific targeting signal is not localized to the extreme carboxyl terminus of the protein. Instead, targeting of the protein to mitochondria may be the result of the relaxation of an ER-specific targeting mechanism.

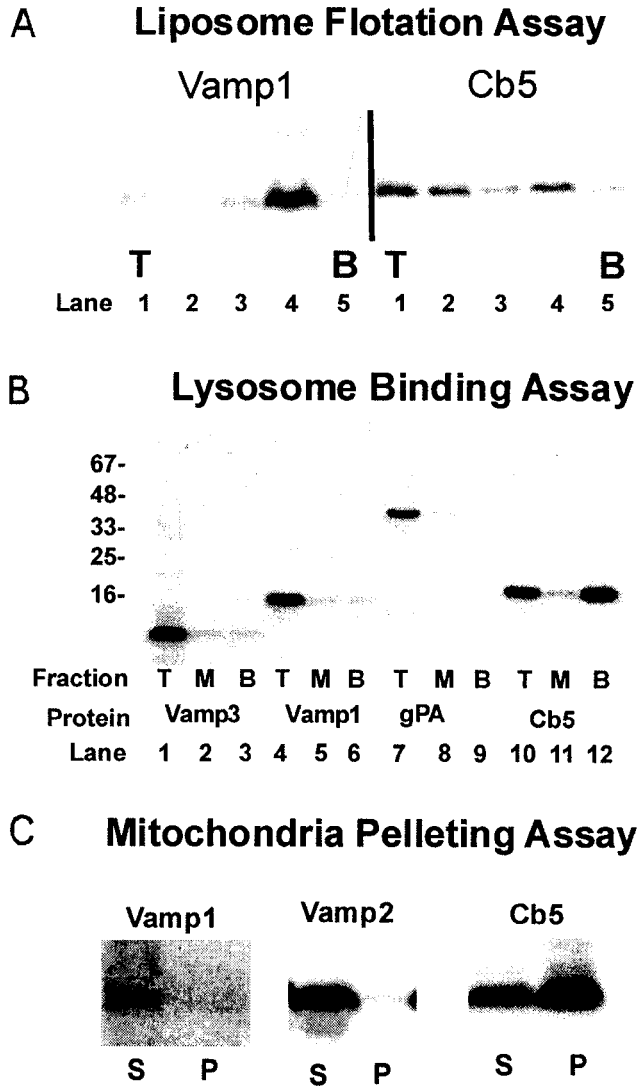

Figure 3.3. Vamp does not bind to other membranes.

A) Phospholipid vesicles were added to translation reactions. After incubation at 24 °C for 1 hour sucrose was added to a final concentration of 0.84 M, the samples (70 µl) were transferred to airfuge tubes, and 110 µL of 0.34 M sucrose in translation buffer and 40 µL translation buffer were sequentially layered on top of the sample. After centrifugation gradients were fractionated from the top (T) into five fractions (55 µL each), with the solubilized pellet as the bottom (B) fraction. Proteins are identified above the panels.
B) In vitro translated proteins were incubated with purified lysosomes (20 µg/ ml of total protein) for one hour. Membranes were separated from the reaction by centrifugation over sucrose step gradients. Gradients were divided into three fractions (T) top, (M) middle and (B) bottom or pelleted fraction. Proteins are identified below the panels.
C) In vitro translated proteins were incubated with purified mitochondria from rat liver (20 µg/ml of total protein) for one hour at 30 °C. Membranes were separated from the reaction by centrifugation over a 0.25 M sucrose gradient. S – Supernatant; P – pellet fraction. Proteins are identified above the panels.

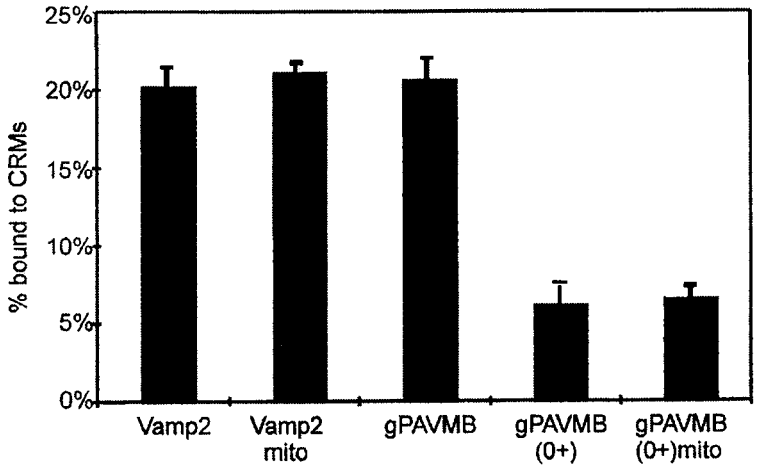

Figure 3.4. Lysine mutants do not bind to canine ER microsomes.

In vitro translated Vamp2 mutants and fusion proteins were incubated with 1 equivalent of microsomes for 1 hour at 24 °C. The microsomes were separated from the reaction by centrifugation over sucrose step gradients as above. gPAVMB, gPA fused to the minimal binding domain of Vamp2. Molecules indicated "mito" contain the Vamp mitochondrial targeting sequence. (0+) indicates that the 6 positively charged amino acids amino-terminal of the hydrophobic core of the insertion sequence have been replaced with non-charged hydrophilic amino acids.

3.2.3. 16 amino acids preceding the transmembrane domain are required for membrane binding.

The requirement for a receptor on the ER membrane for Vamp binding suggests that there may be a specific sequence in the Vamp family of proteins that is not present in insertion sequence proteins whose targeting is promiscuous. Recently the α-helix region (residues 31 to 60) was found to be essential for the targeting of Vamp2 and Vamp3 to synaptic vesicles (Grote et al 1995). However, it is not known whether this sequence is required for the initial targeting to membranes. Therefore, to elucidate the ER targeting sequence on the Vamp family of proteins, N-terminal deletion mutants of Vamp2 were screened for membrane binding. As shown in the schematic in figure 3.5A, various N-

terminal Vamp2 deletion mutants were fused onto the C-terminus of the cytosolic fusion protein gPA. The fusion proteins containing amino acid deletions up to residue 80 bound efficiently to microsomes (figure 3.5B, compare gPAVMB with Vamp2), suggesting that the synaptic vesicle targeting signal is not the ER binding signal. However, the ER binding signal may be the sequence following residue 80 as deletion beyond this residue resulted in a decrease in binding (figure 3.5B, gPAV2 (90-116) & gPAV2 (96-116)). Therefore, the gPA fusion protein containing residues 80-116 was designated gPAVMB (Vamp Minimal Binding) (figure 3.5). Neither the TM domain (residues 96-116) alone (Figure 3.5B, gPAV2 (96-116)) nor the TM truncated Vamp2 (data not shown) was not able to bind to membranes much above background, suggesting that the TM is necessary but not sufficient to target the protein to ER microsomes. The binding of both gPAV2 (full length Vamp2) and gPAVMB to ER microsomes was ATP dependent (figure 3.5C), suggesting that the mechanism of binding of the fusion proteins is similar to that of the wild type Vamp2.

We have designated the 16 residues preceding the transmembrane domain as the N-terminus sequence or NTS. A close examination of the sequence of all three Vamp proteins reveals a high sequence similarity in their NTS. Within this region are five positively charged amino acids, two tryptophans, and an asparagine that are all conserved in the three Vamps (figure 3.6A). Several secondary structure analyses predict this region to have a high helical propensity (data not shown). In fact, the crystal structure of the cytosolic domain of Vamp2 reveals that the whole domain (residue 1 to 95) is highly helical which is required to form a coil-coiled formation with its binding partners SNAP-

25, and Syntaxin (Sutton et al 1998). The helical wheel plot of the 16 residues preceding the TM reveals an amphipathic helix with four of the five conserved positively charged residues lining up on one face of the helix (figure 3.6b). Since Vamps 1, 2, and 8 all targeted similarly to ER it seemed likely that the conserved residues are involved in the specific recognition of this region by a membrane receptor at the ER membrane. It is also equally possible that targeting requires only the hydrophobic domain and that the NTS is necessary only for correct spacing between the hydrophobic and the cytosolic domains of gPAVMB. To test these hypotheses, all the positive charges on gPAVMB were changed to non-hydrophobic residues such as serine, threonine, glutamine and asparagine (figure 3.6A). The resulting fusion protein, gPAVMB-(0+), did not bind to microsomes much above background, suggesting that the positively charged residues are necessary for binding (figure 3.4).

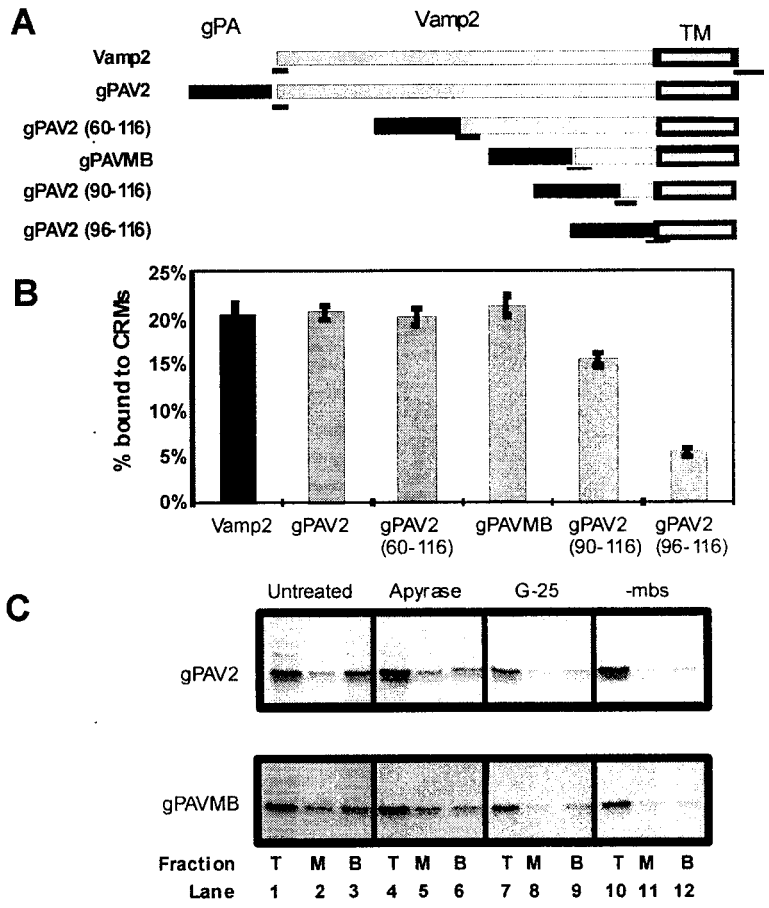

Figure 3.5. A sixteen amino acid sequence amino-terminal of the insertion sequence is required for Vamp1 membrane binding.

A) Schematic and nomenclature of the gPAVamp2 fusion proteins. Black bar, gPA. Gray bars, regions of Vamp2 fused to gPA. Numbers below represent the corresponding positions in Vamp2. Enclosed box, hydrophobic putative transmembrane domain of Vamp2 (TM). The fusion protein gPAVMB includes residues 80-116 of Vamp2 that contain the endoplasmic reticulum minimal binding region.

B) Binding assays for gPAVamp2 fusion proteins averages from three independent experiments. The error (one standard deviation) ranged from 1-1.6 % for the different mutants.

C) Membrane binding assays for gPAV2 and gPAVMB. Apyrase; after translation for one hour, 5 units of Apyrase was added to the translation reactions and incubation was allowed to continue for another 30 minutes and microsomes were added. G-25; after translation for one hour small molecules were removed from the translation reactions by centrifugation through a column of Sephadex G-25. – mbs; microsomes were not added to the translation reaction. After in vitro translation of ^{35}S Met labeled proteins, 1 equivalent of microsomal membranes was added to 10 µl of the translation reaction and incubated for one hour at 24 °C. Membrane bound proteins were separated by pelleting through a sucrose cushion. Gradients were divided into three fractions (T) top, (M) middle and (B) bottom or pelleted fraction.

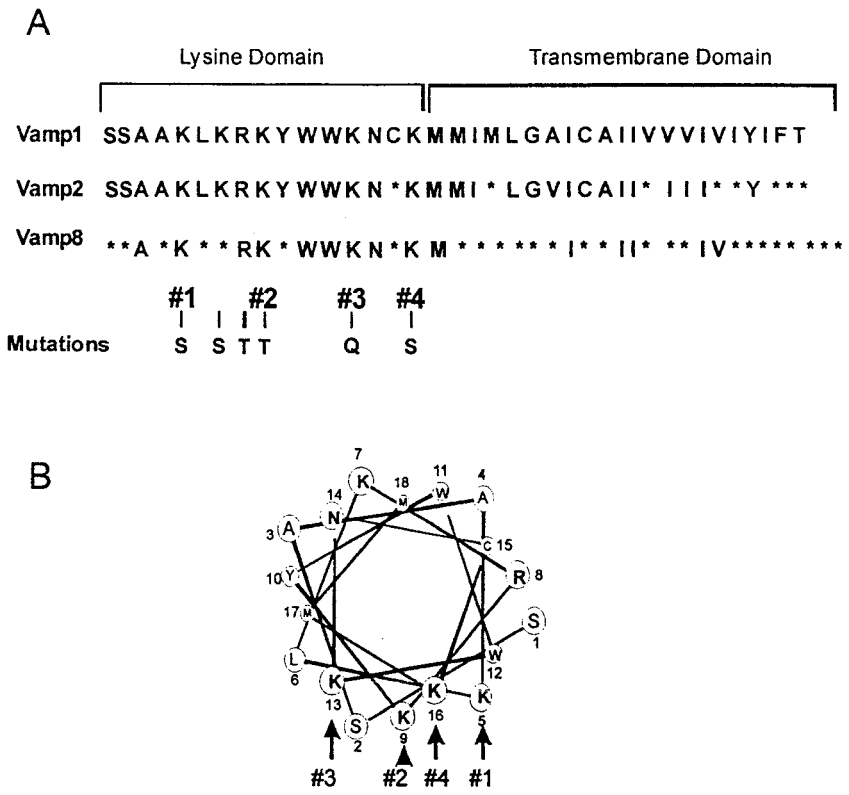

Figure 3.6. The minimal binding region of Vamps modelled as an amphipathic helix.

A) Amino acid sequence of the minimal binding region from Vamps1, 2, and 8. Non-conserved residues in Vamp2 and Vamp8 are indicated as *. Residues analyzed by mutagenesis are boxed with positively charged residues in stippled boxes. The four lysines indicated in (B) are numbered. Substitution mutations for the charged amino acids are shown below the sequence.

B) The putative amphipathic helix from Vamp1 presented as a helical wheel. The four lysines on the same face are numbered as in (A).

To determine if the four positively charged residues on the same face of the amphipathic helix is sufficient for binding to membranes, lysine-85 and arginine-86, which are not on the same side of the helix, were changed to serine and threonine respectively. This fusion protein, named gPAVMB-(4K), bound to membranes as

efficiently as wild type (figure 3.7A). Furthermore, the binding was ATP dependent, and sensitive to trypsinization of the membrane (data not shown). To determine if any one of the four lysines is important for membrane binding, a series of mutants were constructed containing various combinations of the four residues. No single residue was shown to have a significant effect on the binding. Therefore, membrane binding was averaged for mutants with the same number of positively charged amino acids. As figure 3.7A reveals, there is a direct relationship between the number of lysines in the protein and the amount of protein bound to membranes. Similar results were obtained for Vamp8 (figure 3.7B). The corresponding region on Vamp8 was sufficient to bind gPA to ER microsomes (figure 3.7B, gPAV8MB). Removal of all the positively charged residues in this minimal region abolished the binding of gPA to membranes (figure 3.7B, gPAV8MB (0+)). gPAV8MB was resistant to carbonate extraction and required ATP for binding to ER microsomes (data not shown). Finally, to discredit the unlikely possibility that the passenger protein gPA is in some way obstructing the transmembrane domain from inserting into the membrane once the positive charges are removed, the four lysines in both full length Vamp2 and Vamp8 were changed to non-charged non-hydrophobic residues. As expected, the binding of these two mutants to membranes was greatly diminished (data not shown).

The above results suggest that the putative hydrophobic transmembrane domain and the NTS together constitute a Vamp targeting signal for ER membranes. However, since the mitochondrial targeting isoform, Vamp1B, contains this ER signal, it is possible that the changes in the extreme carboxyl-terminus in Vamp1b might render the targeting

to the ER insensitive to the lysines in the amphipathic helix. However, as seen in figure 3.4, the Vamp1b targeting signal was unable to confer ER binding when added to gPAVMB (0+) (compare gPAVMB to gPAVMB (0+)mito in figure 3.4). Thus, together, these results suggest that the four lysines on the same face of the amphipathic helix are necessary for targeting Vamps to the ER. Furthermore, it suggests that the changes in the extreme carboxyl-terminus that permit binding to mitochondria do not alter the mechanism of binding to ER. Thus it appears that in whole cells the mitochondrial targeting signals is the dominant signal in Vamp1b.

To determine if either of the two conserved tryptophans or the asparagine also plays a role in the binding of Vamp2 to membranes, they were changed to other amino acids by cassette mutagenesis using doped oligonucleotides. These oligonucleotides were designed to encode one of ten amino acids at the site of tryptophan and asparagine. Of all the single mutants tested for binding, only the mutants in which the asparagine was changed resulted in significantly reduced membrane binding (figure 3.8). In contrast, with the exception of one mutant in which the tryptophan was changed bound to membranes as well as the wild type. Changing a tryptophan to a proline resulted in a marginal decrease ($p = 0.02$), which is most likely due to disruption of the local secondary structure.

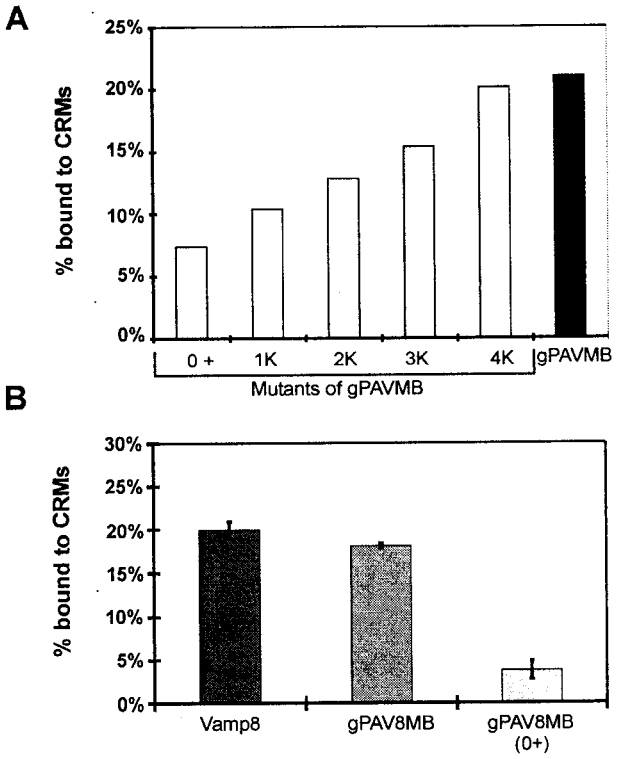

Figure 3.7. Four conserved lysines within the minimal binding region are necessary for membrane association.

A) Membrane binding gPAVMB point mutants. The five lysines and one arginine were changed to non-charged hydrophilic residues as K83S, K85S, R86T, K87T, K91Q and K94S where the first amino acid indicates the original residue, the number the amino acid position in Vamp2 and the final letter indicates the amino acid substitution. 0+ contains all of the mutations and therefore contains zero charged residues. The remaining mutants all contain the K85S and K86T mutations. Numbers beneath the bars indicate the number of charges restored at positions 1-4 indicated in Figure. 5. Values for membrane binding for the mutants with the designated number of charges were averaged. 1K, four mutants K83S, K87T, K91Q, K94S. 2K, six mutants (K83S, K87T), (K83S, K91Q), (K83S, K94S), (K87T ,K91Q), (K87T, K94S), (K91Q, K94S). 3K, three mutants (K83S, K87T, K91Q), (K83S, K87T, K94S), (K87T, K91Q, K94S). 4K, one mutant containing all four lysines. Each mutant was analyzed in three independent experiments.

B) Membrane binding by the Vamp8 minimal binding domain requires the 4 lysines on the same face of the putative amphipathic helix. Membrane binding is equivalent for Vamp8, and gPA fused to the conserved region (sixteen amino acids) and the TM domain from Vamp8 (gPAV8MB) in single letter code (TAQKVARK FWWKNVKMIVLICVIVFIIILFIVLFATGAFL). Substitution of the four conserved lysines in GPAV8MB (K83S, K87T, K91Q, K94S, numbering according to Vamp2) abolished binding to microsomes (gPAV8MB(0+)). Results are from 4 independent experiments. Error bars indicate one standard deviation.

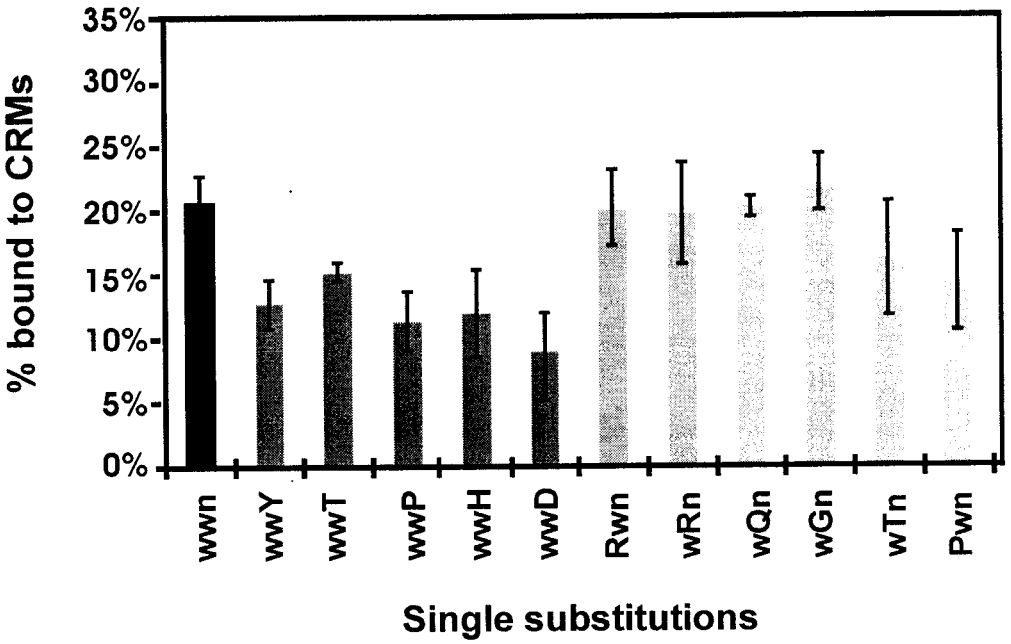

Figure 3.8. Mutation of the conserved asparagine within the minimal binding region reduced membrane binding.

The codons for W89, W90, and N92 in gPV2MB were mutated to encode different amino acids and the resulting mutants were assayed for binding to 2 equivalents of microsomes. The x-axis shows the relevant amino acid sequence for each mutant in single letter code. Lower case letters (wwn) indicate the wild type sequence. The capitalized letters denote the individual mutations. Results are averages from 4-5 independent experiments for each mutant. Error bars indicate one standard deviation.

3.2.4. Sec61γ and Sec61β also require ATP and a membrane receptor for binding to ER microsomes.

Sequence comparison predicted Sec61γ and Sec61β (both of which are components of the ER translocon (Kalies et al 1994)) to contain an amphipathic helical region preceding a TM domain similar to that seen in the Vamps. Therefore the targeting mechanism of these proteins may be similar to that of the Vamps. To compare the targeting mechanism of these proteins to the Vamps, post-translational membrane binding experiments were done under several different conditions. Both proteins bound to ER microsomes (figure 3.9 compare control vs. –mbs). When ATP was removed from

the binding reaction by either Apyrase treatment or G25 chromatography of the translation reaction, the binding of both Sec61γ and Sec 61β was greatly diminished (figure 3.9A). Binding was re-established by the addition of 0.5 mM ATP to the G25-treated reaction, suggesting that ATP is required for binding to ER microsomes (figure 3.9A). Furthermore, Sec61β and Sec61γ did not bind to trypsinized ER membranes, suggesting they also require an ER membrane protein to bind to the ER (figure 3.9A). The receptor for these proteins is also found only on the ER, as they did not bind to other organelles such as mitochondria or lysosomes (data not shown).

To further verify that both Sec61β and Sec61γ require a membrane-bound receptor, they were incubated with liposomes. Sec61β did not bind to either acidic liposomes (figure 3.10) or to liposomes of similar composition to ER (data not shown). Only a very small amount of Sec61γ (10%) bound to liposomes composed of an ER-like lipid composition; however, approximately 43% of total protein bound to liposomes rich in acidic phospholipids (figure 3.10). The binding of Sec61γ to acidic liposomes was not just an electrostatic interaction like that of mT. Instead, its resistance to high salt extraction suggests the protein was integrated into the lipid bilayer. The ability of Sec61γ to bind to liposomes rich in anionic phospholipids is not surprising as Sec61γ is rich in cationic residues. However, since the membrane bilayer is artificial, the observed targeting may be an artifact of the *in vitro* assay. Several small polypeptides that contain an amphipathic and hydrophobic domains are known to spontaneously bind lipid bilayers, especially those rich in anionic phospholipids (Bechinger 1997, Bechinger 2000). Although Sec61γ is larger than most peptides studied that spontaneously inserts into

membranes, Sec61γ is a relatively small polypeptide consisting of only 68 residues. Thus, it is possible that due to its small length, Sec61γ may behave very much like a membrane inserting peptide in certain environments. However, the inability of Sec61γ to bind to biological membranes, such as trypsinized ER microsomes or liposomes with an ER-like lipid composition, suggests that in general Sec61γ targets by a mechanism more similar to Vamps than to small amphipathic helical peptides. To test this hypothesis, the size of the polypeptide was increased by fusing Sec61γ behind gPA and the ability of gPASec61γ to bind acidic liposomes was examined. The inability of a gPASec61γ fusion protein to bind to anionic liposomes is consistent with the hypothesis that Sec61γ binding to anionic liposomes is an artifact of the *in vitro* assay (figure 3.10). Similar to Sec61γ the gPASec61γ fusion protein bound readily to ER microsomes (figure 3.9B & 3.9C), and its binding was ATP dependent (data not shown),

To further determine if the targeting mechanism of Sec61γ is similar to Vamp2, deletion mutants were made to test whether the lysine domain preceding the TM domain is required for the ATP-dependent binding to ER microsomes. Similar to the study on Vamp2, various lengths of the C-terminal end of Sec61γ were fused to the C-terminus of gPA. Figures 3.9B & 3.9C show that the putative transmembrane domain alone is not sufficient to bind gPA to membranes. However, the fusion protein containing both the lysine domain and the transmembrane domain (residues 20-68) of Sec61γ was sufficient to allow binding to ER membranes. Furthermore, the binding was ATP-dependent (figure 3.9C). These results suggest that the mechanism of membrane targeting of Sec61γ is similar to that of the Vamps.

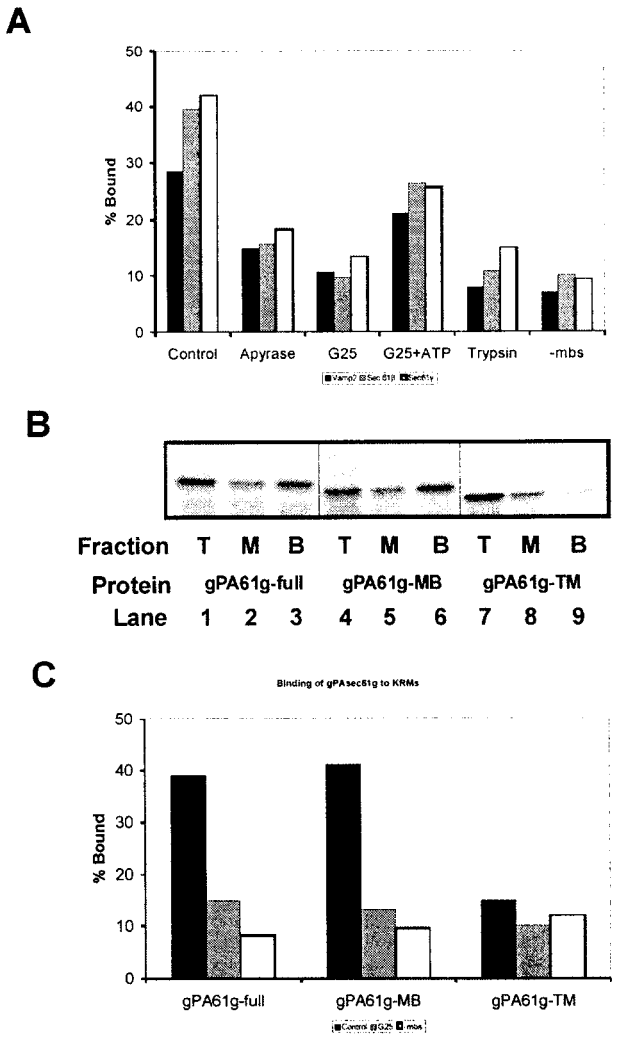

Figure 3.9. Sec61β and Sec61γ binding to ER microsomes is ATP dependent.

A) Reticulocyte lysate translation reactions of Vamp2, Sec61β and Sec61γ were incubated with microsomes (control) or treated as follows: Apyrase treated, G-25 treated, G-25 + 0.5 mM ATP, Trypsin treated potassium treated canine ER microsomes or – mbs. Equivalent amounts of (T) top, (M) middle and (B) bottom/pelleted fraction were analyzed by SDS-PAGE. The bands were quantified on a Phosphorimager station. The percent of the total protein at the B fraction was calculated and presented on a bar graph.
B) Various gPA Sec61γ fusion proteins were examined for binding to ER microsomes gPA61g, full length of Sec61γ fused to the C-terminus end of gPA. gPA61g-MB, gPA fused to the minimal binding domain of Sec61γ. gPA61g-TM contains only the TM of Sec61γ on gPA. Equivalent amounts of each fraction were analyzed by SDS-PAGE and visualized on by autoradiography.
C) Same as above. The percent of the total protein at the B fraction was calculated and presented on a bar graph. Solid black bar; 1 equiv. of microsomes were added to the translation reaction as above. Gray bar; G-25 treated translation reaction. Blank bar; no membrane control.

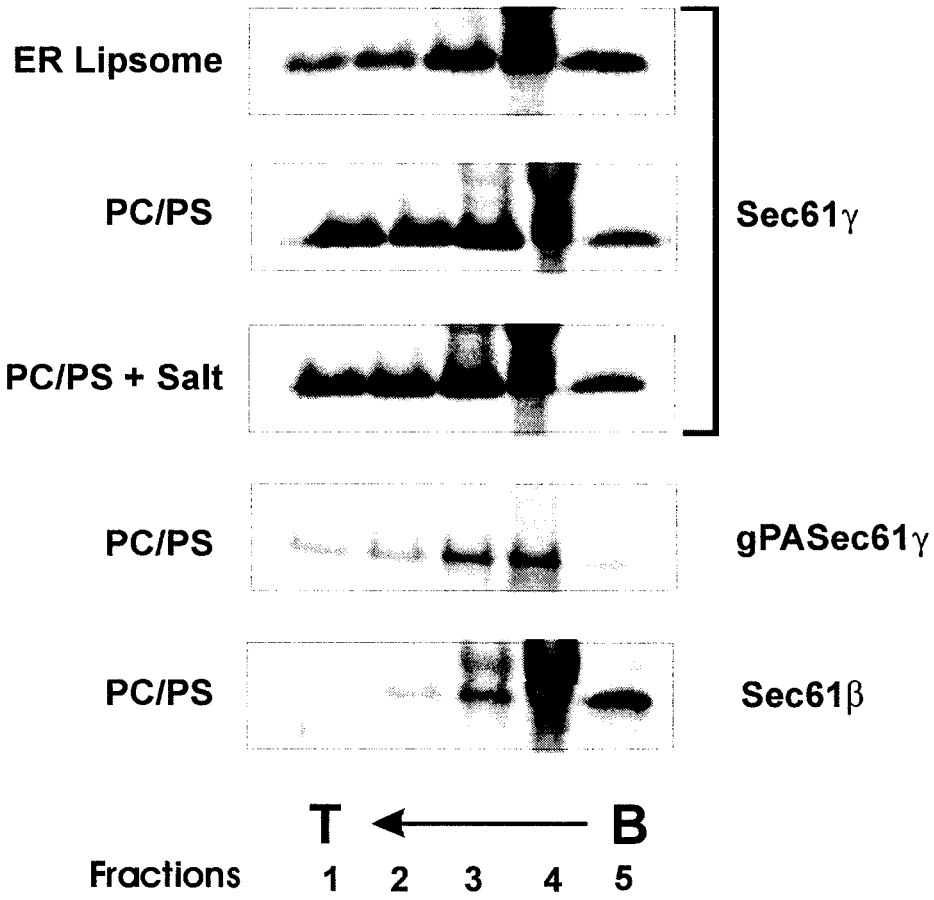

Figure 3.10. Sec61β and Sec61γ do not bound to liposome of ER composition. Phospholipid vesicles were added to translation reactions. After incubation at 24 °C for 1 hour sucrose was added to a final concentration of 1.6 M, the samples (70 µl) were transferred to TLA100 tube, and 100 µL of 1.3 M sucrose in translation buffer and 30 µL of 0.8 M sucrose were sequentially layered on top of the sample. After centrifugation in a TL-100 (Beckman Instruments) for 2 hours at 55,000 rpm (120,000 xg), gradients were fractionated from the top (T) into five fractions (50 µL each), with the solubilized pellet as the bottom (B) fraction. ER liposome: (7:8:1:4 phosphatidylcholine/ phosphatidylethanolamine/ phosphatidylserine/ cholesterol) were prepared by extrusion in 10 mM Tris-HCl buffer, pH 7.5 (24). PC/PS (2:1 phosphatidylcholine/ phosphatidylserine).

3.3. Discussion

Vamp1 and Vamp2 require ATP and a membrane component to bind to ER microsomes (chapter 2) (Kutay et al 1995). Similarly, the distantly related Vamp, Vamp8, also requires both ATP and a membrane component (figure 3.1) to bind to membranes. Furthermore, Vamp8 exhibited an identical binding curve to the other two Vamps (figure 3.2). Together, these results suggest that all three of these Vamps share the same mechanism of targeting to canine ER microsomes, where they are then sorted through the secretory pathway to their ultimate destinations. In fact, all three proteins share a unique targeting signal located N-terminal to their putative transmembrane domain which is necessary for ATP-dependent binding to ER microsomes (figure 3.7).

Deletions and point mutations in both Vamp2 and Vamp8 have demonstrated that the amphipathic α-helix region just N-terminal to the TM is necessary for binding of the Vamps to ER microsomes. Therefore, these results suggest that the 16 amino acids preceding the TM domain make up the ER targeting signal of these Vamps. The four lysines, which make the helix amphipathic, were found to be necessary for binding. However, no individual lysine was found to be critical for membrane binding. Instead, binding correlated with the number of positively charged residues on the same side of the helix (figure 3.8). Neither the lysine region without the TM (data not shown), nor the TM membrane domain alone (figure 3.5B), was sufficient to allow membrane binding. Therefore, these results suggest that the two sequences together are necessary and sufficient for binding to membranes (figure 3.4 and figure 3.5). However, it is unlikely a specific targeting signal is located in the TM domain as the replacement of the TM of

Vamp2 with a hydrophobic segment composed of 11 to 17 leucines and one valine resulted in a molecule that bound to canine microsomes *in vitro* (Whitley et al 1996).

Recently, the NTS of Vamp2 has been implicated in binding to calmodulin in a Ca^{2+}-dependent fashion, and to acidic phospholipids vesicles (Quetglas et al 2000). Using surface plasmon resonance, the TM truncated Vamp2 still containing the NTS or a 23 residue peptide of the NTS (residue 77-90 of Vamp2) has been shown to bind to both calmodulin and phospholipids vesicles rich in phosphoserine in a mutually exclusive fashion (Quetglas et al 2000). Furthermore, the peptide has been shown to inhibit exocytosis, suggesting the NTS of Vamp2 plays a role in vesicle-plasma membrane fusion. In terms of protein targeting, it is possible that phospholipid binding is one of the steps in the targeting of Vamps to the ER microsomes, however it alone is not sufficient to allow integration of the protein since Vamps were not able to bind to trypsin-treated membranes or to phospholipid vesicles (figure 3.10A & figure 3.3). In fact, in our vesicle flotation experiments we did not observe any electrostatic interaction between the protein and the liposomes. In the previous chapter we have shown that mT binds to liposomes through an electrostatic interaction with phospholipids. However, none of the Vamp proteins bound to liposomes even in the absence of high salt. Similarly, it is unlikely that the ER Vamp receptor is similar to calmodulin as our translation system is virtually free of Ca^{2+} and no increase in Vamp binding was observed when Ca^{2+} was added up to 3 mM (data not shown). Nevertheless, our finding that Vamps require trypsin sensitive saturable component on the ER membrane together with the recent finding that the NTS binds specifically to calmodulin, strongly suggests that the lysine

region preceding the TM is most likely required to mediate a protein-protein interaction rather than binding non-specifically to acidic phospholipids.

Along with the four lysines, five additional amino acids (lysines, arginine, asparagines, and two tryptophans) are conserved in those Vamp proteins that contain a hydrophobic putative transmembrane domain. Of these five, only the asparagine residue contributed to the binding to ER microsomes *in vitro* (figure 3.8). This suggests that the asparagine may also contribute to some undefined role in membrane binding. The two tryptophans, along with the positively charged residues, have been suggested to be important in interacting with phospholipids during membrane fusion (Takagaki et al 1983). However the tryptophans do not appear to be required bind the protein to ER membranes *in vitro* (figure 3.8).

Our data suggesting a common receptor-mediated, ATP-dependent mechanism used by different Vamps to target to the ER establishes this as a *bona fide* targeting mechanism for tail-anchored proteins. Sequence analysis revealed that a number of tail-anchored proteins have a lysine-rich NTS region preceding the putative TM sequence (Chapter 1, table1). The structures of these proteins are not known, however helical wheel analysis shows that most of these lysine-rich regions are potential amphipathic helices (Chapter 1, table 1). It is not known whether the amphipathic helix is a prerequisite for the ATP dependent/ receptor mediated binding to ER microsomes. Of the proteins with lysine rich NTS, only the Vamps (Elferink et al 1989a), Ascorbate peroxidase (APX) from cotton seed (Mullen et al 1999, Mullen & Trelease 2000), Sec61β (figure 3.9), and Sec61γ (figure 3.9) were shown to require ATP for membrane binding.

Both Sec61β and Sec61γ did not bind to trypsinized canine microsomes (figure 3.9A). Furthermore, the NTS is required for binding Sec61γ to membranes suggesting that its membrane targeting mechanism is similar to that of the Vamps (figure 3.9C). Several other proteins such as UBC6, syntaxin and microsomal aldehyde dehydrogenase all have an amphipathic helix preceding the TM. It is not known if these proteins require ATP or a membrane component to bind to the ER. Binding studies that include mutagenesis studies on these proteins are required to determine if their amphipathic helix is required in ER membrane binding.

Whether a universal receptor facilitates the binding of all of these tail-anchored proteins is not known, however there is some evidence to suggest that some of these unrelated proteins may share a common receptor. Sec61β and Vamp2 may share a similar receptor, as the binding of both Sec61β and Vamp2 to ER microsomes was abolished at the same trypsin concentration, while for Sec61γ and APX; a higher concentration of trypsin was required to abolish binding (data not shown). Unlike the low trypsin concentration required to block binding of Sec61β and Vamp2, the trypsin concentration required to prevent the binding of Sec61γ and APX, many of the translocon complex proteins were cleaved (data not shown). Thus, there is large number of candidate receptors for Sec61γ and Apx. However to determine if any of these proteins use the same receptor competition experiments are necessary.

Since the translocon is the ultimate destination for Sec61γ it is possible that the translocon itself may be the receptor for Sec61γ. To elucidate whether the translocon is required for the binding of Sec61γ, immunodepletion experiments are required. By

immunodepleting solubilized ER membranes of translocon components and reconstituting them back to vesicles, these vesicles can be used to determine whether the translocon, particularly Sec61α, is required for the binding of Sec61γ.

The NMR structural analysis on the first 47 residues of Sec61γ, which includes the entire cytoplasmic domain and the first 7 residues of the putative transmembrane domain, reveals a helix-loop-helix structure in the presence of phospholipid micelles (Beswick et al 1996). The lysine domain was located on the second helix. The first helix has been suggested to lie on the lipid-water interface in a micelle, while the second helix does not interact with the micelle. This result suggests that the first helix may be involved in the targeting of Sec61γ to membranes by binding with the head groups of phospholipids. However, since the deletion of the first helix did not effect targeting of the gPASec61γ fusion protein to membranes, we suggest that it is not involved in the targeting of the protein to the ER (figure 3.10C). Instead, the lysine rich sequence, located in the second helix, and the hydrophobic sequence putative TM is responsible for the targeting of Sec61γ, as both were required to target the fusion protein gPA to membranes in an ATP-dependent fashion (figure 3.10C).

It is not known whether the putative receptor for Vamp targeting requires ATP. It is possible that ATP is used by the receptor to overcome the thermodynamic barrier of translocating the transmembrane domain across the lipid bilayer. Alternatively, it may be required by the receptor to cause a conformational change of the proteins which makes the insertion sequence protein available for membrane insertion. Bax, a protein that regulates apoptosis is one example of a tail-anchored protein that requires a

conformational change to bind to membranes. In its inactive form, it remains in the cytosol. However, it is believed that other apoptotic regulators bind Bax to cause a conformational change in the protein that allows the TM to insert into the lipid bilayer of membranes (Eskes et al 2000, Wolter et al 1997, Goping et al 1998). A membrane receptor that causes the conformational change of the tail-anchored proteins would allow organelle specific targeting of the protein.

ATP may also be required by chaperones in the membrane targeting of these tail-anchored proteins. Recently, it has been shown that a plant tail-anchored protein, peroxisomal ascorbate peroxidase (APX), requires both ATP and Hsp70 to bind to the ER (Mullen et al 1999). Chaperones are most likely required to prevent the aggregation of Apx proteins via the hydrophobic TM before insertion into the lipid bilayer. The involvement of Hsp70 or other chaperones like the constitutively expressed Hsc70 suggest a possible role for the ATP. Exchange of ADP for ATP is required for the release of the substrate from the chaperone (Zhang et al 2002, Hartl & Hayer-Hartl 2002). Therefore, removing ATP from the reaction mix may prevent the dissociation of the chaperone/protein complex, thus preventing the integration of the protein into the lipid bilayer. If this is true then perhaps the receptor acts as the nucleotide exchange factor to release the chaperone from the tail-anchored protein.

Chaperones may also play a role in distinguishing the differential targeting of the Vamp1 isoform to either the mitochondria or the ER. Recently it has been shown that for Vamp1b, the mitochondrial isoform of Vamp1, targeting to the mitochondria is saturable but not dependent on ATP (Lan et al 2000). Together with our results, this suggests that

two different chaperones may be involved in the targeting of these Vamp isoforms to their respective locations, one that requires ATP and one that does not. The mitochondrial signal does not mask the ER signal, as the mitochondrial isoform readily binds to the ER in an ATP-dependent fashion *in vitro* (figure 3.3). Therefore, it is most likely that the mitochondrial isoform has a higher affinity toward the mitochondrial chaperone than the ER chaperone.

3.4. Conclusion

Here, we have presented the first evidence of a membrane targeting signal for tail anchored proteins that is located N-terminus of the putative transmembrane domain that is required for ATP-dependent targeting to the ER. The identification of this sequence has aided in predicting the membrane-binding mechanism of other insertion sequence proteins. For example, Sec61β and Sec61γ have been shown here to bind to ER membranes in an ATP-dependent and membrane receptor-dependent fashion. These results will be useful for identifying the molecule(s) that mediate the membrane targeting and insertion of these proteins. Furthermore, our findings may aid in predicting the various targeting mechanisms employed by tail-anchored proteins.

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Chapter 4

The Dynamic Membrane Topology of Bcl-2 during Apoptosis

Preface.

All the data was produced by Peter K. Kim except for figure 4.7C. The Annexin V staining assay was produced by Matthew G. Annis. The data presented here were not previously published up to the date of the submittion of this thesis.

4.0. Introduction

The position of the TM domain at the C-terminal end in tail-anchored proteins suggests an $N_{cytosol}$-C_{lumen} topology with the majority of the protein in the cytosol (Wattenberg & Lithgow 2001). This membrane topology has been observed for all the tail-anchored proteins examined to date (Grote et al 1995, Vergeres et al 1995, Yang et al 1997). However, due to the structural similarity of Bcl-2 to the pore-forming domains of diphtheria toxin and colicin, Bcl-2 has been suggested to form a multi-TM integral membrane protein (Muchmore et al 1996, Petros et al 2001).

Bcl-2 prevents or delays apoptosis in many cell lines. Since its discovery in 1985, it has become the founding member of a group of apoptosis regulating proteins called the Bcl-2 family (Cleary & Sklar 1985, Bakhshi et al 1985, Tsujimoto et al 1985, Vaux et al 1988). The Bcl-2 family of proteins all share sequence similarity in at least one of four regions called Bcl-2 homology (BH) domains (Diaz et al 1997). The family is broken up into three groups based on their activity and sequence similarities. The first group consists of the anti-apoptotic proteins which all have four BH domains, BH 1 to BH 4. The second group of proteins promotes apoptosis and has three domains, BH1, BH2 and BH3. The third group of proteins is also pro-apoptotic, but only contains the BH3 domain.

The structures of TM-truncated proteins of several of the Bcl-2 family of proteins have been solved, including that of Bcl-2 (Petros et al 2001). The N-terminal portion of Bcl-2 consists of 7 α-helices, where two hydrophobic helices are surrounded by five amphipathic helices (figure 4.1) (Petros et al 2001). The structure formed by the two

hydrophobic helices, α-helix 5 and 6, is similar to the pore forming domain of diphtheria toxin (Petros et al 2001). This region has been postulated to insert into the lipid bilayer to form a subunit for a pore complex (Muchmore et al 1996).

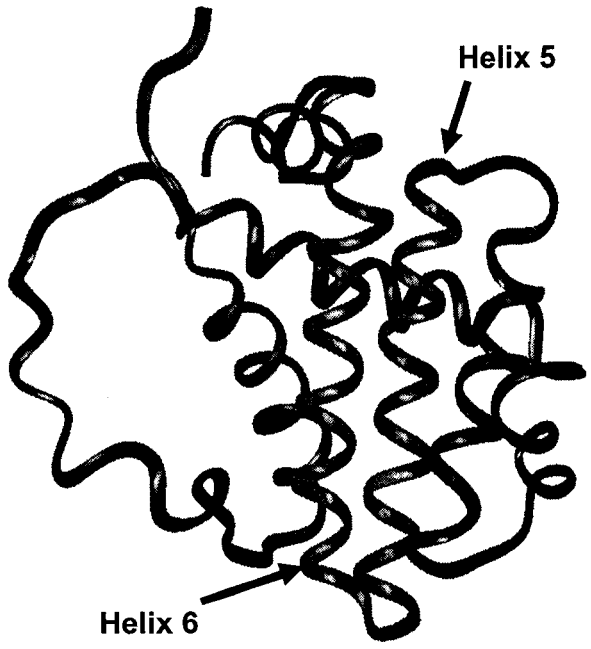

Figure 4.1. NMR of the Bcl-2/Bcl-xl fusion protein.
Ribbon depiction of the average minimized structure of Bcl-2(1). Bcl-2(1) is a fusion of Bcl-1 residues 1-34, 35-50 of Bcl-xl and 51-218 of Bcl-2. The last 10 amino acids of Bcl-2 have been excluded. Helix 5 & 6 are indicated. Modified from Petros et al PNAS 2001 98:6 (3012-3017). The image was created using the program RasMol V2.5.

There is some *in vitro* evidence that suggests Bcl-2 forms ion conducting channels. Work with purified TM-truncated Bcl-2 has shown that it can form channels in artificial membranes. Similar to bacterial pore forming proteins, the channels formed by Bcl-2 are pH and voltage dependent, and have low ion selectivity (Schlesinger et al 1997). One major criticism of the *in vitro* pore forming evidence is the physiological relevance of

these studies; only TM truncated proteins were used in these studies and all of the proteins tested conducted ions only at pH lower than 5.5. Thus, to begin to understand the role of ion channel formation by the Bcl-2 family of proteins during regulation of apoptosis, *in vivo* evidence of such formation is required.

Taking the pore-forming hypothesis of Bcl-2 into account, Bcl-2 may take one of two possible topologies when it is inserted into membranes. The first is the tail-anchored topology, where Bcl-2 is anchored to the membrane solely by its carboxyl-terminal transmembrane domain (figure 4.2A). The second is the pore-forming topology where α-helix 5 and α-helix 6 are inserted into the lipid bilayer along with the transmembrane domain (figure 4.2B). These two topologies can be distinguished by the use of the hydrophilic cysteine modifying reagent 4-acetamido-4'-((iodoacetyl)aminostilbene-2,2'-disulfonic acid) (IASD), which does not modify cysteines in the lipid bilayer (Krishnasastry et al 1994). Bcl-2 has two cysteines, cysteine 158 in α-helix 5 of the putative pore-forming domain, and cysteine 229 which is located in the transmembrane domain (see figure 4.2). Here, IASD was used to determine the membrane topology of Bcl-2 in an *in vitro* system and in membrane fractions of Rat 1 cells. In the tail-anchored topology only cysteine 158 will be accessible to IASD, whereas both cysteines would be inaccessible to IASD in the pore-forming topology (figure 4.2).

Using the hydrophilic cysteine modifying reagent (IASD), we demonstrate in normal cells, that Bcl-2 is in the classical tail-anchored topology. However during apoptosis, Bcl-2 changes conformation to a topology that is consistent to a multi-membrane spanning integral protein. This topology change in Bcl-2 represents the first

example of an integral membrane protein that undergoes a topology change due to an internal cellular signal.

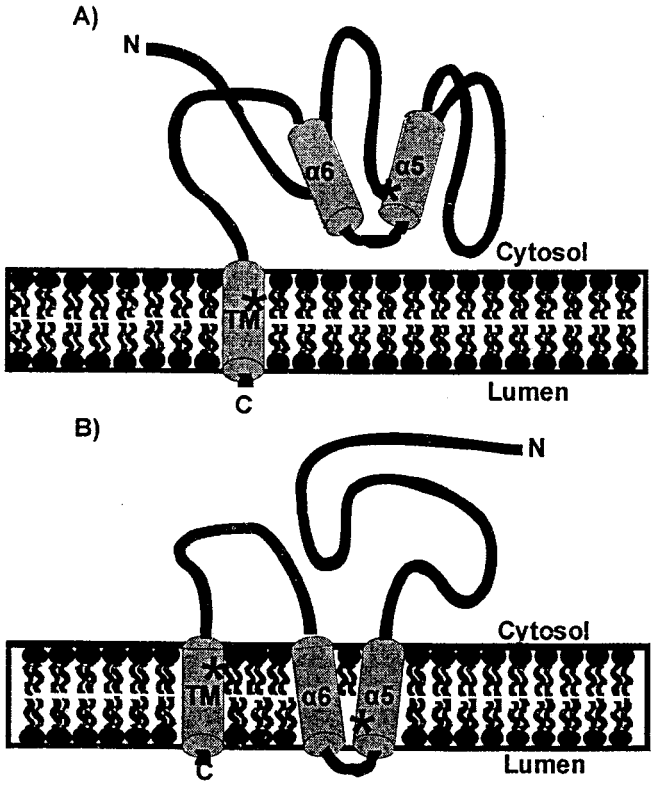

Figure 4.2. Bcl-2 membrane topology schematic.
A) Tail anchor topology where the protein is anchored to the membrane solely by the putative carboxyl-terminal transmembrane domain. Since the putative transmembrane domain is at the carboxyl-terminus, the majority of the protein is on the cytosolic side of the mitochondria or the endoplasmic reticulum membrane. B) Pore forming topology. In this topology, the tail-anchor and the putative pore forming domains, α-helix 5 and α-helix 6, are inserted into the lipid bilayer of the membrane. α5 = α-helix 5 and α6 = α-helix 6.

* the proximal location of cysteine 158 and cysteine 229 are indicated.

4.1. Methods

4.1.1. Plasmid Construction

Restriction enzymes were supplied from either New England Biolabs or MBI Fermentas. All constructs were inserted into both the pSPUTK and pBabe Hygro vectors. The construction of Bcl-2, Bcl-Cb5 and Bcl-Acta has been previously described (Zhu et al 1996). Cysteine 158 in pSPUTK-Bcl-2 (plasmid number 445) was changed to serine using the QuikChange™ method by Stratagene. The primers used to change cysteine 158 codon to a serine codon were TTC GGT GGG GTC ATG AGT GTG GAG AGC GTCA and TGAC GCT CTC CAC ACT CAT GAC CCC ACC GAA (the nucleotide changes are displayed in bold font). The single codon change also adds a new recognition site for the restriction enzyme BspH1. After 25 cycles, the thermal cycling reaction was treated with the restriction enzyme *Dpn*1 for 1 hour at 37 °C to digest the parental template vector and then transformed into *E.coli.* DH 5α. Plasmids isolated from ampicillin resistant single *E.coli.* DH 5α colonies were first screened with *BspH*1, and the positive plasmids were verified by sequencing the whole coding region of Bcl-2. The resulting plasmid was named pSPUTK-Bcl-2 C158S.

4.1.2. Cell culture and cell lines

Rat 1 Myc-ERTM cell line expressing Bcl-2, Bcl-Cb5 and Bcl-Acta were generated by Erinn Soucie in the laboratory of L.Z. Penn. Bcl-2 and the various mutants were transfected into Rat 1 Myc ERTM as previously described (Soucie et al 2001). In

short, retroviral vector pMNiresGFP containing the coding sequences for various Bcl-2 were used to transfect the retroviral packaging cell line Phoenix-Eco. Viral supernatants from Phoenix-Eco were used to infect Rat1 Myc-ERTM in the presence of Polybrene (8 μg/ml (Sigma)) for 18 h. Infected cells were selected using a fluorescence-activated cells sorting to collect cells expressing the green fluorescent protein. Rat-1 Myc-ERTM fibroblast cells were maintained in α-MEM with 10%Fetal Bovine Serum (Clontech).

4.1.3. Apoptosis induction

Apoptosis was induced in the Rat 1 cells by tamoxifen induced activation of Myc together with treatment of the cells with either etoposide or ceramide. Cells grown to 70% confluence in 100 mm Petri-dishes were washed with phosphate-buffer saline (PBS) and the medium was replaced with media containing either etoposide (6 μM etoposide + 100 nM 4-hydroxytamoxifen), or ceramide (50 μM ceramide + 100 nM 4-hydroxytamoxifen). Etoposide treated cells were harvested at 12 hours and 18 hours. Ceramide treated cells were harvested at 15, 30 and 48 hours.

4.1.4. Sub-cellular fractionation.

Cell disruption was performed as described (Annis et al 2001). Briefly, six dishes of Rat-1 cells were mechanically harvested and pelleted in a clinical centrifuge. Cells were washed twice with cell buffer (250 mM Sucrose, 20 mM HEPES-Na pH 7.5, 2 mM $MgCl_2$, 1 mM NaEDTA, 1mM PMSF, 1 mM DTT and a cocktail of protease inhibitors). The final pellet was resuspended in 400 μL of cell buffer. This suspension was held at 150 psi for 15 minutes in a 45 mL Nitrogen Bomb (Parr Instruments). The cells were

lysed by releasing the pressure and collecting the lysate into a microcentrifuge tube. Nuclei and cell debris were removed by centrifugation in a microcentrifuge at 500 x g for 2 minutes to yield approximately 350 μL of whole cell lysate (WCL). This resulting WCL was subjected to centrifugation at 1000 xg for 25 minutes using a TLA100.2 rotor (Beckman) to yield S1 and P1. The S1 fraction was further separated into a S2 and P2 by centrifugation at 213 000 x g for 30 minutes using a TLA100.2 rotor. The WCL, P1 and P2 fractions were quantified for total protein using the Bradford Assay (BioRad).

4.1.5. Gel-Shift Assay.

The gel-shift assay was adapted from that described by Krishnasastry et al (1995). Cysteine residues in the polypeptides were modified by incubation with IASD (Molecular Probes). For analysis *in vitro* polypeptide was synthesized using rabbit reticulocyte lysate in the presence of [^{35}S]-Met as previously described (Andrews et al 1989). The binding of polypeptide to canine microsomes was previously described (Chapter 2). In short, following termination of translation by adding cycloheximide to a final concentration of 2 μg/mL, 1 equivalent of microsomes was added to 10 μL of the translation reaction and incubated at 24 °C for one hour. Microsome was separated from the reaction by centrifugation over a sucrose cushion (0.5 M sucrose, 10 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM $MgCl_2$) at 100,000 xg for 10 min in a TLA 100 rotor (Beckman). The resulting pellet was resuspended in 30 μL of resuspending buffer (0.3 M Tris-HCl (pH 8.5) and 1 mM DTT) and then diluted with 30 μL of urea buffer (8 M urea, 0.3 M Tris-HCl (pH 8.5), and 1 mM DTT) to give a final urea concentration of 4 M. 14

μL was removed as a control before adding IASD to a final concentration of 20 mM from a 100 mM stock solution in deionized filtered water. 17 μL was removed at 5 min, 10 min and 15 min into 5 μL of 1M DTT to quench the reaction. As a membrane free control, 1 μL of translation product was incubated with IASD as described above. Samples were analyzed by SDS-PAGE on a 16%-17% gradient (Laemmli 1970). The radioactive polypeptide was visualized using a phosphorimager (Storm™ - Molecular Dynamics) and quantified using the accompanying software, ImageQuant ™ (Molecular Dynamics).

Proteins in the P1 and P2 fractions from Rat1 cells were assayed as follows. 40 μg of total protein from either the P1 or P2 fraction in 30 μL of cell buffer (see above) was diluted with Urea-cell buffer (4M urea, 250 mM sucrose, 20 mM HEPES pH 7.5, $MgCl_2$, 1 mM NaEDTA, 1mM PMSF, 1mM DTT and protease inhibitor). Labelling with IASD was carried out as above. Samples (approximately 10 μg of total protein) were also analyzed by SDS-PAGE on a 16%-17% gradient and immunoblotted with rabbit anti-Bcl-2 (Stan, 1:10,000 dilution), followed by donkey anti-rabbit conjugated to horseradish peroxidase (Jackson Laboratories, 1:10,000 dilution). Proteins on the blots were then visualized using enhanced chemiluminescence (NEN-life Science). To quantify the bands, three different exposures of the same blot were recorded and analyzed using Imagequant ™ software (Molecular Dynamics).

4.1.6. Cell death assay

Rat 1 cells grown in 100 mm dishes to 70 % - 80 % confluency were mechanically harvested and pelleted in a clinical centrifuge. Cell were then washed two times with PBS before adding 100 µL of 1 % SDS/10 mM Tris-HCl pH7.5 heated to 90 °C. The lysate was heated for 5 minutes at 90 °C. The genomic DNA was mechanically sheared by passing the lysate through a 27 gauge needle three times using a 1 mL syringe. Total protein of each lysate was determined using BCA assay (Pierce). 0.5 µg of total protein of each sample was separated by SDS-PAGE and the bands corresponding to PARP were identified by immunoblotting with an anti-PARP mouse antibody and visualized using donkey anti-mouse HRP and chemiluminescence. To assay translocation of Bax to membrane, five 100 mm dishes of cells at approximately 70 % confluency were lysed by nitrogen cavitation as described above. The nucleus and unbroken cells were removed by centrifugation at 500 xg for 2 minutes generating a whole cell lysate (WCL). Membranes were pelleted from the WCL by centrifugation at 100,000 xg in a TL 100 centrifuge (TLA 100.2 rotor - Beckman) for 1 hour. The supernatant was removed and the pellet was resuspended in the same volume as the supernatant with hot 1% SDS/ 10 mM Tris-HCl pH 9.5 buffer (approximate at 80 °C). The protein concentration of WCL was determined by Bradford assay (Biorad). Equal volume of the supernatant and pellet that corresponded to 1 mg of total protein in the WCL was analyzed by SDS-PAGE using a Tricine gel system (Schagger & von Jagow 1987) and immunobloting for Bax using a mouse mono-clonal antibody (1D1). Bands on the blot were visualized using a secondary antibody, donkey anti-mouse HRP and ECL.

Externalization of phosphatidylserine was assayed using FITC conjugated Annexin V. Twenty thousand cells were plated into 4 well Chamber Coverglass dishes (Lab-Tek) and allowed to adhere overnight. The next day the cells were treated with 50 μM ceramide and 100 nM 4-hydroxytamoxifen. After the appropriate incubation the media was removed and the cells were washed once with PBS and then incubated with 5 μL of Alexa Fluor 488 Annexin V in 500 μL of Annexin-Binding Buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$ pH 7.4, Molecular Probes). Fluorescence was imaged using a Zeiss LSM 510 Confocal microscope.

4.2. Results

4.2.1. Characterization of IASD modification of Bcl-2.

To probe the topology of Bcl-2 on membranes IASD was used (Chapter 2 figure 2.2A). Like other iodoacetamides, IASD readily reacts with cysteine residues (Krishnasastry et al 1994). In the absence of free thiols, IASD can react with methionine, histidine, tyrosine, and some unprotonated amines. However these reactions are very slow and occur only at extreme pH, whereas cysteine modification occurs readily at neutral pH (Jullien & Garel 1981, Musci & Berliner 1986, Chung & Lewis 1986, Krishnasastry et al 1994). IASD is also unable to cross lipid bilayers due to its two charged sulphate groups. These characteristics of IASD have made it useful in analyzing the topology of membrane proteins (Krishnasastry et al 1994, Walker & Bayley 1995, Chang & Cronan, Jr. 1997, Bernardo et al 2000). Modification of a protein with IASD results in a band shift in SDS-PAGE (Creighton 1980). In most cases, the mobility of the protein is retarded proportionally to the number of modified cysteines (Creighton 1980).

However, in some cases a downward shift (increased mobility) is observed (Chapter 2 figure 2.2B).

The IASD labelling profile of *in vitro* synthesized Bcl-2 in the absence of membranes was first examined (figure 4.3). Comparison of IASD labelling for native and denatured Bcl-2 is relatively straight-forward because Bcl-2 contains only the two cysteine residues. Cysteine 158, is located in helix 5 (the putative pore forming domain), and Cysteine 229 is in the C-terminus helix 9 (putative transmembrane domain). Based on the structure determined for the cytoplasmic portion of Bcl-2 by NMR, Cysteine 158 of Bcl-2 is expected to be buried within the hydrophobic core of the protein (Petros et al 2001).

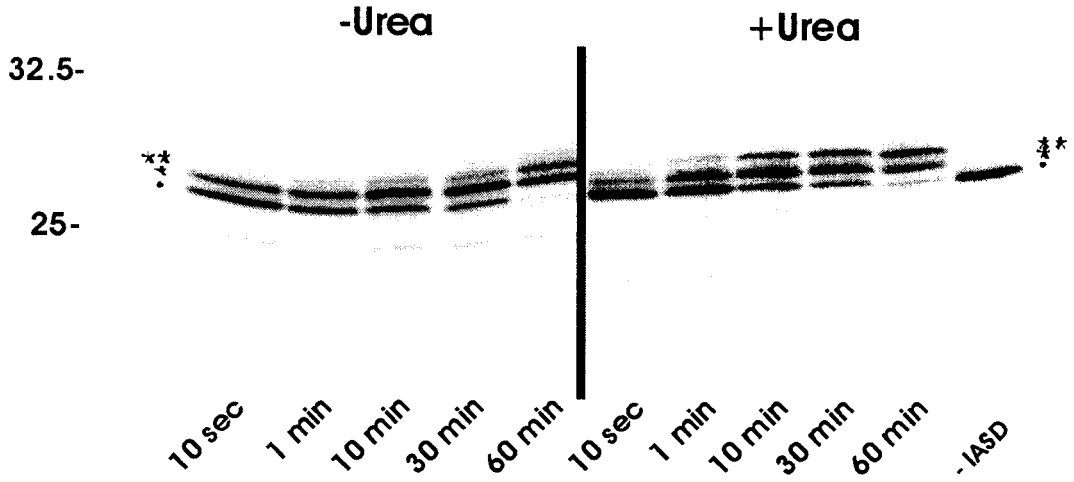

Figure 4.3. Modification of cysteines in native and denatured molecules by incubation with IASD.

Denaturation in 8M urea increased the rate of IASD labelling of one of the two cysteines in Bcl-2. Unmodified, singly and doubly modified molecules are indicated by a dot, * and ** respectively.

As expected, IASD labelling of both native and denatured Bcl-2 resulted in two discrete band shifts (figure 4.3). The first shift (figure 4.3 *) represents protein with only a single cysteine modification and the second shift (figure 4.3, **) results from proteins in which both cysteines were modified by IASD. To determine if the cysteines are being specifically labelled by IASD, another cysteine modifying agent, N-ethylmaleimide (NEM) was used. NEM does not react with methionine, histidine or tyrosine. When *in vitro* synthesized proteins were treated with NEM and excess NEM was removed by gel filtration chromatography, no labelling by IASD was detected (data not shown) suggesting that in our reaction conditions IASD reacts specifically with cysteines.

In non-denaturing conditions, the two cysteine residues in Bcl-2 were labelled with very different kinetics (Figure 4.3). Ten seconds after adding the reagent, 30% of the molecules were labelled on one of the two cysteine residues (figure 4.3, *). The second cysteine was not appreciably labelled before 30 minutes incubation with IASD, and was labelled to only 20% after incubation for a total of 60 minutes. As expected, after denaturation in 4 M urea, the rate of double labelling of Bcl-2 dramatically increased as significant labelling of the less-reactive cysteine was observed after 10 minutes. Even though incubation in 4 M Urea increased the exposure of this residue to the reagent, labelling of the two cysteines was not identical, presumably due to differences in the local chemical environment (sequence context) of the two cysteines.

To identify which of the two cysteines in Bcl-2 were exposed by denaturation, we determined the labelling kinetics for two fusion proteins (gPABcl-2 and Bcl-Cb5) each containing only one of the cysteines. In gPABcl-2, the sole cysteine residue (cys 158) in the

molecule is located in the C-terminal helix 9 (insertion sequence domain) of Bcl-2 which, in this fusion protein, is fused to the carboxyl-terminus of gPA. As mentioned in chapter 2, gPA has been previously characterized as an independently folded passive protein domain, thus it does not interfere with the folding or function of the insertion sequence domain of Bcl-2 (Janiak et al 1994). Bcl-Cb5 is a fusion between the cystolic domain of Bcl-2 and the insertion sequence domain of Cb5. Since the insertion sequence domain of Cb5 does not have any cysteines, this fusion protein only has the helix 5 cysteine (cysteine 158) of Bcl-2. Similar to the gPABcl-2 fusion, the folding and function of both domain (the N-terminus cytosolic domain and the insertion sequence) are independent of each other as this fusion proteins was shown to target exclusively to the ER and is still able to protect cells from the onset of apoptosis (Zhu et al 1996). These proteins were used to characterize the IASD labelling profile of the individual cysteines of Bcl-2. When gPABcl-2 was labelled with IASD, the cysteine was labelled very rapidly and labelling was not affected by denaturation with urea, both results consistent with this cysteine being exposed to the aqueous environment in gPABcl-2 (figure 4.4A, 4.4B). Furthermore, this result demonstrates that the local sequence context of this residue is compatible with rapid labelling. In contrast, the single cysteine from the cytoplasmic portion of Bcl-2 (helix 5) in the fusion protein Bcl-Cb5 labelled more slowly even when the protein was denatured with urea (figure 4.4A, 4.4B). The similar rate of labelling of the internal cysteine in Bcl-Cb5 and the more slowly labelled cysteine in wild type Bcl-2 indicates that the local sequence around cysteine 158 makes this residue less reactive with IASD than the cysteine 229 in helix 9. In addition the fact that this residue is very poorly labelled in the absence of urea strongly suggests that cysteine 158 is

less accessible to IASD than in residue 229 in wild type Bcl-2. Therefore, we conclude from these experiments that, in native Bcl-2, the cysteine in the putative transmembrane domain is more accessible to IASD than the cysteine in the cytoplasmic portion of the molecule.

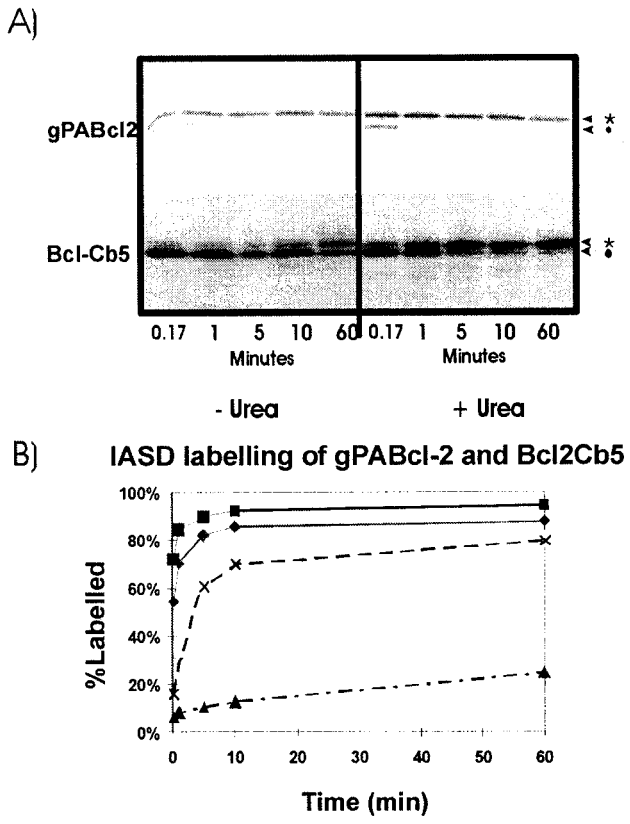

Figure 4.4. IASD labelling of gPABcl-2 and BclCb5.

A) IASD labelling of gPABcl-2 and BclCb5 either in the presence or absence of 8M urea. Unmodified is indicated by a dot, and modified molecules are indicated by a *.
B) The rate of labelling of the cysteine in BclCb5 is increased by denaturation in 8M urea. BclCb5 is labelled with IASD more rapidly with than without urea, Xs and triangles, respectively. Labelling for gPABcl-2 is the unchanged with and without urea, squares and diamonds, respectively. Reticulocyte lysate translation reactions (10 µl) were diluted to 40 µl with 0.3 M Tris/HCl pH 8.5, 1 mM DTT and 4 µl of 100 mM IASD was added. After incubation at 24 °C for the indicated times, an aliquot equivalent to 1 µl of the translation reaction before denaturation and labelling was added to 20 ml of SDS-PAGE loading buffer containing 0.25 M DTT and analyzed by SDS-PAGE on a 12%-18% gradient gel.

4.2.2 In vitro-synthesized Bcl-2 adopts the tail anchor topology.

To elucidate the topology of Bcl-2 in membranes, Bcl-2 was synthesized in vitro in rabbit reticulocyte lysate, in the presence of 35S-methionine, and targeted to ER microsomes. Membrane bound Bcl-2 was isolated from unbound molecules by centrifugation over a sucrose cushion and then the membrane fraction was treated with IASD in the presence of 4 M urea. As a control, unbound Bcl-2 was used. As expected, in the absence of membranes IASD treatment resulted in two band shifts in SDS-PAGE (figure 4.5A, Bcl-2: compare lane 4 with lane 1). The lower band shift (*) represents modification of a single cysteine, while the higher band shift (**) indicates proteins in which both cysteines were modified. When Bcl-2 was bound to ER microsomes, only a single cysteine was modified (indicated by a single band shift - figure 4.5A Bcl-2, lanes 2 and 3). This suggests that one of the two cysteines of Bcl-2 is in the lipid bilayer. To determine which of the two cysteines of Bcl-2 is in the lipid bilayer, Bcl-Cb5 was incubated with membranes and then labelled with IASD. When ER microsome-bound Bcl-Cb5 was incubated with IASD, a single shift was observed, demonstrating that cysteine 158 located in helix 5, did not insert in to the lipid bilayer (compare figure 4.5A Bcl-Cb5 lane 1 with lanes 2 &3). To verify in wild type Bcl-2 that cysteine 229 is in the lipid bilayer, cysteine 158 was changed to a serine residue to create Bcl-2 C158S. As expected, in control reactions without added ER microsomes, a single shift was observed, thus demonstrating that the single cysteine present in Bcl-2 C158S is efficiently labelled after 15 min incubation with IASD (Figure 4.5A, Bcl-2 C-S, lane 4). When this mutant was bound to ER membranes, Cysteine 229 was protected from modification by IASD,

suggesting that the cysteine is in the lipid bilayer (Figure 4.5A, Bcl-2 C-S). Taken together, these results suggest that synthesized Bcl-2 in vitro adopts the tail-anchored topology (figure 4.2A) with cysteine 158 in the cytosol and cysteine 229 in the bilayer. Therefore, Bcl-2 is anchored to the membrane solely by the carboxyl-terminal transmembrane domain when synthesized in reticulocyte lysate and incubated with ER microsomes (figure 4.2A).

Since Bcl-2 is found in both ER and mitochondria, it is possible that it may adopt a different topology in each membrane. To test this possibility in vitro-synthesized Bcl-2 was bound to mitochondria and then incubated with IASD. Similar to the result obtained with ER microsomes, Bcl-2 bound to purified rat liver mitochondria was modified at only a single cysteine (Figure 4.5B, Bcl-2 (*)). The topology of Bcl-2 in mitochondria was verified using a mitochondrial-specific Bcl-2 mutant, Bcl-Acta. Bcl-Acta has been shown to protect cell from apoptosis indistinguishable from wild type Bcl-2, yet it targets specifically to mitochondria in cells (Zhu et al 1996, Annis et al 2001). Similar to Bcl-Cb5, Bcl-Acta has a single cysteine that is located at position 158. When the mitochondria bound Bcl-Acta was incubated with IASD in 4 M urea, cysteine 158 was readily modified (figure 4.5B, Bcl-Acta), suggesting that, Bcl-2 Act and the wild type, Bcl-2 adopt the tail-anchored topology (figure 4.2A). Thus, in vitro-synthesized Bcl-2 adopts the same topology at the ER and mitochondria.

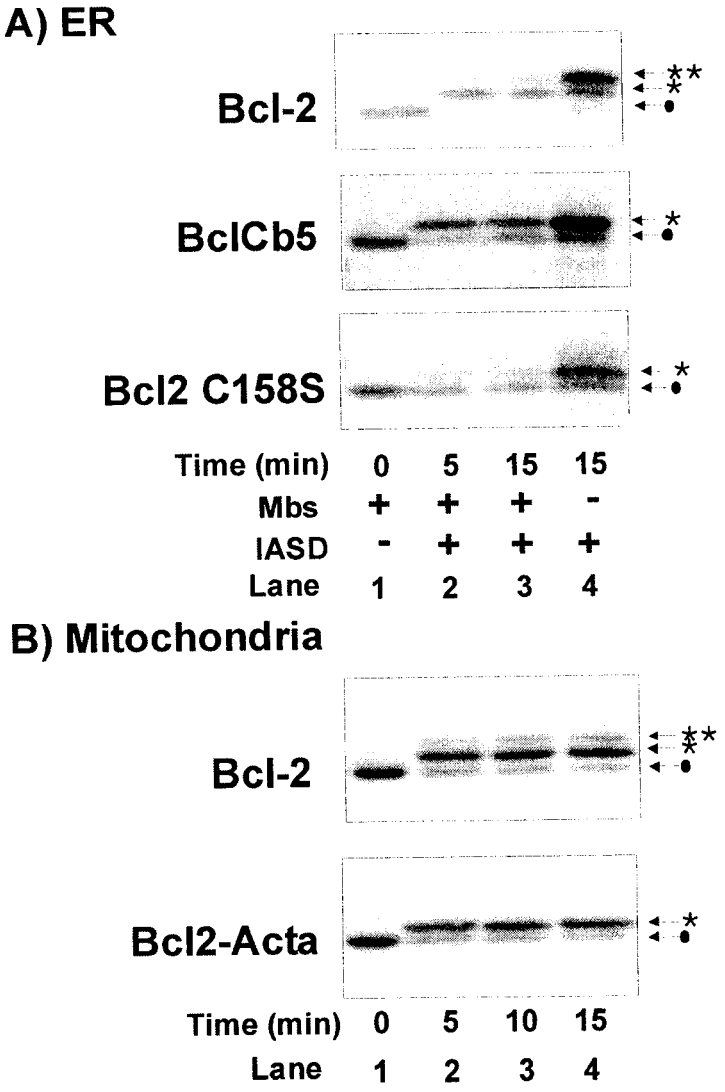

Figure 4.5. IASD labelling of *in vitro* synthesized Bcl-2 and Bcl-2 mutants.
A) Polypeptides are synthesized in rabbit reticulocyte lysate in the presences of 35-S Met. The translation reaction was stopped by the addition of cycloheximide (2 μM final). The reaction mix was incubated with 1 equiv. of canine ER microsomes per 10 μL of translation reaction for 1 hour at 24 °C. Membranes were isolated by centrifugation over sucrose cushions, and resuspended in 4 M Urea labeling buffer. IASD was added and samples were removed at the indicated times and IASD labeling was quenched with DTT. Lane 4 Membrane free polypeptide incubated with IASD for 15 minutes.
B) *In vitro* synthesized Bcl-2 and Bcl-Acta were targeted to mitochondria and then isolated membranes were incubated with IASD for 5, 10 and 15 minutes (lane 2 to 4). Lane 1 is polypeptide in the absences of IASD. All samples were analysized by western blotting after separation by 16%-17% gradient SDS-PAGE. * - indicates single cysteine modification gel shift. ** - indicates modification of two cysteines.

4.2.3. Bcl-2 expression in Rat1 cells delays apoptosis caused by etoposide and ceramide treatment.

Although in vitro-synthesized Bcl-2 adopts the tail-anchored topology, its topology in cells may differ. The structural similarity of Bcl-2 to diphtheria toxin suggests that Bcl-2 may assume a topology which helix 5 and 6 are inserted into the lipid bilayer (figure 4.2). Diphtheria toxin undergoes regulated insertion into endosomal membranes from the lumen side in response to a decrease in pH (Falnes & Sandvig 2000). Reorganization of one integral membrane topology for another has not been observed in native cellular proteins. However, a pro-apoptotic member of the Bcl-2 family of proteins, Bax, undergoes regulated insertion into mitochondria membrane during apoptosis (Desagher et al 1999, Antonsson et al 2000). The topology of Bax in membranes in not known, however, it is also believed to be similar to that of diphtheria toxin and colicin (Roucou & Martinou 2001). Although not predicted previously it is possible that undergoes reorganization with the membrane that converts the protein from the tail-anchored topology to the multi-spanning pore forming topology.

To determine the topology of Bcl-2 in live cells before and during apoptosis, Rat 1 fibroblast cells stably expressing Bcl-2 were assayed with IASD before and after adding an apoptotic agonist. It was previously shown that apoptosis can be induced in Rat 1fibroblasts by up-regulating c-myc expression together with treatment with either etoposide or ceramide (Soucie et al 2001, Annis et al 2001). Stable expression of Bcl-2 in these cells dramatically delayed the onset of apoptosis (Soucie et al 2001, Annis et al 2001). Furthermore when apoptosis was induced in these cells by either ceramide or

etoposide, the initial cellular changes suggest that these drugs initiate apoptosis by distinct pathways (Soucie et al 2001, Annis et al 2001).

The Rat1 fibroblast cell line used was the Rat1 myc ERTM. These cells stably express myc-ERTM, a fusion protein between full-length c-myc and a mutant estrogen receptor (ERTM) (Littlewood et al 1995). ERTM is not activated by estrogen but by a synthetic analogue 4-hydroxytamoxifen, it allowing synchronized activation of myc. Previously a difference in anti-apoptotic function between Bcl-Acta and Bcl-Cb5 was demonstrated in cells expressing myc-ER in the absence of myc activation (Soucie et al 2001, Annis et al 2001). However, for IASD labelling, the coordinated induction of apoptosis was required. This was achieved by activating myc simultaneously with drug treatment.

Apoptosis was monitored by several different assays. Induction of apoptosis by etoposide was monitored by examining the cleavage of PARP and the translocation of Bax from cytoplasm to mitochondria following etoposide treatment (figure 4.6). For cells treated with ceramide, apoptosis was monitored by examining PARP cleavage, Bax translocation, and the appearance of phosphatidylserine on the cell surface (figure 4.7). In cells expressing the wild type Bcl-2, or either of its membrane-specific mutants, apoptosis induced by etoposide was delayed by at least 6 hours (figure 4.6). Substantial PARP cleavage was not observed until 18 hours after the addition of 6μM etoposide in cells expressing Bcl-2, Bcl-Cb5, or Bcl-Acta (figure 4.6A). In the vector control cells, PARP cleavage was observed after 12 hours of etoposide treatment. Similarly, Bax translocation to membranes was observed 18 hours after the addition of etoposide to cells

expressing Bcl-2 or its mutants, compared to 12 hour in the vector control cells (figure 4.6B).

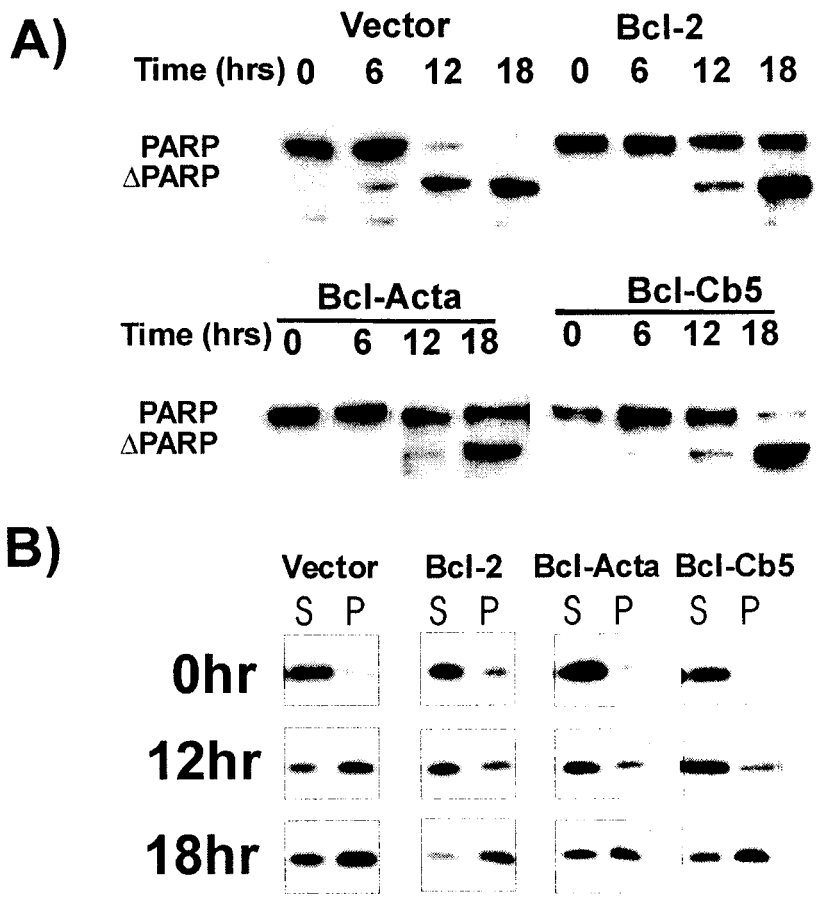

Figure 4.6. Apoptosis death assay.

Rat 1 Myc-ERTM cells stably transfected with either vector, Bcl-2, Bcl-Acta or Bcl-Cb5 were treated with 10 μM Etoposide and 4-hydroxytamoxifen for 6, 12 and 18 hours before harvesting.
A) PARP cleavage assay. Cells were mechanically harvested and lysed with 1 % SDS. For each time point, 0.5 μg of total protein was separated by SDS-PAGE and immunoblotted with mouse anti-PARP.
B) Bax translocation assay. Cells mechanically harvested were lysed by nitrogen cavitation. Membranes were separated from the cytoplasm centrifugation of the whole cell lysate at 100,000 xg for 1hr in a Beckman TL 100 centrifuge. The supernatant (S) and the pellet (P) were separated by SDS-PAGE and immunoblotted with mouse anti-bax (1D1).

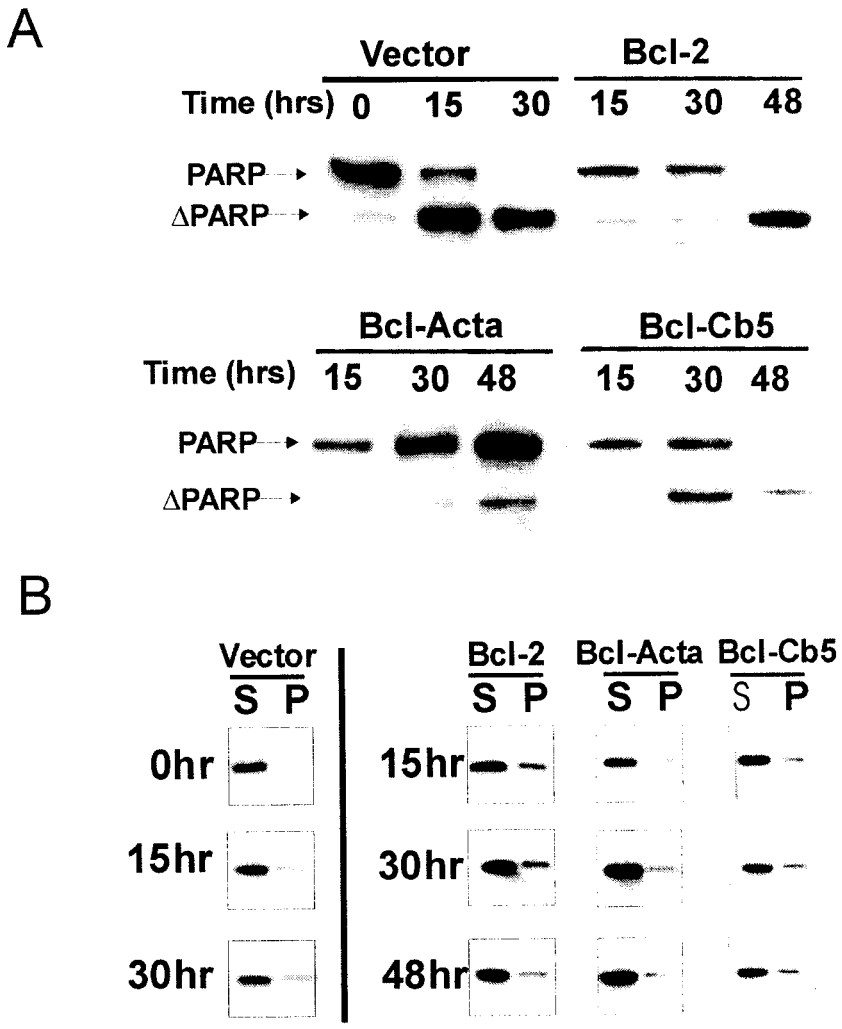

Figure 4.7. Apoptosis death assay of Ceramide treated Rat 1 Myc-ERTM

Rat 1 Myc-ERTM cells stably transfected with Bcl-2, Bcl-Acta or Bcl-Cb5 are treated with Ceramide and 4-hydroxytamoxifen for 15, 30 and 48 hours before harvesting. The vector control cells were harvested at 15 and 30 hours after treatment Ceramide 4-hydroxytamoxifen.

A) PARP cleavage assay. See Figure 4.6A.

B) Bax translocation assay as in figure 4.6B.

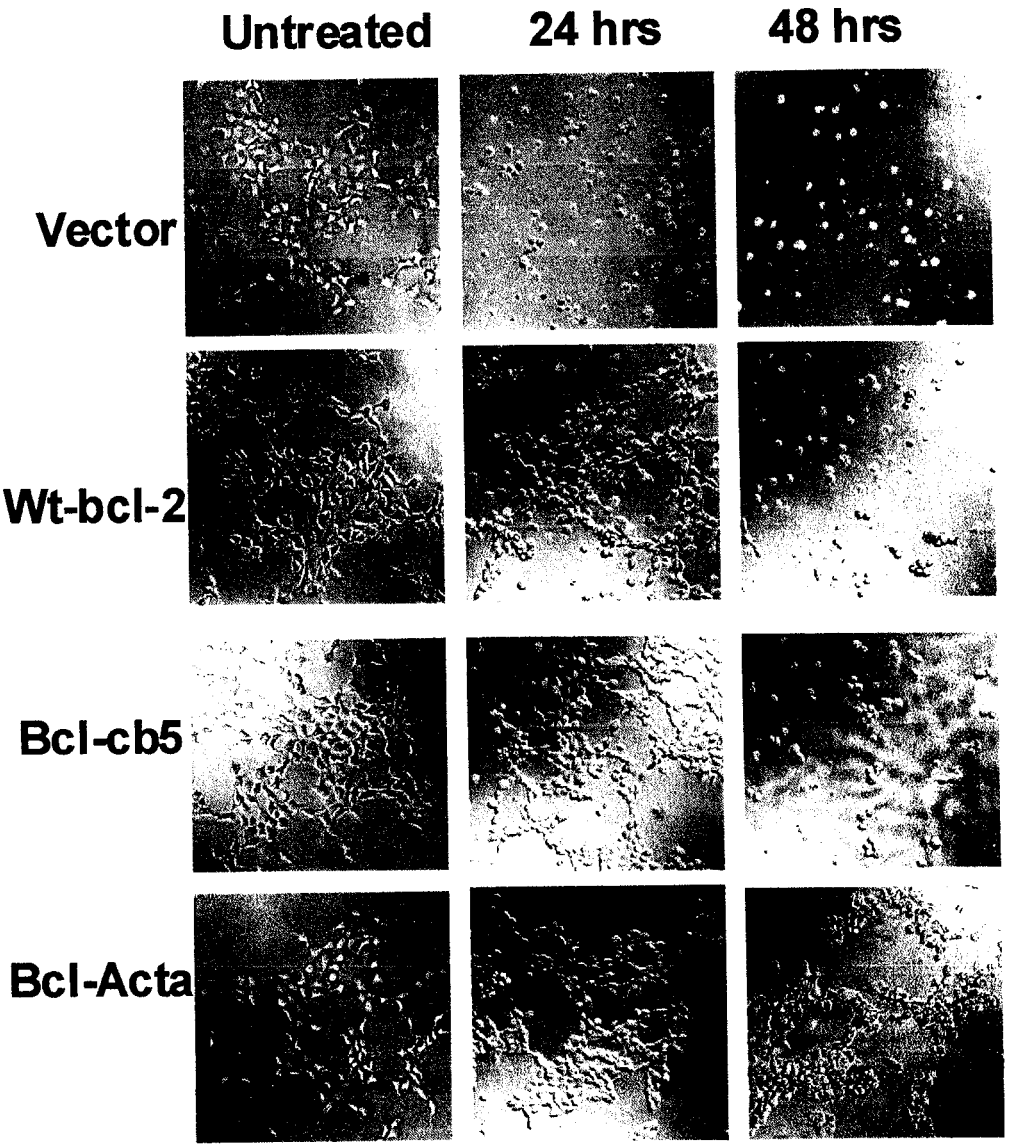

Figure 4.7C. Annexin V staining assay.
Twenty thousand cells were plated into 4 well Chamber Coverglass dishes (Lab-Tek) and allowed to adhere overnight. The next day the cells were treated with 50 :M ceramide and 100 nM 4-hydroxytamoxifen. After the appropriate incubation the media was removed and washed once with PBS and then incubated with 5 :L of Alexa Fluor 488 Annexin V in 500 :L of Annexin-Binding Buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$ pH 7.4, Molecular Probes). Samples were analyzed on the Zeiss LSM 510 Confocal microscope. Data collected by Matthew G. Annis.

In cells treated with ceramide, PARP cleavage was observed after 15 hours of treatment in the vector control cells. However, in cells expressing Bcl-2 or Bcl-Cb5 cleavage was not observed until 48 hours of growth after ceramide treatment. In Bcl-Acta expressing cells, PARP cleavage was not observed, although 80% of the cells were either showing signs of apoptotic death or floating on the dish (data not shown). Furthermore, Bax translocation was not observed in any of the cell lines including the vector control cells (figure 4.7B). This result supports the finding that the apoptotic pathway induced by ceramide treatment is different from etoposide induced apoptosis (Annis et al 2001). To verify that apoptosis and not necrosis was occurring due to ceramide treatment, cells treated with ceramide were stained with Annexin V (figure 4.7C). Annexin V binds to phosphatidylserine (PS), which is externalized on the cell surface of in cells undergoing apoptosis (Zamzami et al 1995). In the vector control, Annexin V staining was observed after 24 hours of treatment with ceramide, whereas substantial cell staining was not observed until 48 hours of ceramide treatment in cells expressing Bcl-2 or its mutant (figure 4.7C). Therefore we conclude that ceramide induced apoptosis is mechanistically different from that induced by etoposide and that Bcl-2 can prevent both cell death pathways from either ER or mitochondria.

4.2.4. Membrane topology of Bcl-2 in cells is dynamic.

To determine the topology of Bcl-2 in these cells, the membrane fraction from cells lysed by nitrogen cavitation was isolated at different time points during treatment with either ceramide or etoposide. The topology of Bcl-2 was assayed by incubating the

membrane fraction with IASD. Before the addition of any drugs, the majority of Bcl-2 (approximately 80%) was singly labelled (figure 4.8A, 4.8C - 0Hr), indicating a tail-anchored topology (figure 4.2). Interestingly, after 12 hours exposure to etoposide some of the Bcl-2 proteins were protected from IASD (figure 4.8A, 4.8C -12 hr). By 18 hours, only 65% of Bcl-2 was completely protected from IASD (35 % was singly modified), suggesting that both cysteine residues in the majority of the Bcl-2 molecules adopts a topology in which both cysteines are embedded in the lipid bilayer. In figure 4.8C, the protein modified in four separate experiments is presented graphically. The result indicates that as the cells enter apoptosis, cysteine 158 becomes less accessible to IASD, suggesting that cysteine 158 enters the lipid bilayer.

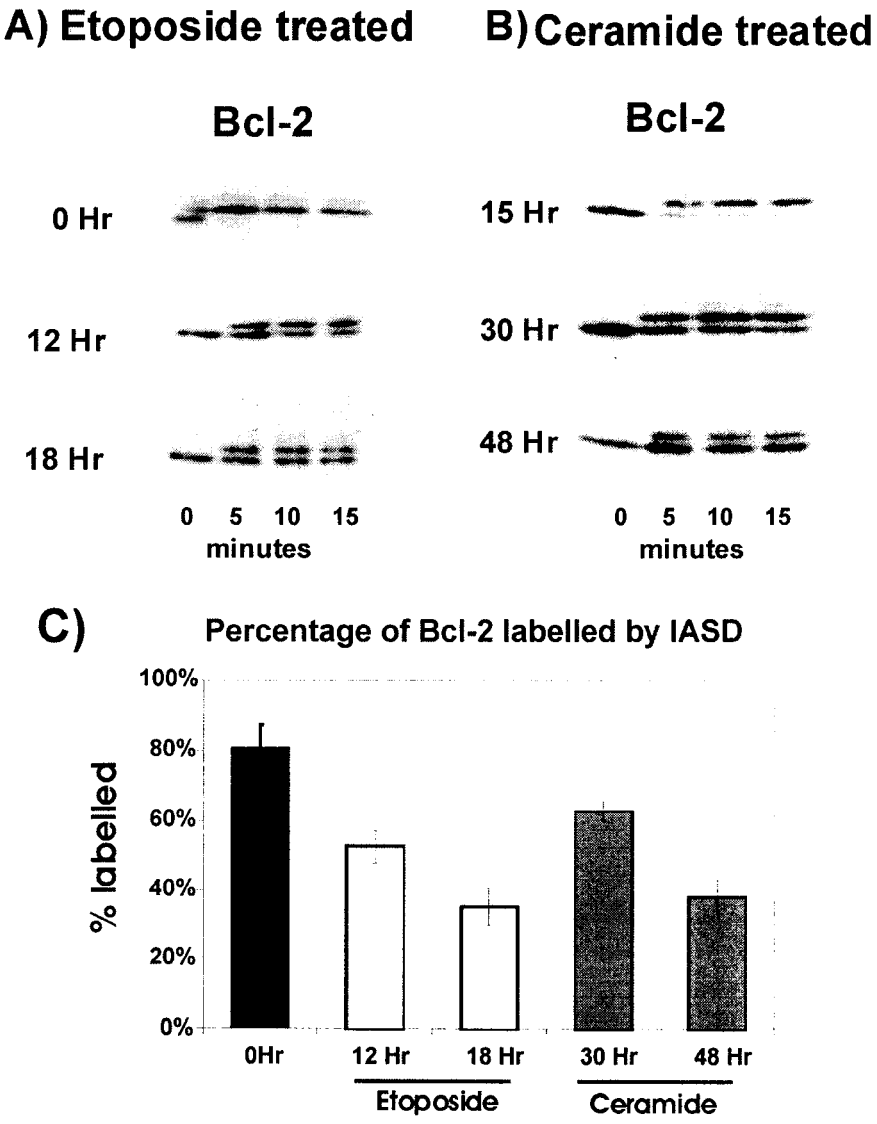

Figure 4.8. IASD labelling of Bcl-2 in cells undergoing apoptosis.

A) Cells were treated with Etoposide for either 12 or 18 hours in the presence of 4-hydroxytamoxifen before harvesting for nitrogen cavitation. Membranes from cells lysed by nitrogen capitation were incubated with IASD for 5, 10 or 15 minutes.
B) Cells were treated with Ceramide for 15, 30 and 48 hours in the presence of 4-hydroxytamoxifen. The membrane fractions were treated with IASD for 5, 10 and 15 minutes.
C) Histogram illustrating the extent of protein labeling after incubation IASD for 15 minutes. Error bars indicate the standard deviation (n=3).

4.2.5. The Conformational change of Bcl-2 is independent of the apoptotic pathway.

To determine if ceramide treatment causes a similar topology change in Bcl-2, Rat 1 myc-ERTM cells were treated with ceramide for 30 hours or 48 hours before harvesting. When the membrane fractions of these cells were assayed with IASD, a similar trend was observed (figure 4.8B, 4.8C). As cells undergo apoptosis, cysteine 158 becomes increasingly protected from IASD modification, indicating that it inserts into the lipid bilayer of the membrane during apoptosis (figure 4.8C).

4.2.6. Bcl-2 conformational change is independent of cellular localization.

To determine if the conformational change in Bcl-2 observed when cells are drug treated occurs at both the ER and the mitochondria, organelle-specific mutants of Bcl-2 assayed *in vitro* were analyzed after expression in Rat-1 cells. Membrane fractions from cells expressing either the mitochondrial-specific (Bcl-Acta) or the ER-specific (Bcl-Cb5) were assayed as above. Similar to wild type, the onset of apoptosis in both Bcl-Cb5 and Bcl-Acta was delayed for about 6 hours when treated with etoposide, as shown by PARP cleavage and bax translocation (figure 4.6). PS externalization was used to detect apoptosis in ceramide treated cells (figure 4.7C). Figure 4.7C shows that after 48 hours of treatment with ceramide, the majority of cells expressing either Bcl-Cb5 or Bcl-Acta show Annexin V staining, indicating that these cells are undergoing apoptosis. This is in sharp contrast to the vector control, which shows a similar amount of Annexin V staining after 24 hours of treatment with ceramide.

The membrane fractions of both Bcl-Cb5 and Bcl-Acta expressing cells were isolated at various time points after treatment with either etoposide or ceramide, and then subjected to IASD assay. Similar to wild type Bcl-2, cysteine 158 is accessible to IASD before the onset of apoptosis (figure 4.9A Bcl-Acta, 4.9B Bcl-Cb5, 15 hrs). As cells enter apoptosis, cysteine 158 becomes protected in cells expressing either of the two Bcl-2 mutants (figure 4.9, 48 hrs). Together, these results suggest that the topology change exhibited by Bcl-2 is independent of its cellular location.

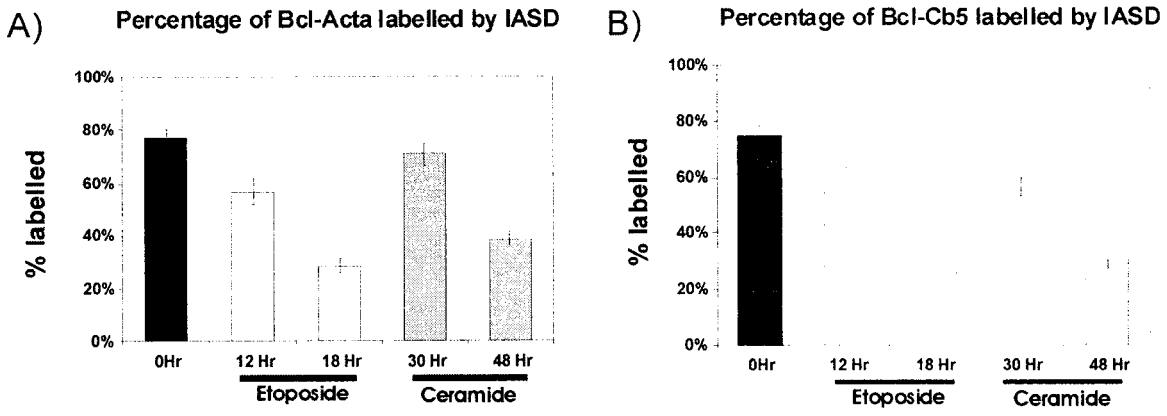

Figure 4.9. IASD labelling Bcl-Acta and Bcl-Cb5 during apoptosis in Rat1 Myc-ERTM.

Rat1 Myc-ERTM cells expressing Bcl-Acta (A) or Bcl-Cb5 (B) were treated with either etoposide or ceramide together with 4-hydroxytamoxifen. At the indicated times cells were harvested and lysed by nitrogen cavitation. Membrane fractions from each lysate were labeled with IASD. The extent of labeling of cysteine 158 is illustrated. Error bars indicate 1 standard deviation (n=3).

4.3.Discussion

The data presented here is the first *in vivo* evidence that demonstrates a membrane protein that changes its membrane topology due to an internal cellular signal. We have shown that in cells Bcl-2 changes its topology from a classical single transmembrane domain tail-anchored conformation to a multi-transmembrane conformation during apoptosis. Furthermore, the results here are the first *in vivo* evidence that shows that Bcl-2 adopts a pore-forming conformation *in vivo*.

To date, the pore-forming model for the regulation of apoptosis by Bcl-2 family is based on *in vitro* experiments using purified proteins and purified organelles/artificial membrane. *In vitro* electrophysiological studies suggest pore forms but provide no insight into Bcl-2 topology. Instead topology has been inferred based on work done on diphtheria toxin, in which the equivalents of helix 5 and helix 6 of Bcl-2 insert into the lipid bilayer when it forms a channel.

A simple pore-like membrane topology of Bcl-2 is illustrated in figure 4.2. In such a topology, the cysteine residue in helix 5 should be buried in the lipid bilayer where it will be protected from modification by the hydrophilic iodoacetamide, IASD. In normal Rat1/ERTM cells expressing Bcl-2, cysteine 158 was readily accessible to IASD suggesting a tail anchored topology (Figure 4.8C). However, when the cells were committed to the apoptotic pathway, cysteine 158 was significantly less accessible to modification by IASD (Figure 4.8C). This change in IASD labelling profile suggests a conformation of Bcl-2 where cysteine 158 is buried in the lipid bilayer.

Cysteine 229 is an internal control for both IASD labelling and membrane integrity. Cysteine 229 is located in the hydrophobic domain of the putative TM of Bcl-2. This C-terminal hydrophobic domain is necessary to bind Bcl-2 to both ER and mitochondria (Martinou et al 1999, Montessuit et al 1999, Diaz et al 1997). Furthermore Bcl-2 is resistant to both high salt and high pH extractions (Janiak et al 1994) suggesting that it is integrated into the lipid bilayer. When Bcl-2 is not bound to membranes cysteine 229 is readily modified by IASD (figure 4.3, 4.4 and 4.5), however when Bcl-2 is bound to membranes it is no longer accessible thus demonstrating that cysteines within the lipid bilayer are protected from IASD (figure 4.5). The lack of IASD modification of cysteine 229 in membrane bound Bcl-2 is not due to steric hinderance by protein-protein interaction since all IASD labelling assays were carried out in 4 M urea. Furthermore, when the IASD labelling experiments were carried out in a higher urea concentrations (6 M and 7 M urea), similar results were observed (data not shown). However the higher urea concentration also resulted in a slight increase in the doubly-labelled band (data not shown), suggesting an increased loss of membrane integrity which exposes cysteine 229. Therefore these results further suggest that the inaccessible of cysteine 158 in apoptotic cells is unlikely due to protein-protein interaction but rather is due to integration into the lipid bilayer of the membrane.

The decrease in cysteine modification was not due to translocation of Bcl-2 into the lumen of the microsomes since IASD has been shown to label cysteines in the lumen of the ER (Falcone et al 1999). In cell free system, preprolactin is translocated into the lumen of ER microsomes through the Sec61 translocation complex where it is cleaved by

signal peptidase to produce prolactin. When these microsomes where incubated with IASD, prolactin was labelled within 5 minutes (data not shown). It is unlikely that IASD was able to cross the lipid bilayer through the phospholipids, as encapsulated IASD in lipid vesicles does not leak out of the vesicles (data not shown). Instead, it is most likely that IASD crosses the ER bilayer through one of the multiple channels in the ER membrane. Therefore, our data suggest that Bcl-2 changes its topology during apoptosis, such that cysteine 158 in the hydrophobic helix 5 region of Bcl-2 is inserted into the lipid bilayer.

The topological change in Bcl-2 was independent of both the apoptotic pathway and subcellular location of Bcl-2, suggesting that the conformational change is a general response of Bcl-2 to apoptosis. Etoposide and ceramide have previously been shown to activate apoptosis by two distinct pathways in Rat1/myc cells lines (Soucie et al 2001, Annis et al 2001). Here, we have shown that in Rat1/ ERTM cell lines, etoposide and ceramide also appear to induce apoptosis by different pathway as demonstrated by the Bax translocation data (figure 4.6 & 4.7). The induction of apoptosis by etoposide resulted in the translocation of Bax whereas in ceramide treated cells, Bax translocation did not occur. Nevertheless Bcl-2 was able to delay both ceramide and etoposide induced apoptosis in Rat 1/ETRM (figure 4.6, 4.7). How Bcl-2 promotes cell survival in response to either of the drugs in Rat 1 cells is not known; however we have shown that in response to both drugs, a conformational change in Bcl-2 occurs during apoptosis.

The conformational change in Bcl-2 was also independent of its cellular location. There is growing body of evidence that suggests that apoptosis can be initiated in

different organelles in the cell, such as the mitochondria, ER, lysosomes and the Golgi apparatus (Ferri & Kroemer 2001). The initiation of apoptosis in each of these organelles is distinct from each other (Ferri & Kroemer 2001). Bcl-2 has been shown to regulate apoptosis from two of these organelles, the ER and the mitochondria (Zhu et al 1995, Annis et al 2001, Hacki et al 2000). How Bcl-2 regulates apoptosis in these organelles is not known. However, using organelle specific mutants of Bcl-2 we have shown that a similar conformational change in Bcl-2 occurs in both the ER and the mitochondria.

To date no other integral membrane protein has been shown to undergo a topology similar to that observed for Bcl-2 *in vivo*. A number of protein toxins from bacteria are known to spontaneously insert into the bilayer of mammalian cells to forming a multi-transmembrane topology. However none of these toxins are integral proteins prior to forming a pore in the lipid bilayer. Also, they either insert directly into the lipid bilayer upon contact, or they require a change in pH to insert into the lipid bilayer (Falnes & Sandvig 2000).

One such bacterial toxin is the diphtheria toxin (DT) which belongs to the AB family of toxins due to the presence of two moieties: A and B (reviewed in (Falnes & Sandvig 2000)). The B domain of DT is made up of an N-terminal translocation domain (also known as the pore-forming domain) and a C-terminal receptor binding domain. The translocation domain of DT is structurally homologous to several Bcl-2-family of proteins, including Bcl-2 (Muchmore et al 1996, Petros et al 2001). Although the precise insertion mechanism is not known, it is believed that low pH (approx pH of 4.5) is required to unfold DT and exposed the hydrophobic α-helices of DT such that they

interact with the lipid bilayer (Falnes & Sandvig 2000). Upon the exposure of these hydrophobic helices to the lipid bilayer, the insertion of the translocation domain of DT is spontaneous (Falnes & Sandvig 2000).

Although the mechanism of the insertion of the putative pore-forming domain of Bcl-2 may be similar to DT, the initiation of insertion is probably different. Much like DT, a TM truncated Bcl-2 was shown to insertion into lipid bilayer to form channels with low ion selective at pH below 5.5 (Antonsson et al 1997, Schlesinger et al 1997). However, a decrease in pH of that magnitude has not observed in cell undergoing apoptosis (Matsuyama & Reed 2000, Antonsson 2001). Instead there must be another mechanism to initiate the unfolding of Bcl-2 to cause its pore-forming domain to be exposed during apoptosis to allow insertion into the bilayer.

One such mechanism may involve interaction with other apoptosis regulating proteins such as the BH3-only proteins. BH3-only proteins are also members of the Bcl-2 family of proteins. However, these proteins share sequence homology only in the BH3 domain. Several BH3-only proteins, such as Bim and Bid, are known to bind and inhibit Bcl-2 (Wang et al 1996, Strasser et al 2000, Bouillet et al 1999). How Bim and Bid inhibit Bcl-2 is not known. During apoptosis, Bid is cleaved to form tBid. tBid has been shown to induce a conformational change in Bax resulting in the translocation, oligomerization and the release of cytochrome c from mitochondria (Desagher et al 1999, Eskes et al 2000). Therefore it is possible that during apoptosis the binding of Bim or tBid to Bcl-2 may unfold Bcl-2 allowing the insertion of the putative pore-forming domain. One approach to determining whether Bim, or tBid binding to Bcl-2 changes the

conformation of Bcl-2 to a multi-TM conformation may be elucidated by incubating peptides containing the BH3 domain of Bim, or Bid with the membrane fraction of Bcl-2 expressing cells and assaying localization of cys 158 by IASD labelling.

4.4.Conclusion

In summary, we have shown the first evidence that Bcl-2 in live cells changes the topology of Bcl-2 from a classical tail-anchored topology to a pore-like topology during apoptosis. This change in conformation is independent of both the pathway of apoptosis and the cellular location of Bcl-2. Furthermore, we present the first example of an integral membrane protein that can adopt a different membrane topology depending on a cellular signal.

These findings further support the pore-forming theory of apoptosis. Several pro-apoptotic proteins such as Bax have been suggested to form channels in mitochondria to allow the translocation as several apoptotic factors (reviewed in Antonsson et al 2001). Due to the structural similarity of Bcl-2 and Bax within the putative pore-forming domain, it is possible that Bax also adopts a pore-forming topology. The technique of examining the accessibility of a specific amino acid position to a chemical labelling reagent will be useful to determine if there is a topology change in Bax similar to that observed in Bcl-2. Furthermore, this technique can be used to determine if the conformational change in Bcl-2 does indeed form a pore by using Bcl-2 with cysteine placed at various places along the putative pore forming domain.

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Chapter 5

Conclusions and Future Directions

The last ten years have seen a renewed interest in tail-anchored proteins. The appeal of understanding tail-anchored proteins was partly fueled by the identification of new tail-anchored proteins, and partly by the important roles these proteins play in many cellular functions (such as apoptosis, and vesicle transport and fusion). Novel findings on the targeting mechanisms and topology of tail-anchored proteins have been presented in this thesis. Firstly, evidence for the existence of at least two different targeting mechanisms was presented (Chapter 2). In the chapter that followed (Chapter 3) the characterization of one of the targeting mechanisms was described. Lastly, the dynamic topology of tail-anchored proteins was examined (chapter 4).

5.1. Tail-Anchor membrane targeting

5.1.1. Two Targeting Mechanisms to the ER

The post-translational targeting and integration of tail-anchored proteins was believed to occur spontaneously and non-saturably (Kutay et al 1993). Exemplified by Cb5, tail-anchored proteins exist as monomers and multi-oligomers in the cytoplasm however they most likely integrate into the bilayer as monomers (figure 5.1). The targeting was thought to be only governed by the kinetics of the TM integrating into the lipid bilayer, which is dependent on bilayer lipid composition and physiology. However, with the discovery of other tail-anchored proteins, the spontaneous/kinetic model as a general model for all tail-anchored proteins has been seriously challenged.

The data presented in this thesis demonstrate that not all tail-anchored proteins integrate into the ER in a fashion similar to Cb5. Exemplified by Vamp1, in this new

model (figure 5.1B), the integration of these proteins into the ER is not spontaneous, but requires both a cytoplasmic and membrane component (Chapter 2). Unlike Cb5, the binding of Vamp1 to ER microsomes was saturable, which suggested a rate limiting receptor binding step (Chapter 2). One of the cytoplasmic components required for binding was identified as ATP (Chapter 2), however, it not known whether a cytoplasmic protein component such as a chaperone is also involved. Evidence for a role for chaperone proteins has been presented by other groups (Mullen et al 1999, Lan et al 2000). Using deletion mutants and site-directed mutagenesis, the ATP dependent targeting signal on Vamp2 and Vamp8 was found to include four lysines present an amphipathic helix located within the NTS (the sequence preceding the putative TM) (Chapter 3). The TM domain and the positively charged NTS together were found to be sufficient and necessary to bind tail-anchored proteins to ER microsomes (Chapter 3). A positively charged amphipathic NTS is also found in other tail-anchored proteins. Similar to the Vamps, their targeting to ER microsomes was also ATP-dependent, suggesting this mechanism for targeting tail-anchored proteins is not unique to the Vamps (Chapter 3). Furthermore, these results provide the first evidence that demonstrates a targeting signal that is located within the NTS of tail-anchored proteins.

5.1.2. ER targeting signal of Tail-anchored proteins.

The CTS (C-terminal sequence) is located at the carboxyl-terminal end just after the putative TM domain of tail-anchored proteins and has been found to be important in the targeting of these proteins to the mitochondria, as well as targeting the ER isoform of Cb5 to the ER (Mitoma & Ito 1992, De Silvestris et al 1995, Isenmann et al 1998, Horie

et al 2002). In particular, the charged residues in the CTS were necessary for organelle-specific targeting (Borgese et al 2001, Mitoma & Ito 1992, Isenmann et al 1998). For mitochondrial targeting, at least two positively charged residues were found to be necessary. Without these charged amino acids the protein was found in both the ER and the mitochondria (Mitoma & Ito 1992, Berglund et al 1999, Borgese et al 2001, Horie et al 2002).

In Chapter 1, Table 1, the TM length and the number of charged resides on either side of the TM is presented. It is not known whether there is a correlation between the location of the charged residues (NTS or CTS) and the targeting mechanism employed by tail-anchored proteins. Close examination of Table 1 suggest that there may be some trend. Spontaneous insertion into membranes may occur with proteins that do not have a positively charged, amphipathic, helical NTS that mediates integration of the TM via an interaction with a receptor. My experimental data suggest that similar rules apply to both Sec61γ and Bcl-2. Similar to Cb5, Bcl-2 does not have an amphipathic NTS helix and it does not require ATP or a trypsin-sensitive membrane receptor to target to membranes. On the other hand, Sec61γ which requires ATP and a trypsin-sensitive membrane receptor has a positively charged amphipathic helix located in the NTS. To determine if the correlation between an amphipathic helical NTS and Vamp-like targeting exists for other tail-anchored proteins, other tail-anchored proteins must be examined. A relatively rapid method of screening other tail-anchored proteins would be to test for ATP-dependent binding and binding to trypsinized membranes using the *in vitro* assay in Chapter 2.

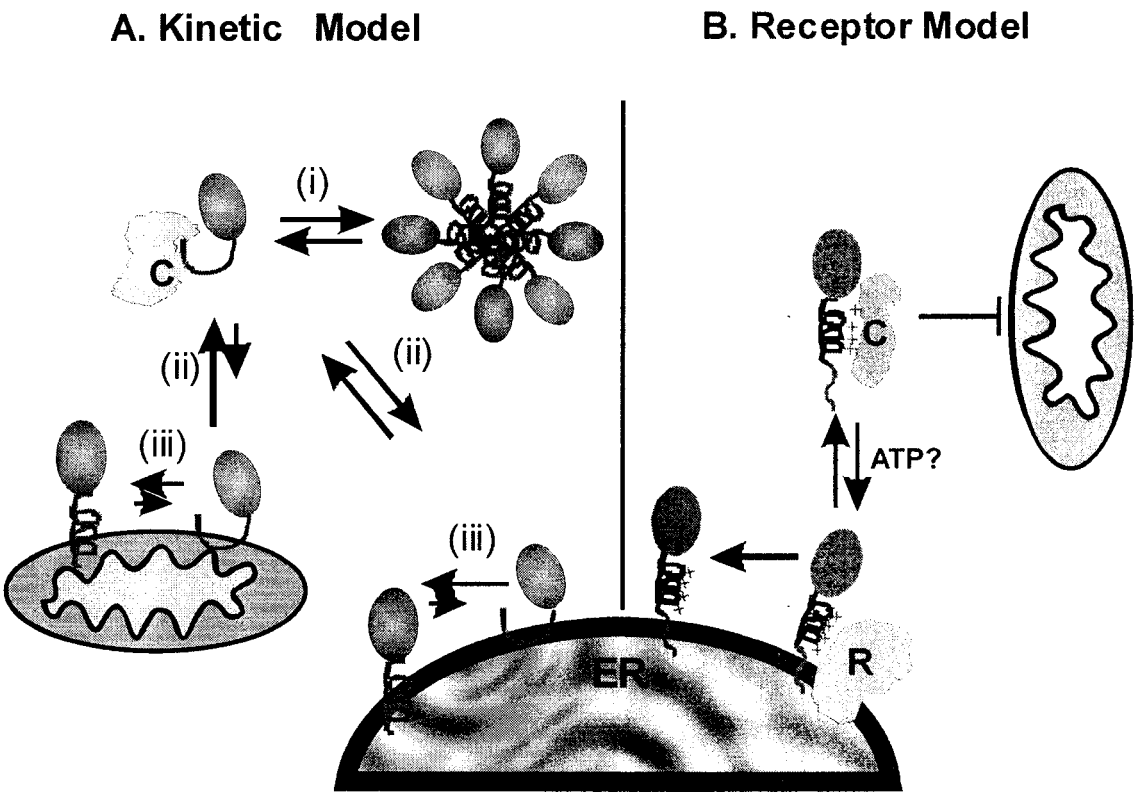

Figure 5.1. Kinetic and Receptor mediated ER membrane targeting.

Two models of targeting tail-anchored protein to the ER membrane are presented. A) In the Kinetic model there are several equilibrium states. First is equilibrium (i) between the oligomeric and the monomeric forms for the protein. Only the monomeric form can bind to membranes. The aggregation of the monomeric form is probably prevented by a chaperone (C, yellow). A second equilibrium exists between the unbound and 'loose' membrane bound form (ii). The 'loose' bound form can also change its conformation to form the 'tight' bound form (iii). The 'tight' bound is the end point as it can not be removed from the lipid bilayer. Membrane specificity is determined by the kinetic preference of the protein for the ER membrane. The steps (ii) and (iii) is greater for the ER than it is for the mitochondria.
B) In the Receptor model, membrane binding is regulating by a receptor (R, blue) on the target membrane. The receptor may interact with the positively charged residues on the NTS of the tail-anchored protein. It is also possible a chaperone (C, green) is also involved in the targeting of these tail-anchored proteins. These proteins do not bind to the mitochondria as it does not have a receptor for these proteins.

5.1.3. ER signal vs. Mitochondrial signal.

Recently several groups have demonstrated that the mitochondria targeting signal is localized in the CTS and the TM domain. The mitochondria targeting signal consists of a shortened TM (less then 18 residues) and at least two positive charges in the CTS (Isenmann et al 1998, Borgese et al 2001, Horie et al 2002). Vamp1b (the mitochondrial isoform of Vamp1) can be localized to the ER by either lengthening the TM or removing the two lysines (Isenmann et al 1998). Interestingly, in chapter 3 figure 3.4 shows that the mitochondria localization signal does not interfere with the targeting of Vamp2 to the ER *in vitro*. In fact, Vamp2-mito (Vamp2 with the mitochondria targeting signal) bound to ER microsomes at similar levels to the wild type protein (Chapter 3, figure 3.4) and the binding was ATP-dependent (data not shown) in an *in vitro* assay. Furthermore, the removal of the NTS targeting signal in Vamp2-mito abolished the binding of the protein to ER microsomes (Chapter 3, figure 3.4), suggesting that the NTS alone is required for ER binding. Furthermore, using an *in vitro* assay, Borgese et al 2001 have shown that ER-Cb5 with the mitochondrial signal and an N-glycosylation sequence at the C-terminus is efficiently glycosylated in the ER. However, *in vivo* this protein was targeted exclusively to mitochondria without it being glycosylated, suggesting that *in vivo,* the targeting of the protein to the mitochondria occurs directly without going through the ER. Together these results suggest that the mitochondrial signal does not prevent the targeting of tail-anchored proteins to the ER. Instead, if both signals are present in the protein, the mitochondrial signal appears to be preferred over the ER signal. It remains to be determined whether organelle preference is due to a kinetic advantage for a receptor on

mitochondria versus ER, or if there is a mitochondrial specific targeting protein in cytoplasm.

5.1.4. Future prospects on the targeting mechanism.

The data in this thesis can be used as the basis for further study of the mechanism of targeting tail-anchored proteins to the ER. To test the membrane targeting model, three areas need closer examination. They include the identification of the ER targeting signal in other ER-targeted tail-anchored proteins; the identification of the ER receptor; and the identification of the cytoplasmic chaperones.

The charged residues in the NTS of Vamps are necessary for ER targeting. However, it is not known whether these charges are required for ER localization of other ER-targeted tail-anchored proteins. As mentioned above, a number of ER-targeted tail-anchored proteins have positively charged residues in the NTS. Using the cell-free membrane binding assays presented in this thesis, the membrane binding of these proteins can be easily compared to that of the Vamps. Furthermore, mutagenesis studies are required to determine the specific role of the NTS in the targeting of these proteins.

Experimental evidence for the existence of a membrane receptor required for the targeting of Vamp was presented in Chapter 2. However, the identity of the receptor(s) has remained elusive. Several approaches can be used to isolate and identify the receptor, including mutational analysis in yeast and reconstitution experiments using canine ER microsomes. The latter is most feasible in the lab of Dr. David Andrews' because of his expertise in cell-free systems. In order to conduct experiments with solubilized and reconstituted ER microsomes, several criteria need to be fulfilled. First, a tail-anchored

protein is required that does not bind to trypsinized membranes, nor to liposomes of ER-like lipid composition. Second, the protein should readily bind to reconstituted ER vesicles. Finally, solubilization and reconstitution conditions need to be determined that will maximize the reconstitution of membrane proteins without the loss of tail-anchor protein binding.

Although there is some evidence for the requirement of a cytoplasmic chaperone for tail-anchored protein targeting, it remains largely a hypothesis (Mullen & Trelease 2000, Lan et al 2000). The identification of the cytoplasmic chaperone(s) for targeting proteins to mitochondria and ER would aid in determining if the targeting distinction occurs primarily in the cytoplasm or at the membrane. If the same chaperone binds to both the ER and mitochondrial targeted tail-anchored proteins, it would suggest that targeting occurs at the level of the membrane. However, if there are distinct chaperones for each membrane than it suggests that targeting can occur in the cytoplasm.

5.2. Membrane topology of Tail-Anchored proteins.

Tail-anchored proteins are generally believed to be in a $N_{cytoplasmic}$-C_{Lumen} (Tail-Anchored) topology (Kutay et al 1993, Wattenberg & Lithgow 2001). In this thesis, evidence is presented that suggests that some tail-anchored proteins can adopt more than one topology. The membrane topology of Bcl-2 changes from a tail-anchored topology to a multi-membrane spanning topology in response to an inter-cellular signal (Chapter 4). To my knowledge, there are no other published results that demonstrate a change in the membrane topology of an integral membrane protein from a single TM to a multi-TM integral protein. Others have shown that several members of the Bcl-2 family of proteins

form ion conducting channels at low pH *in vitro* (Antonsson et al 1997, Minn et al 1997, Schlesinger et al 1997). However, the membrane topologies of these proteins were not elucidated. Several bacterial toxins are known to bind to membranes via an interaction with a membrane receptor, and then insert into the lipid bilayer to form a channel in response to a pH change (Eskes et al 2000). However, none of the known bacterial toxins bind to the bilayer via its own transmembrane domain. Therefore, the data presented suggest that Bcl-2 is the first constitutively TM protein that has been demonstrated convert between two distinct membrane topologies in response to a cellular signal.

5.2.1. Future prospects: Elucidating the function of the topology change of Bcl-2.

The reason for the topology change in Bcl-2 is not known. One explanation for the conformational change may be to interact with other proteins in the mitochondria and/or the ER. Bcl-2, an anti-apoptotic protein, has been claimed to bind to a number of membrane proteins including Bax (Hsu & Youle 1997, Brenner et al 2000), adenine nucleotide translocator (ANT) (Kuo et al 1998), SERCA (Kobrinsky & Kirchberger 2001), and voltage-dependent anion channel (VDAC) (Shimizu et al 1999). In the mitochondria, Bcl-2 has been postulated to prevent mitochondrial membrane permeability (MMP) by sequestering pro-apoptotic proteins in heterodimeric complexes (Desagher & Martinou 2000). Bax, a pro-apoptotic protein, is believed to initiate apoptosis by releasing apoptotic factors, such as cytochrome c, by either forming a channel via homo-oligomerization, or by binding to other proteins that form a channel such as VDAC and/or ANT (Desagher & Martinou 2000). Bax has also been suggested

to induce apoptosis by releasing Ca^{2+} stores from the ER (Nutt et al 2002b, Nutt et al 2002a). In our assay, we have observed that Bax translocation to membranes correlates with the insertion of Bcl-2 into membranes. Therefore, it is possible that Bcl-2 is inserted into the lipid bilayer to form heterodimers with Bax. This might prevent oligomerization of Bax, thus inhibiting pore formation, or sequester it from interacting with mitochondrial pore complexes to release apoptotic factors such as cytochrome c, and apoptosis-inducing factor (AIF).

The hetero-dimerization between Bax and Bcl-2 is mediated by the binding of the BH3 domain of Bax with the hydrophobic pocket formed by the BH1, BH2, and BH3 domains of Bcl-2 (Sattler et al 1997, Diaz et al 1997). A conformational change in Bax is required for this heterodimerization to occur (Hsu & Youle 1997). However, it is not known whether a similar change in Bcl-2 is required for it to bind to Bax. For Bax to immunopreciptate with Bcl-2, non-ionic detergent was required which presumably exposes the BH3 domain of Bax (Hsu & Youle 1997). Due to the structural similarities between Bax (Suzuki et al 2000) and Bcl-2 (Petros et al 2001), it is possible that the non-ionic detergent also causes a conformational change in Bcl-2. The structural change in Bax also leads to the translocation of Bax from the cytosol to the membrane where it is postulated to form a pore to release cytochrome c (Eskes et al 2000, Desagher et al 1999). Although the membrane structure of Bax is yet to be solved, based on its structural similarity with the bacterial pore forming proteins, it likely adopts a multi-membrane spanning topology in membranes. Therefore, Bcl-2 may also be required to change its

topology from a tail anchor topology to a multi-membrane spanning topology to interact with Bax.

If a conformational change in Bcl-2 is required for it to dimerize with Bax, Bcl-2 may have a dual effect on Bax. I propose two functions for Bcl-2, a direct and an indirect effect on Bax. First, it has been observed in many systems that Bcl-2 functions by preventing the translocation of Bax to membranes (Antonsson et al 1997, Annis et al 2001). Bax is found in the cytosol in non-apoptotic cells, therefore it will not be able to interact directly with Bcl-2. However, Bcl-2 is able to prevent Bax translocation in response to etoposide treatment (Chapter 4, figure 4.6B). The exact mechanism is not known, however the effect must be indirect and Bcl-2 may be involved in inhibiting the activation of BH3-only proteins, and/or sequestering them by binding to them. BH3-only proteins have been shown to be required to be sufficient cause a conformational change in Bax and it is this conformational change that results in Bax translocating to membranes (Wang et al 1996, Roucou et al 2002, Desagher et al 1999).

The direct effect Bcl-2 has on Bax is by binding to it. Since Bax and Bcl-2 are found in both the mitochondria and the ER (Nutt et al 2002a),(Zhu et al 1996) and both proteins have been shown to be able to bind to each other (Hsu & Youle 1997), it is possible that Bcl-2 inhibits Bax-induced apoptosis by binding to Bax on the membrane and prevent its pro-apoptotic function. It might be possible to determine if the direct interaction of Bcl-2 with Bax prevents apoptosis by making membrane specific mutants of Bax. By co-transfecting the Bax membrane-specific mutants into Bax -/- cells with membrane-specific mutants of Bcl-2, one can determine if Bcl-2 needs to be in the same

membrane to prevent Bax function. As well, the same cells could be used to determine if the interaction between Bax and Bcl-2 induces the conformational change in Bcl-2. It may be possible to use Bax BH3 domain peptides as another way to determine if the binding of the BH3 domain of Bax to the BH1, BH2, and BH4 hydrophobic pocket of Bcl-2 causes a conformational change in Bcl-2 on cell lysate from cells expressing Bcl-2.

Bcl-2 also prevents apoptosis from the ER. The ER-specific Bcl-2, Bcl-Cb5, can prevent both cytochrome c release and the loss of mitochondrial potential (Hacki et al 2000, Annis et al 2001). One hint on how ER-localized Bcl-2 influences mitochondrial apoptosis is its effect on ER Ca^{2+} levels. Cells over-expressing Bcl-2 increase the uptake of Ca^{2+} by the ER through up-regulating the expression of SERCA (Kuo et al 1998). Furthermore, Bcl-2 and SERCA mutually co-precipitate in detergent-solubilized cell lysate (Kuo et al 1998). Although Bcl-2 and SERCA co-precipitated from non-apoptotic cells, since non-ionic detergent was used to lyse the cells, it is not known if Bcl-2 constitutively binds SERCA or if it binds only during apoptosis. Therefore, whether a conformational change in Bcl-2 is required to bind to SERCA needs to be examined.

Alternatively, the conformational change in Bcl-2 may be a mechanism to inhibit the anti-apoptotic activity of Bcl-2. The insertion of the helix 5 region into the lipid bilayer may prevent Bcl-2 from binding to other proteins. Several BH3-only proteins (such as Bim and Bid) are known to bind and inhibit Bcl-2 (Wang et al 1996, Strasser et al 2000, Bouillet et al 1999). How Bim and Bid inhibit Bcl-2 is not known. A cleaved form of Bid, tBid has been shown to induce a conformational change in Bax, resulting in translocation, oligomerization and the release of cytochrome c from the mitochondria

(Desagher et al 1999, Eskes et al 2000). Does the binding of Bim or tBid to Bcl-2 change its conformation? This question may be answered by incubating a peptide containing the BH3 domain of Bim or Bid with the membrane fraction of Bcl-2 expressing cells to determine if a conformational change in Bcl-2 occurs.

Previously, Bcl-2 has been found to have some pro-apoptotic activity (Uhlmann et al 1998). It is equally possible that the insertion of the helix 5 and helix 6 domains of Bcl-2 is involved in this pro-apoptotic function of Bcl-2. Pro-apoptotic activity of Bcl-2 resulted either from the overexpression of Bcl-2 or from the expression of N-terminally deleted Bcl-2. The expression of our Bcl-2 was not at the levels observed by Uhlmann et al (1998), were any deletion products observed in our cells during apoptosis. However, it is possible that, during apoptosis, Bcl-2 may be converting from an anti-apoptotic protein to a pro-apoptotic protein.

In vitro experiments with purified Bcl-2 have shown that it can conduct ions in artificial vesicles (Antonsson et al 1997, Minn et al 1997, Schlesinger et al 1997). Thus it is possible that Bcl-2 may be aiding in the formation of channels in both the mitochondria and the ER. The data presented in this thesis does not explicitly show that Bcl-2 forms a channel, however it does show that a topology change that is consistent with a pore-forming topology occurs in Bcl-2 during apoptosis. To determine if Bcl-2 forms a channel that is sufficiently large to translocate cytochrome c or other proteins to the cytoplasm, cysteine-scanning mutagenesis experiments are being carried out. The native cysteine 158 is being removed and a cysteine residue placed at various positions within both helix 5 and helix 6, the putative pore forming domain. Assaying these mutant

proteins with IASD may provide new evidence for the formation of pore-forming complexes by Bcl-2.

5.3. Reference

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