Evidence for Multiple Mechanisms for Membrane Binding and Integration via Carboxyl-Terminal Insertion Sequences[†]

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ABSTRACT: Subcellular localization of proteins with carboxyl-terminal insertion sequences requires the molecule be both targeted to and integrated into the correct membrane. The mechanism of membrane integration of cytochrome b_5 has been shown to be promiscuous, spontaneous, nonsaturable, and independent of membrane proteins. Thus endoplasmic reticulum localization for cytochrome b_5 depends primarily on accurate targeting to the appropriate membrane. Here direct comparison of this mechanism with that of three other proteins integrated into membranes via carboxyl-terminal insertion sequences [vesicle-associated membrane protein 1(Vamp1), polyomavirus middle-T antigen, and Bcl-2] revealed that, unlike cytochrome b_5, membrane selectivity for these molecules is conferred at least in part by the mechanisms of membrane integration. Bcl-2 membrane integration was similar to that of cytochrome b_5 except that insertion into lipid vesicles was inefficient. Unlike cytochrome b_5 and Bcl-2, Vamp1 binding to canine pancreatic microsomes was saturable, ATP-dependent, and abolished by mild trypsin treatment of microsomes. Surprisingly, although the insertion sequence of polyomavirus middle-T antigen was sufficient to mediate electrostatic binding to membranes, binding did not lead to integration into the bilayer. Together these results demonstrate that there are at least two different mechanisms for correct membrane integration of proteins with insertion sequences, one mediated primarily by targeting and one relying on factors in the target membrane to mediate selective integration. Our results also demonstrate that, contrary to expectation, hydrophobicity is not sufficient for insertion sequence-mediated membrane integration. We suggest that the structure of the insertion sequence determines whether or not specific membrane-bound receptor proteins are required for membrane integration.

Integral proteins are inserted into membranes through several different types of mechanisms. In eukaryotes the best characterized of these is the signal recognition particle-(SRP-)[1] dependent pathway (Walter & Johnson, 1994), in which membrane targeting is initiated cotranslationally by a signal sequence encoded near the amino terminus of the nascent peptide. For these proteins integration occurs via a complex multistep process ending with release of the polypeptide into the lipid membrane coincident with the completion of protein synthesis (Andrews & Johnson, 1996). Another small, but rapidly growing, class of membrane proteins, lacks an amino-terminal signal sequence and instead is targeted by a carboxyl-terminal hydrophobic domain termed an insertion sequence [reviewed in Kutay et al. (1993)]. The carboxyl-terminal location of insertion sequences dictates that these proteins are targeted to and integrated into the bilayer of membranes posttranslationally (Enoch et al., 1979; Rachubinski et al., 1980; Sabatini et al.,1982). Therefore, neither SRP nor SRP receptor is involved in the targeting of proteins via insertion sequences (Anderson et al., 1983).

Very little is known about the mechanisms involved in insertion sequence-mediated membrane integration. An important unresolved aspect of the process is the mechanism-(s) that regulate membrane selectivity. Previous studies of membrane integration of the endoplasmic reticulum form of cytochrome b_5 (Cb5) suggested that membrane integration is spontaneous and promiscuous (Enoch et al., 1979; Rachubinski et al., 1980; Anderson et al., 1983; Takagaki et al., 1983a,b). Nevertheless, when expressed in cells, both Cb5 and fusion proteins containing the Cb5 carboxyl-terminal insertion sequence accumulate specifically in the ER membrane (Mitoma & Ito, 1992). Therefore, Cb5 insertion sequence-mediated subcellular localization must be regulated by targeting the molecule to the endoplasmic reticulum membrane. Subsequent to correct targeting, membrane integration probably occurs spontaneously. Although it is widely believed that other molecules with insertion sequences are targeted to and integrate into subcellular membranes by mechanisms similar to that of Cb5, currently published results are difficult to interpret because membrane assembly of these molecules has not been directly compared using the same

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[1] Abbreviations: SRP, signal recognition particle; ER, endoplasmic reticulum; Cb5, endoplasmic reticulum specific rat liver cytochrome b_5; mT, polyomavirus middle-T antigen; Vamp, vesicle-associated membrane protein; DTT, dithiothreitol; IASD, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid; OAc, acetate.

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assay system. Therefore, we have examined the mechanisms of membrane integration for four different proteins for which there is published evidence for insertion into endoplasmic reticulum. We have used this information to determine the relative importance of targeting and membrane integration in subcellular localization of these proteins.

Based on sequence comparisons, membrane proteins other than Cb5 that are predicted to contain insertion sequences include middle-T antigen (mT), the transforming protein of polyomavirus (Treisman et al., 1981; Rassoulzadegan et al., 1982), the protooncogene product Bcl-2 (Vaux et al., 1988; Chen-Levy et al., 1989; Lui et al., 1991), and v-snares such as the synaptic *v*esicle-*a*ssociated *m*embrane *p*roteins, Vamp1 and Vamp2 (synaptobrevin) (Elferink et al., 1989; Sollner et al., 1993; Bennet et al., 1994). All of these proteins have been suggested to interact with ER membranes by a mechanism similar to that of Cb5 (Kutay et al., 1993). Although mT, Bcl-2, and Vamp1 may be initially targeted to ER membrane(s), the final destinations of these proteins are not the same. Both plasma membrane and intracellular membrane locations have been reported for mT (Ito et al., 1977; Segawa & Ito, 1982; Zhu et al., 1984; Dilworth et al., 1986). The subcellular localization of Bcl-2 is also controversial. Bcl-2 is aberrantly expressed in human follicular lymphoma and has been shown to prevent apoptosis in a wide variety of cell types (Vaux et al., 1988; Hockenbery et al., 1990). In experiments with cell-free systems, Bcl-2 has been reported to insert into the ER (Chen-Levy et al., 1989; Chen-Levy et al., 1990), the outer membrane of mitochondria (Nguyen et al., 1993), or to both ER and mitochondria (Janiak et al., 1994b). Recent functional studies suggest that dual localization (ER and mitochondria) increases the range of targets that Bcl-2 can interact with to prevent apoptosis in transfected cells (Zhu et al., 1996). Finally, Vamp1 and Vamp2 appear to cycle between the plasma membrane and synaptic vesicle membranes (Grote et al.,1995; Grote & Kelly, 1996). However, data obtained using transfected cells (Grote et al., 1995) and cell-free systems (Kutay et al., 1995) suggests that Vamp2 is first targeted to the ER and then transported along the secretory pathway to synaptic vesicles. Membrane integration has not been examined for the Vamp2 homologue, Vamp1. Therefore, we have examined targeting of Vamp1 to determine whether homologous proteins such as Vamp1 and Vamp2, that are normally expressed in different cell types (Elferink et al., 1989; Trimble et al., 1991; Rossetto et al., 1996), are targeted by the same or a different mechanism. To determine whether subcellular localization is established by the initial targeting of these molecules to an appropriate membrane (similar to cytochrome b_5) or if they will integrate into only a *bona fide* target membrane (suggesting that prior to membrane integration they could diffuse freely in the cytoplasm), we have compared directly the mechanism(s) of membrane binding of Cb5, mT, Bcl-2, and Vamp1 using a cell-free system supplemented with either microsomes or liposomes. Furthermore, we determined the role of the putative insertion sequences from these proteins in membrane integration by fusing them to common passenger proteins. We show that fusion proteins containing the mT insertion sequence bind electrostatically to, 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 into lipid vesicles. We also show that although Cb5, Bcl-2, and Vamp1 synthesized in reticulocyte lysate all insert into ER membranes, ATP and a trypsin-sensitive membrane protein are required for membrane integration of Vamp1.

Finally, quantitative measurements for the binding of Cb5, Bcl-2, and Vamp1 synthesized in reticulocyte lysate to ER microsomes revealed that binding of Vamp1 to microsomes is saturable, with ER containing roughly 20 fmol of Vamp1 binding sites/100 fmol of signal recognition particle receptors. In contrast, binding of both Bcl-2 and Cb5 to ER microsomes appears to be nonsaturable. Thus, our results suggest that, unlike Cb5, the mechanisms of membrane integration for Vamp1, Bcl-2, and mT contribute to the correct subcellular localization of these proteins.

EXPERIMENTAL PROCEDURES

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 (Elferink et al., 1989; Andrews et al., 1993; Janiak et al., 1994a,b). We used a polypeptide called gPA 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*HI site at the 3′ end of the coding region of gPA and an *Xho*I 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 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. All of the fusion junctions and coding regions for the putative insertion sequences were confirmed by DNA sequencing. Space constraints make a complete description of the construction of all 11 of the plasmids impractical; however, the plasmids, construction details, and the complete sequence for each plasmid is available from the authors upon request.

Salt-extracted canine pancreatic ER microsomal membranes (microsomes) were prepared as described by Walter and Blobel (1983). Each batch of microsomes was tested for cotranslational translocation of preprolactin before use (Andrews, 1989). In addition, batches of microsomes were

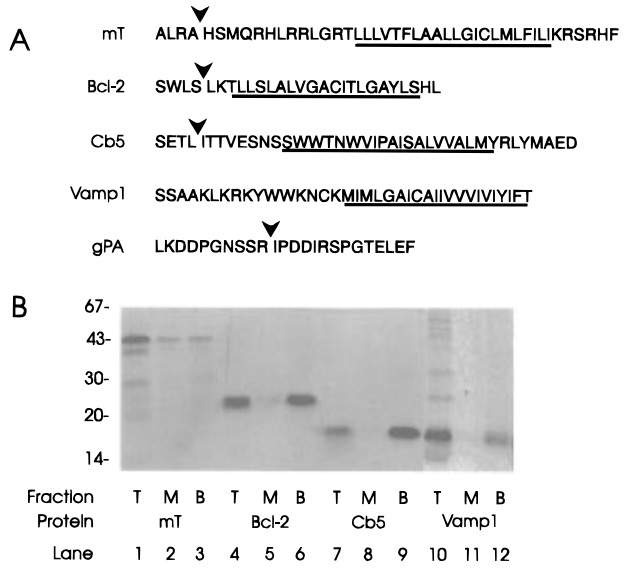

FIGURE 1: (A) Amino acid sequences of the putative insertion sequences are given in one-letter code. mT, polyomavirus virus middle-T antigen; Cb5, the endoplasmic reticulum-specific form of rat liver cytochrome b_5. The hydrophobic core of each sequence is underlined. The junction for the fusion proteins 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 kilodaltons) are indicated at the left of the figure.

standardized by measuring the amount of signal recognition particle receptor α-subunit by western blotting. One equivalent of microsomes is defined as containing 100 fmol 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, 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 (Hope et al., 1985). Transcription of the plasmids using SP6 polymerase was as described by Gurevich et al. (1991).

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 30 psi (170,000*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.

Microsomes were added to terminated translation reactions, and the samples were incubated at 24 °C for the times indicated. 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 h 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 labeled 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).

Liposome Binding. Samples containing liposomes were analyzed as described previously (Janiak et al., 1994a; Andrews et al., 1989), 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. The gradient was fractionated into five fractions (55 μL each), with the pellet, solubilized as above, as the bottom fraction.

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. (1995). 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.

RESULTS

Binding of Proteins with Putative Insertion Sequences to Microsomal Membranes. In cells Cb5 targets specifically to endoplasmic reticulum membranes (Mitoma et al., 1992; Zhu et al., 1996), yet in an *in vitro* assay containing a single membrane target, Cb5 will integrate into a variety of different membranes including liposomes (Enoch et al., 1979). The promiscuous membrane integration of Cb5 seen *in vitro* appears to be due to an uncoupling of targeting and integration in cell-free assays because they contain only a single type of membrane. When both ER and mitochondria are added together into the same cell-free assay, Cb5 accumulates preferentially in ER membranes (Janiak et al., 1994b). Therefore, by providing only a single target membrane in a cell-free assay, it is possible to examine insertion sequence-mediated membrane integration independent of targeting.

The four proteins Cb5, mT, Bcl-2, and Vamp1 share no sequence homology, but all contain a single contiguous sequence of hydrophobic amino acids near the carboxyl terminus of the polypeptide, characteristic of an insertion sequence (Figure 1A). When these proteins were translated in a reticulocyte lysate cell-free system with 2 equiv of ER microsomes added cotranslationally (data not shown) or posttranslationally (Figure 1B), all but mT pellet with microsomes during centrifugation over a sucrose cushion. The small amount of mT detected in the bottom fraction (Figure 1B, lane 3) represents only 5% of the total mT used and can be attributed to electrostatic binding to the microsomes (see below). As expected, the control molecule Cb5 bound to microsomes very efficiently (Figure 1, lanes 7−9). Bcl-2 and Vamp1 also bound to ER membranes posttranslationally albeit with lower efficiency (61% and 20%, respectively) than Cb5 (82%; Figure 1, compare lanes 7−9 with lanes 4−6 and 10−12). When membranes were resuspended in translation buffer and incubated for 20 min in buffer containing 1 M salt, only mT was observed to be released from the membranes, demonstrating that for the experimental conditions used here membrane binding is irreversible (data not shown). Extraction of the resuspended membranes with 0.1 M sodium carbonate (pH 11.5) confirmed that for Cb5, Bcl-2, and Vamp1 the molecules observed to bind to membranes were integrated into the microsomal membranes (data not shown). Although extraction with sodium carbonate is the standard assay for membrane integration, there are exceptional examples of carbonate-resistant peripheral membrane proteins (Young et al., 1996). Therefore, membrane integration was also assayed for Bcl-2 and Vamp1 using the sulfhydryl-modifying reagent IASD. The putative insertion sequences from Bcl-2 and Vamp1 both contain a single cysteine residue that in the absence of membranes can be modified by IASD. This modification results in a change in electrophoretic mobility on SDS−PAGE. Although IASD has been reported to not cross the plasma membrane (Krishnasastry et al., 1995), it rapidly crosses the ER membrane. In control experiments we have labeled both lumenal proteins and the lumenal domains of integral membrane proteins with this reagent (Falcone and Andrews, unpublished results). However, residues buried in the lipid bilayer of the membrane are not modified, presumably due to the two negative charges on IASD. After membrane binding, the cysteine residue in the Bcl-2 and Vamp1 insertion sequences was not labeled by IASD (data not shown), consistent with our other evidence that these sequences integrated into the bilayer. Finally, to confirm that all of the molecules were processed correctly by our cell-free system, the topology of the molecules was determined by accessibility to added protease. After membrane binding, all of the molecules remained accessible to added protease and were thus anchored with the expected topology such that the bulk of the molecule faced the cytosol [data not shown; see also Janiak et al., (1994a,b)].

Effect of ATP on Membrane Targeting. Binding of Vamp2 (synaptobrevin) molecules synthesized in reticulocyte lysate was previously reported to be ATP-dependent (Kutay et al., 1995). Membrane binding of purified cytochrome b_5 has not been reported to depend on ATP. However, ATP dependence was not examined when membrane binding was previously analyzed for Cb5 synthesized in reticulocyte lysate (Rachubinski et al., 1980; Anderson et al., 1983). Therefore, it is equally possible that ATP is a general requirement for insertion sequence-mediated membrane integration of molecules synthesized in reticulocyte lysate or is specifically required for Vamp2 membrane binding. To distinguish these possibilities, we investigated whether ATP is required for membrane binding of Vamp1, Cb5 or Bcl-2 molecules synthesized in reticulocyte lysate. To remove ATP from the *in vitro* translation reaction, 5 units of Apyrase was added. After incubation with Apyrase, Vamp1 membrane binding was reduced from 30% to 7% (Figure 2, compare lanes 1−3 with lanes 4−6). Furthermore, when all nucleotide triphosphates were removed by passing the reaction mix through a column of Sephadex G-25, binding of Vamp1 was severely impaired (3% bound) (Figure 2, lanes 10−12). Binding was

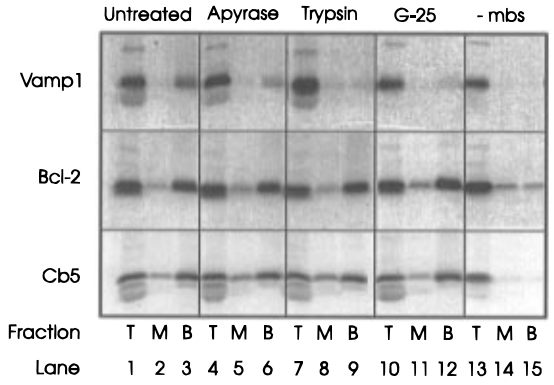

FIGURE 2: Only Vamp1 membrane binding requires ATP and a trypsin-sensitive membrane component. Reticulocyte lysate translation reactions (10 μL) for Vamp1, Bcl-2, and Cb5 were incubated with 2 equiv of ER microsomes for 20 min at 24 °C (untreated) or treated as follows. (a) Apyrase: after translation for 1 h, 5 units of apyrase was added to the translation reactions. After another 30 min incubation, 2 equiv of microsomes was added. (b) Trypsin: microsomes were digested with 5 μg/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.

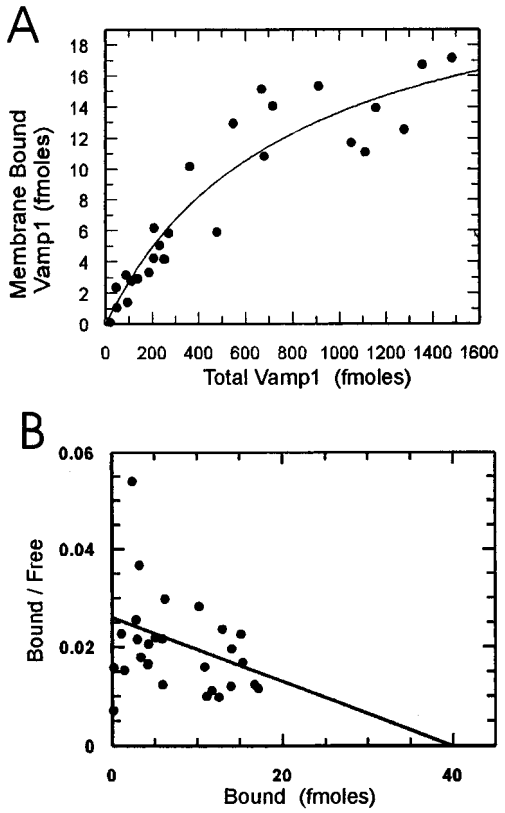

FIGURE 3: Binding of Vamp1 to microsomes. (A) Increasing amounts of the individual proteins in translation reactions were incubated with 2 equiv of microsomes for 2 h at 24 °C. Translation buffer (30 μL) was added to the reaction mixtures and they were layered on top of a 0.5 M sucrose cushion (110 μL). 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 fmol/equiv of microsomes.

also abolished for Vamp1 when the membranes were preincubated with a small amount of trypsin (5 μg/mL) for 1 h at 0 °C (Figure 2, lanes 7−9). The similarity of these results to those previously reported for Vamp2 (Kutay et al., 1995) suggests that the mechanism of membrane integration may be similar for Vamp1 and Vamp2. In contrast, neither nucleotide triphosphate depletion nor *mild* trypsin digestion affected membrane binding for either Bcl-2 or Cb5 (Figure 2). These results indicate that membrane integration of Vamp proteins differs from that of Bcl-2 and Cb5.

The characteristics of Vamp protein membrane integration strongly suggest the involvement of a membrane-bound receptor protein. If Vamp proteins remain bound to such a protein, membrane binding of Vamp1 should be saturable. To examine saturation of membrane binding and to measure the number of binding sites for Vamp1, aliquots of Vamp1 were incubated with 2 equiv of microsomes (approximately 200 fmol of SRP receptor). Preliminary control experiments demonstrated that after 2 h of incubation, binding had reached equilibrium, was irreversible, and was diagnostic of membrane integration (data not shown). Therefore, we assayed Vamp1 for membrane binding rather than integration to limit the number of processing steps that unavoidably increase the amount of error in the measurements. To measure membrane binding the molecules were incubated with microsomes for 2 h before the microsomes were pelleted through sucrose step gradients as above. To permit direct comparison of the binding characteristics of Cb5, Vamp1, and Bcl-2 (see below), the radioactivity recorded on the phosphorimager plate was normalized for the number of methionines in each molecule. These values were converted to femtomoles of protein by calibrating the phosphor screen by scintillation counting of TCA-precipitable material (protein) in a duplicate sample. The assumptions made to convert counts per minute to femtomoles (see Materials and Methods) are expected to result in a slight underestimate of total radioactive protein.

Given the requirement for both ATP and a trypsin-sensitive ER membrane component for Vamp1 binding to microsomes (Figure 2), it was not surprising that binding of this molecule to microsomes was saturable (Figure 3). Near the saturation point of the binding curve, only 6% as many Vamp1 molecules bound to 1 equiv of microsomes when compared to Cb5 (see below). The reticulocyte lysate used for these experiments was depleted of endogenous methionine by gel-filtration chromatography prior to use. The unlabeled free methionine in the lysate is negligible (22 μM) and the only other source of methionine in the extracts is the small amount of methionyl-tRNA in the nuclease-treated lysate. For this reason, measurement of the amount of methionine incorporated into the molecules can be used to calculate a rough (under)estimate of the number of binding sites for these molecules on microsomes. Using this approximation and Scatchard analysis of two independent data sets (one of which is shown in Figure 3), we determined that 1 equiv of microsomes contains approximately 20 ± 13 fmol of Vamp1 binding sites. Although the data clearly demonstrate satura-

tion, we were unable to synthesize enough Vamp1 in vitro to extend the Scatchard analysis far enough-and there is too much noise to rule out an additional low-affinity binding site. In the previous study that reported that Vamp2 membrane binding was ATP-dependent, Vamp2 was not examined for saturable binding to microsomes (Kutay et al., 1995). Nevertheless, our results suggest that the mechanism of membrane integration of the two Vamp proteins is similar but unlike that of Cb5 or Bcl-2.

Comparison of Membrane Binding Properties of the Putative Insertion Sequences. In contrast to the other molecules examined (Figure 1), mT did not stably bind to ER microsomes. If the mechanism of membrane integration of mT is similar to that of Cb5, we would expect that when microsomes were the only membranes added to the reaction, that mT would integrate into the microsomes even if they are not an authentic target membrane for mT. However, if the mechanism of mT membrane integration is similar to that of Vamp1, it would integrate only into an authentic target membrane. Alternatively, if mT binding does not lead to integration and is reversible, there may be significant dissociation during the time needed for isolation of the microsomes by centrifugation. For this reason inefficient pelleting cannot be unambiguously interpreted as lack of binding. Nevertheless, if transient binding occurs, it does not lead to detectable membrane integration. Finally, mT molecules synthesized in reticulocyte lysate may fold such that the insertion sequence is not available for membrane binding or integration. To determine if masking of the carboxyl terminal sequence is likely within the context of wild-type mT, the carboxyl terminus of mT was replaced with the corresponding region derived from either Bcl-2 or Cb5. These fusion proteins bound tightly to ER membranes (data not shown), suggesting that the lack of binding between the carboxyl terminus of mT and microsomes is not due to inaccessibility of the sequence in the wild-type molecule.

To examine this possibility further, we constructed gPA fusion proteins containing the putative insertion sequence from mT, Bcl-2, or Cb5 (Figure 1A). The gPA fusion partner was selected for this purpose because it has been shown previously that gPA contains no intrinsic targeting information and that targeting signals placed at the carboxyl terminus are efficiently presented and processed by a variety of membrane systems (Janiak et al., 1994a). As expected, the control molecule gPA (without an insertion sequence) did not bind to microsomes (Figure 4A, lanes 1−3). Addition of the putative insertion sequence from either Bcl-2 or Cb5 to the carboxyl terminus of gPA resulted in efficient membrane binding of the fusion proteins, 73% and 61% pelleted, respectively (Figure 4A, lanes 7−12). Furthermore, membrane binding resulted in integration of these molecules as determined by carbonate extraction and IASD labelling (data not shown). In contrast, the fusion protein with the mT hydrophobic tail bound very poorly to microsomes, (17%; Figure 4A, lanes 4−6). Thus, the insertion sequences of Bcl-2 and Cb5 are sufficient for membrane integration, whereas in the same context the carboxyl terminus of mT binds gPA to microsomes very poorly.

The small amount of gPAmT pelleted with microsomes appears to be bound to the microsomes electrostatically rather than inserted into the lipid bilayer because raising the ionic strength of the buffer to 1 M NaCl at the end of the incubation reduced membrane binding of gPAmT to a

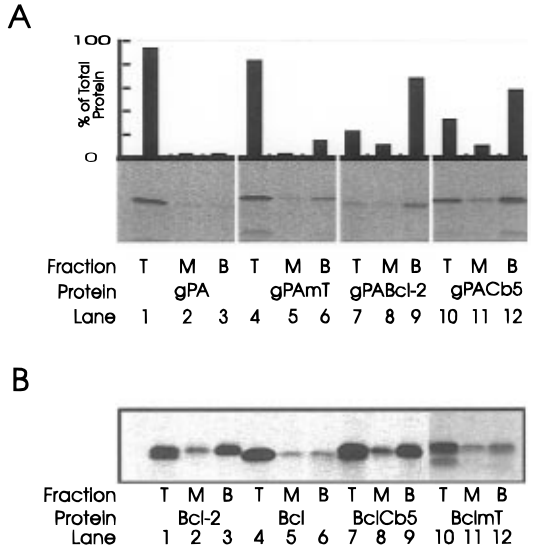

FIGURE 4: The carboxyl-terminal hydrophobic sequence of mT will not bind fusion proteins to microsomes. (A) Membrane binding assays for gPA and the gPA fusion proteins. As a convenient nomenclature for the fusion proteins, the first part of the name represents the fusion partner and the second part of 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.

negligible amount but did not reduce membrane binding of gPABcl-2, gPACb5, Bcl-2, Vamp1, or Cb5 (data not shown). Comparison of the amount of material pelleted (Figure 4A, lanes 3 and 6) suggests that pelleting of gPAmT with membranes depends on the presence of the putative mT insertion sequence. Therefore, it is likely that gPAmT folds such that the carboxyl-terminal sequence from mT is exposed and available for membrane binding. This result suggests that mT membrane integration is not promiscuous and that ER may not be an appropriate target membrane for mT. Nevertheless, it remains possible that gPAmT folds such that part of the carboxyl-terminal sequence from mT is masked and membrane integration is thereby prohibited.

To demonstrate in yet another context that the carboxyl terminus of mT does not mediate membrane binding of a fusion protein, we replaced the insertion sequence on Bcl-2 with that of mT (Figure 4B, lanes 10−12). The cytoplasmic domain of Bcl-2, here termed Bcl, can present a functional insertion sequence, as both Bcl-2 and Bcl-Cb5 bound to membranes efficiently, 44% and 41% of molecules pelleted with membranes, respectively (Figure 4B, lanes 1−3 and 7−9 compared to lanes 4−6). Nevertheless, Bcl-mT bound to membranes only poorly, 19% pelleted (Figure 4B, lanes 10−12). Therefore, unlike Cb5 and Bcl-2, it appears that the mT sequence itself is not compatible with spontaneous membrane insertion. Furthermore, increasing the amount of microsomes in the reactions from 2 to 4 equiv did not result in an appreciable increase in binding of mT, gPAmT, or BclmT to microsomes. Increasing the amount of microsomes in the reactions did increase membrane binding for Vamp1,

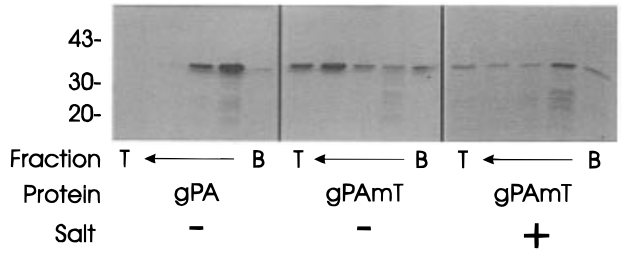

FIGURE 5: Analysis of binding of gPAmT to phospholipid vesicles by flotation in sucrose gradients. Phospholipid vesicles were added to translation reactions and incubated at 24 °C for 15 min. Sucrose was added to a final concentration of 0.86M, the samples (70 μL) were transferred to airfuge tubes and 110 μL of 0.34M sucrose in translation buffer and 40 μL of translation buffer were sequentially 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 the addition of phospholipid vesicles. After centrifugation in an A100−30 rotor for 2 h at 30 psi (170000*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 fusion proteins are identified below the relevant panels. Migration positions of molecular mass markers (in kilodaltons) are indicated at the left of the panels.

Bcl-2, and Cb5 (see below). Taken together, these data suggest that the small amount of gPAmT and BclmT observed to bind to membranes does so via nonspecific electrostatic interactions.

Binding of gPAmT to Phospholipid Vesicles. Our results suggest that the carboxyl terminus of mT differs from the Cb5 insertion sequence in that it binds electrostatically to microsomes rather than integrating into the lipid bilayer. The small amount of electrostatic binding to membranes seen above may result from binding of the insertion sequence to ER proteins or to membrane lipids nonspecifically. Although unlikely, it is also possible that proteins on the ER surface block access of the mT insertion sequence to the lipid bilayer and thereby prevent spontaneous integration. To examine these possibilities, liposomes were added directly to the *in vitro* translation reactions and vesicles and vesicle-bound proteins were recovered from the translation reactions by flotation in a sucrose gradient.

In a buffer of physiologic ionic strength, gPAmT, but not the control molecule gPA, comigrated with vesicles, confirming that the mT tail sequence can mediate lipid binding (Figure 5). However, when these proteins were incubated with lipid vesicles in 1 M KOAc (Figure 5), very little gPAmT bound to the liposomes. The sensitivity to ionic strength of the binding of gPAmT to both liposomes and microsomes strongly suggests that stable binding is due to an electrostatic interaction between the insertion sequence of mT and the lipids. Furthermore, electrostatic binding of gPAmT to lipid vesicles demonstrates that at least some of the residues from the carboxyl terminus of mT are sufficiently exposed to mediate vesicle binding. In these assays only a small amount of gPAmT pellets and is recovered from the bottom of the tube, suggesting that lack of membrane binding is not due to the formation of large gPAmT aggregates. These experiments and the microsome binding experiments described above (Figure 4) suggest that the lack of membrane binding observed for gPAmT is unlikely to result from folding of the protein such that the mT carboxyl-terminal sequence is masked by the gPA domain. Hence,

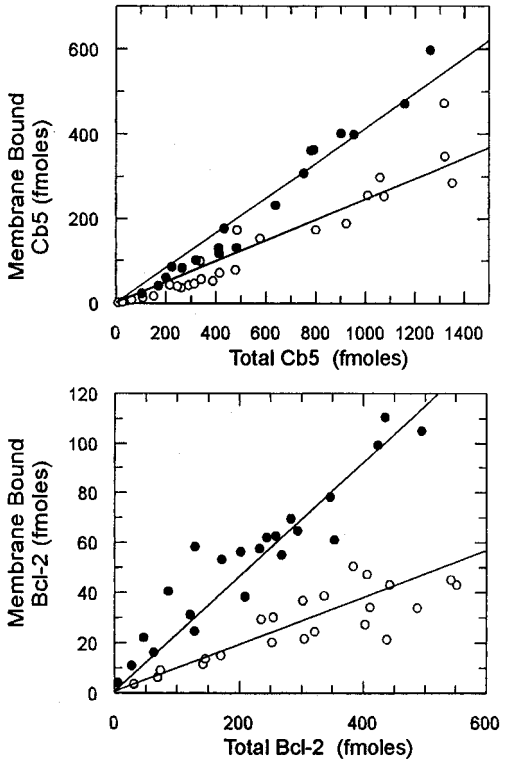

FIGURE 6: Binding of Cb5 and Bcl-2 to microsomes. Translation reactions (60 μL) containing increasing amounts of the individual proteins were incubated with either 1 equiv (○) or 2 equiv (●) of microsomes for 2 h at 24 °C. Membrane binding was assayed as described above (Figure 3).

we conclude that the mechanism of mT membrane integration is different than that of Cb5 or Bcl-2.

Mechanism of Membrane Integration of Bcl-2 Is Similar to, but Distinct from, That of Cb5. Unlike Vamp1 and mT, the membrane binding characteristics revealed here for Bcl-2 (Figures 1, 2, and 4) are very similar to those determined previously for Cb5 (Enoch et al., 1979; Takagaki et al., 1983a,b; Janiak et al., 1994b). Previously it was reported that binding of purified Cb5 to microsomal membranes was not saturable (Strittmatter et al., 1972**)**. To examine saturability of membrane binding for Bcl-2 and Cb5 synthesized in reticulocyte lysate, membrane binding curves were determined as above for Vamp1. Binding of these molecules to microsomes was relatively inefficient with only 23% and 10% of Cb5 and Bcl-2 molecules added to the incubation bound to membranes after 2 h, respectively. It was possible to synthesize only about 1400 and 550 fmoles of Cb5 and Bcl-2 in 100 μL of reticulocyte lysate, respectively. Therefore an analysis of saturation for even moderately abundant binding sites for these molecules is not feasible using reticulocyte lysate. However, binding increased linearly for Cb5 when as little as 40 fmol of Cb5 was added to membranes (9 fmol bound), up to 330 fmol bound/equiv of membranes (Figure 6). Similarly Bcl-2 binding increased linearly from 4 to 55 fmol (Figure 6). Therefore, it is possible that either the number of Bcl-2 binding sites on the microsomes is in excess of 55 fmol/equiv or that, similar to Cb5, the binding of Bcl-2 to microsomes is not saturable. To our surprise, the slope of the binding curves for Cb5 and Bcl-2 doubled when binding was assessed with 2 equiv instead of 1 equiv of microsomes added to the translation reactions (Figure 6). This result suggests that either membrane insertion is a bimolecular event and neither the

membranes nor the Cb5 or Bcl-2 molecules are in sufficient excess to drive the reaction to completion or that an additional equilibrium exists between molecules that are competent (presumed to be monomeric) and not competent (presumed to be oligomeric or aggregated) for membrane binding. If the amount of membranes in the reaction affects the equilibrium between molecules competent and noncompetent for membrane assembly, then the slope of the binding curves would depend on the concentration of microsomes. Because the microsomes are present in what we assume to be a large excess of potential integration sites for these molecules, we favor the latter possibility. However, it is also possible that both processes contribute to the binding curves in Figure 6. Consistent with there being a competing equilibrium in the binding reaction, oligomeric forms of Cb5 were detected in the translation reactions by gel-filtration chromatography (data not shown) and an equilibrium between octamers and monomers has been reported previously for Cb5 molecules in solution (Calabro et al., 1976). Furthermore, gel-filtration chromatography suggests that both monomeric and pentameric forms of Bcl-2 are present in reticulocyte lysate translation reactions (data not shown). Nevertheless, aggregation state is not critical for the studies reported here as both Cb5 and Bcl-2 efficiently bind to microsomes and comparison of the data in Figures 3 and 6 strongly suggests that the mechanism of membrane integration of Bcl-2 is more similar to that of Cb5 than to that of Vamp1. Experiments to address membrane binding of oligomeric and monomeric Cb5 and Bcl-2 molecules are in progress.

Previous work with Cb5 has shown that the carboxyl-terminal insertion sequence integrates into lipid vesicles (Enoch et al., 1979; Takagaki et al., 1983a,b); thus, spontaneous insertion into a lipid bilayer in the absence of other membrane proteins is another characteristic of the prototypic insertion sequence. Therefore, we compared binding of Cb5 and Bcl-2 synthesized in reticulocyte lysate to liposomes by adding the liposomes directly to the *in vitro* translation reactions. As above, vesicles and vesicle-bound proteins were recovered from the translation reactions by floatation in a sucrose gradient.

In a buffer of physiologic ionic strength (Figure 7) or in buffer containing 1 M NaCl (data not shown), Cb5 floated upward with lipid vesicles while Bcl-2 did not bind to the vesicles. Similar results were obtained with liposomes with different lipid compositions (data not shown). Therefore, unlike Cb5, Bcl-2 does not spontaneously insert into liposomes. This result suggests that the posttargeting mechanism of Bcl-2 membrane integration differs from that of Cb5. Moreover, the lack of binding of Bcl-2 to liposomes brings into question the ultimate disposition of the Bcl-2 insertion sequence. Previously it was suggested that Bcl-2 integrates into the bilayer of ER microsomes as it has been shown to be resistant to extraction from membranes with sodium carbonate, pH 11.5 (Janiak et al., 1994b), the standard test for membrane integration. As described above, we have also shown that the cysteine residue in the putative insertion sequence is protected from labelling with IASD when the molecule binds to membranes. Therefore, we think it is unlikely that the carboxyl-terminal insertion sequence of Bcl-2 binds unusually tightly to an abundant ER resident protein (in excess of 55 fmol/equiv) rather than integrating directly into the lipid bilayer.

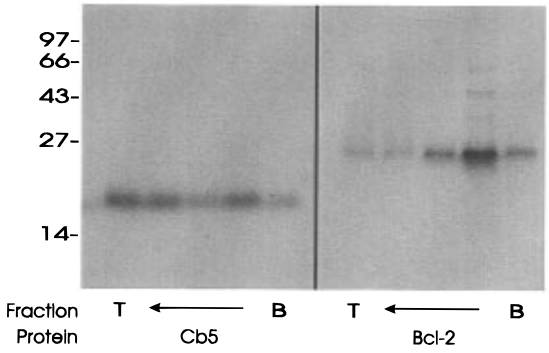

FIGURE 7: Cb5 but not Bcl-2 binds to phospholipid vesicles. Phospholipid vesicles were added to translation reactions and incubated at 24 °C for 15 min. Sucrose was added to a final concentration of 0.86 M, the samples (70 μL) were transferred to airfuge tubes, and 110 μL of 0.34 M sucrose in translation buffer and 40 μL translation buffer were sequentially layered on top. After centrifugation in an A100−30 rotor for 2 h at 30 psi (170000*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.

DISCUSSION

Current models for the membrane integration of proteins with insertion sequences suggest that these proteins spontaneously insert into the target membranes by a mechanism similar to that of Cb5 (Kutay et al., 1993). The driving force for integration is believed to be hydrophobicity. This view is largely based on the observation that insertion sequences are of similar overall hydrophobicity and are invariably located near the carboxyl terminus of the polypeptide. The carboxyl-terminal location of the sequence dictates that membrane binding occurs posttranslationally. However, with the exception of Cb5 there is very little direct experimental data on the mechanism(s) of membrane binding for these molecules. Here we have used microsomes prepared from canine pancreatic ER and phospholipid vesicles to examine targeting and membrane integration for four proteins with putative insertion sequences: Cb5, mT, Vamp1, and Bcl-2. For at least three of these (Cb5, Bcl-2, and Vamp1) there is good evidence that ER is a *bona fide* site for membrane insertion in vivo (Kutay et al., 1995; De Silvestris et al, 1995; Zhu et al., 1997). While there is evidence to suggest that mT also binds to ER membranes, this result is controversial (Ito et al., 1977; Segawa & Ito, 1982; Zhu et al., 1984; Dilworth et al., 1986). Our results suggest that, despite these similarities, the mechanisms for membrane integration of these four proteins are quite different. The archetypal molecule, Cb5, is the only one for which integration into phospholipid vesicles is efficient (Figures 5 and 7). Moreover, Vamp1 and Bcl-2 differ in both the number of binding sites on microsomes (Figures 3 and 6) as well as in the requirement for ATP and a highly trypsin-sensitive membrane component (Figure 2). These results clearly indicate that membrane integration of Vamp1 occurs at unique sites on the ER membrane rather than at a generic insertion sequence binding site (such as a protein that interacts transiently with a variety of insertion sequences to mediate integration or a patch of accessible lipid).

In contrast to these molecules, only electrostatic binding to microsomes was observed for mT (Figure 2). Our results also demonstrate that the carboxyl terminus of mT is sufficient to mediate electrostatic binding to ER microsomes

and phospholipid vesicles (Figures 4 and 5). In addition, we provide two lines of experimental evidence that suggest that the lack of membrane integration for mT and fusion proteins containing the hydrophobic carboxyl terminus of mT is unlikely to result from masking of an otherwise functional insertion sequence by the rest of the molecule. First, when other insertion sequences were used to replace this segment of mT, the resulting fusion proteins (mTBcl-2 and mTCb5) bound to microsomal membranes (data not shown). Thus it seems improbable that (mis)folding of the cytoplasmic domain of mT masks the hydrophobic carboxyl terminus of the protein. Second, when fused to gPA or the cytoplasmic domain of Bcl-2 the mT insertion sequence did not mediate membrane binding of the fusion proteins. Nevertheless, the insertion sequences of Cb5 and Bcl-2, integrated into membranes in the same context, suggesting that lack of integration of gPAmT and BclmT was not due to the rest of the molecule interfering with membrane binding via the mT hydrophobic domain (Figure 4).

The simplest explanation for all of this data is that, unlike Cb5 and Bcl-2, the hydrophobic carboxyl terminus of mT does not directly insert into membranes. The mT sequence is more hydrophobic than that of either Bcl-2 or Cb5 [59 compared to 37 and 31, respectively, using the Kyte−Doolittle hydropathy indices (Kyte & Doolittle, 1982)]. This result is not consistent with the conclusion of Whitley et al. (1996) that hydrophobicity alone governs the membrane integration. In those experiments the membrane binding of Vamp2 was shown to be insensitive to substitution mutations in the hydrophobic portion of the insertion sequence. However, saturation of membrane binding was not examined for Vamp2 or for the mutants. Therefore, it is not clear if the mutants used the same membrane assembly mechanism as the wild-type molecule.

Another difference between the mT sequence and the other insertion sequences is that the mT sequence is flanked with positively charged amino acids (four amino- and three carboxyl-terminal of the hydrophobic core of the putative insertion sequence, Figure 1A). In contrast, Vamp1 contains six positively charged amino acids at the amino terminus, while Cb5 has three charges (two negative and one positive) at the carboxyl terminus of the insertion sequence. It is not clear what role any of these charged residues may have in mediating membrane integration. Nevertheless, it seems likely that either ER is not the appropriate target membrane for mT and that a receptor on the correct target membrane will facilitate insertion of mT directly into a lipid bilayer or that a soluble protein not present in our translation system is required to facilitate targeting and insertion of mT. However, testing these possibilities awaits unambiguous identification and isolation of the site of initial subcellular localization of mT (Zhu et al., 1997).

Unlike mT, Vamp1 bound to microsomal membranes in vitro. Furthermore membrane-bound Vamp1 molecules were not extracted by sodium carbonate, pH 11.5, and membrane binding protected the cysteine in the Vamp1 insertion sequence from labelling with IASD (data not shown), confirming that the insertion sequence is integrated into the lipid bilayer. Vamp1 membrane binding required both ATP and a trypsin-sensitive membrane protein (Figure 2). Relatively few ER proteins are sensitive to degradation with 0.5 μg/mL trypsin at 0 °C (Andrews et al., 1989), yet this amount of trypsin was sufficient to abolish Vamp1 membrane binding, suggesting that the putative Vamp receptor is very sensitive to the protease. Moreover, Vamp1 receptors are moderately abundant as the number of Vamp1 binding sites on ER microsomes was roughly $^1/_5$ that of signal recognition particle receptors on the same membrane (Figure 3). Although the number of binding sites has not been determined for Vamp2, comparison of our results with those previously published for Vamp2 suggests that both Vamps may be integrated into microsomes by a common pathway that is distinct from that used to integrate other proteins with insertion sequences. It is possible that Vamp1 and Vamp2 define what may be a larger family of proteins that share a common receptor on the ER membrane. Studies to address this possibility are underway.

CONCLUSIONS

Together our results suggest that, contrary to current published models, there are at least two different mechanisms responsible for correct subcellular localization of proteins with carboxyl-terminal insertion sequences. At one extreme, exemplified by Cb5 (and to a lesser extent Bcl-2), membrane integration occurs spontaneously and nonsaturably. Therefore, correct subcellular localization of these molecules must be determined primarily by targeting. If Cb5 molecules were to diffuse freely in the cytoplasm, they would undoubtedly integrate into many subcellular membranes. At the other extreme, Vamp molecules could diffuse freely in the cytoplasm because membrane integration is regulated. Therefore, active targeting may improve the efficiency but is predicted not to be essential for correct subcellular localization of Vamp molecules. We suspect that mT targeting falls into this latter category and that ER microsomes are not an authentic target for mT molecules.

Proper intracellular localization as mediated by the insertion sequences is critical to the function of mT, Bcl-2, and Vamp1. Truncation of the mT insertion sequence abolishes both membrane association and the transforming activity of the protein in transfected cells (Carmichael et al., 1982). Furthermore, relocalization of mT to the ER by replacing the insertion sequence with that from Cb5 abolishes the transforming activity of mT (Zhu, Taylor, and Andrews, unpublished results). Similarly, deletion of the Bcl-2 insertion sequence abolishes membrane binding and dramatically reduces antiapoptosis activity (Hockenbery et al., 1990; Zhu et al., 1996). Finally, in its role as a v-snare, appropriate subcellular localization of Vamp1 is believed to be critical for its function in exocytosis [reviewed in Bennett & Scheller (1994)].

The role of specific localization of Cb5 is less obvious. In rat liver, correct targeting of the ER- and mitochondrial specific cytochrome b_5 isoforms is mediated by organelle-specific insertion sequences present on the two molecules (De Silvestris et al., 1995). However, separate ER and mitochondrial isoforms have only been reported in rat liver. Therefore, a certain amount of mistargeting of Cb5 may be tolerated in most cells and the selective pressure for regulation of localization required for the evolution of a more complex mechanism of membrane integration may be absent.

By comparing directly the mechanisms of membrane integration of mT, Vamp1, and Bcl-2 with that of Cb5, we have clearly demonstrated multiple mechanisms for membrane integration of these molecules. However, the similarity

revealed for membrane integration of Vamp1 and Vamp2 also suggests that families of proteins with insertion sequences may follow common pathways. Elucidation of these pathways may lead to new insights into a fundamental cellular process and uncover new possibilities for therapeutic intervention.

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