Identification of the Endoplasmic Reticulum Targeting Signal in Vesicle-associated Membrane Proteins*

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The vesicle-associated membrane proteins (Vamp(s)) function as soluble N-ethylmaleimide-sensitive factor attachment receptor proteins in the intracellular trafficking of vesicles. The membrane attachment of Vamps requires a carboxyl-terminal hydrophobic sequence termed an insertion sequence. Unlike other insertion sequence-containing proteins, targeting of the highly homologous Vamp1 and Vamp2 to the endoplasmic reticulum requires ATP and a membrane-bound receptor. To determine if this mechanism of targeting to the endoplasmic reticulum extends to other Vamps, we compared the membrane binding of Vamp1 and Vamp2 with the distinctly related Vamp8. Similar to the other Vamps, Vamp8 requires both ATP and a membrane component to target to the endoplasmic reticulum. Furthermore, binding curves for the three Vamps overlapped, suggesting a common receptor-mediated process. We identified a minimal endoplasmic reticulum targeting domain that is both necessary and sufficient to confer receptor-mediated, ATP-dependent, binding of a heterologous protein to microsomes. Surprisingly, this conserved sequence includes four positively charged amino acids spaced along an amphipathic sequence, which unlike the carboxyl-terminal targeting sequence in mitochondrial Vamp isoforms, is amino-terminal to the insertion sequence. Because Vamps do not bind to phospholipid vesicles, it is likely that these residues mediate an interaction with a protein, rather than bind to acidic phospholipids. Therefore, we suggest that a bipartite motif is required for the specific targeting and integration of Vamps into the endoplasmic reticulum with receptor-mediated recognition of specifically configured positive residues leading to the insertion of the hydrophobic tail into the membrane.

Despite being discovered more than 30 years ago, the targeting of proteins bound to the cytoplasmic face of membranes by carboxyl-terminal hydrophobic sequences (insertion sequences) remains mysterious (1). Recently, progress has been made in the understanding of the subsequent subcellular trafficking of some of these proteins from the endoplasmic reticulum (ER) to synaptic vesicles and the Golgi. The synaptic vesicle targeting of vesicle-associated membrane proteins (Vamps) is mediated by a region that is distal to the insertion sequence (2). In contrast, the signal for trafficking to the Golgi of Sed5p is related to the length of the hydrophobic segment as sequences that are 4–5 amino acids longer exit the ER (3–5). However, regardless of the final destination of the protein, it is postulated that the hydrophobic sequence provides the thermodynamic driving force for initial integration into the ER membrane. Consistent with this model, many variations are tolerated within the hydrophobic core sequence (6).

The recent cloning of separate isoforms of cytochrome b$_5$ (7) and Vamp1 (8) that are targeted to either ER or mitochondria has led to the identification of the last 9 or 5 amino acids, respectively, as critical for targeting. The results of sequence swapping and mutagenesis experiments demonstrated that at least two positive charges near the carboxyl terminus are essential for insertion into the cytoplasmic face of the outer mitochondrial membrane (7, 8). It remains to be definitively shown whether mitochondrial targeting by these sequences is an active process (i.e., the sequence alterations specify mitochondrial localization) or if the endoplasmic reticulum forms a sequence that prevents mitochondrial localization and that must be altered for more promiscuous targeting.

The original studies on the mechanism of the cytochrome b$_5$ (Cb5) insertion sequence-mediated protein targeting suggested that the integration step was spontaneous, promiscuous, and nonsaturatable (9–13). However, we have demonstrated that there are at least two distinct mechanisms for integration into membranes (14). Vamp1 is the best studied example of a protein for which membrane integration is receptor-mediated (6, 14, 15). Vamp1 is a member of a family of proteins that currently includes eight members (16). Vamps 1 and 2 function as soluble N-ethylmaleimide-sensitive factor attachment protein receptors in the intracellular trafficking of vesicles (14, 15). Both Vamps 1 and 2 require ATP and a membrane-bound receptor to target to the ER (14, 15) 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, we have compared the membrane binding characteristics of distantly related members of the Vamp family (Vamps1 and -2 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. Our results also indicate that there is a common mechanism and probably a single receptor used to target different Vamps to the ER.

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The abbreviations used are: ER, endoplasmic reticulum; Cb5, cytochrome b$_5$; PCR, polymerase chain reaction; Vamp, vesicle-associated membrane protein.
**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Construction of a plasmid expressing Vamp1 (GenBank™ accession no. AAA44232) was described previously (14). Vamp2 (GenBank™ accession no. AAA44232) and Vamp8 (GenBank™ accession no. T63214) cDNA sequences were amplified by polymerase chain reaction (PCR) from rat and human cDNA, respectively, and inserted into phBluescript KS-. For in vitro transcription/translation, the 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 (17). Vamp2 and Vamp8 were subcloned into pSPUTK by PCR. Primer sequences are available from the authors upon request.

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 (18). Gene fusions for gPA and either Vamp2 or Vamp8 were constructed using a unique BamHI site near the 3′-end of the coding region of gPA and a XhoI site 3′ of the gPA termination codon. The required sequences were added to the coding regions of Vamp2 and Vamp8 by PCR amplification. Amino-terminal deletions of the Vamp2 coding sequence in gPAVamp2 were constructed using a whole plasmid PCR method as described (19) 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 gPAPVamp2(96–116), the plasmid encoding gPAPVamp2 was prepared from GM48 cells and cut with BciI (within the Vamp2 coding region) and BamHI (near the carboxyl end of gPA) restriction endonucleases, and the gPA plasmid was recloned by ligation. Plasmids encoding point mutations were generated by cassette mutagenesis using internal HI (near the carboxyl end of gPA) restriction endonucleases, and then the plasmid was reclosed by ligation. Plasmids encoding point mutations were generated by cassette mutagenesis using internal restriction endonucleases, and then the plasmid was reclosed by ligation.

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**RESULTS**

**Common Mechanism of Targeting Vamps to ER Membranes**—Vamp proteins are expressed in all tissue types, and highly conserved in different species (26–28). Among Vamp homologues, Vamps1, -2, and -3 are the most similar (≥71% identical). The other Vamps are 25–33% identical to Vamp1 and to each other (16). In addition, a splice isoform of Vamp1 has been reported that appears to target to mitochondria (29, 30). All transmembrane Vamps contain an insertion sequence that is expected to function as the membrane anchor domain. In addition, both Vamps 1 and 2 require ATP and an ER receptor to bind to ER membranes. Although it is likely that the other family members have similar requirements for membrane targeting it is essential to demonstrate this prior to using sequence comparisons to identify a putative common ER targeting signal for Vamp proteins. Vamp8 was selected as an example of a divergent Vamp because it is only 29% identical to Vamp1 and 33% identical to Vamp2.

Vamp2 and Vamp8 both bind with similar efficiency to microsomes (Fig. 1, lanes 1–3). Although the binding efficiency (20 and 21%, respectively) was much lower than that observed for the control protein cytochrome b5 (67%), the amount of material pelleted was significantly higher than in control reactions without microsomes (lanes 13–15). Specific binding of Vamps never exceeded 25% suggesting that most Vamps do not fold into membrane binding competent conformation in our in vitro system. However, >80% of the membrane-bound material was resistant to extraction with sodium carbonate, pH 11.5, confirming that this material becomes integrated into the bilayer (data not shown). When ATP was depleted from the reaction mix using apyrase or by passing the reaction mixture through a G25 column, binding was abolished for both Vamp2 and Vamp8 (Fig. 1, lanes 4–6 and 7–9). When ATP was added to reactions from which small molecules were depleted by G25 chromatography, membrane binding was restored (Fig. 1, lane 10–12). Other nucleotides were ineffective, confirming the nucleotide specificity (data not shown).

Previously we have shown saturable binding of Vamp1 to microsomes stripped of peripheral proteins by high salt and that this binding can be abolished when membranes are treated with a very low concentration of trypsin (14). Saturable binding was also observed when Vamp1, Vamp2, and Vamp8 binding was examined to nonsalt treated microsomes (Fig. 2). Binding curves for the three proteins are superposable with saturation occurring for 1 equivalent of membranes at 45 fmol.
of protein. Furthermore, none of the proteins bound to trypsinized microsomes (data not shown). Taken together, these results suggest that all three Vamps bind to ER membranes via an interaction with a membrane protein. To formally demonstrate the presence of a common receptor would require competitive binding assays. The amount of protein required for such experiment is not attainable using in vitro translation.

To confirm that unlike membrane binding of Cb5 membrane binding of Vamps requires a membrane protein, Vamp1 was assayed for binding to lipid vesicles with a composition similar to that of the ER membrane (21). In this assay, proteins bound to liposomes float upward in the sucrose gradients (Fig. 3A, fraction 1). Unbound proteins remain in fraction 4, where they were loaded originally, and aggregates pellet to the bottom (fraction 5). Cb5 molecules were recovered from fractions 1 and 2 indicating direct insertion into lipid bilayers. In contrast, neither Vamp1 (Fig. 3A) nor Vamp8 (data not shown) bound to the liposomes, consistent with the presence of a Vamp receptor on the ER.

A Vamp Receptor Is Not Found on Mitochondria or Lysosomes—Vamp1 and -2 are known to cycle between the plasma membrane and internal organelles (30). Therefore, it is possible that Vamp proteins may insert directly into other membranes. However, in vitro, Vamp1 and Vamp8 did not pellet with lysosomes, a membrane system similar to that of endosomes (Fig. 3B). Cb5, a positive control for membrane binding in vitro, bound to lysosomes quite efficiently confirming that the membranes were intact.

Recently, a novel isoform of Vamp1 was identified that appears to target specifically to mitochondria (8). Targeting to mitochondria is mediated by a shortened hydrophobic domain and two positive-charged residues at the carboxyl terminus (8). Similar to lysosomes, authentic Vamp1 and 8 do not bind to mitochondria in vitro (data not shown). It is not known whether the modification at the carboxyl terminus that leads to mitochondrial binding eliminates binding to ER. Therefore, we modified the targeting signal on Vamp2 to match the sequence of the isoform previously shown to bind to mitochondria. This mutant termed Vamp2mito, bound to ER membranes as efficiently as Vamp2 (Fig. 3C) with ATP dependence (data not shown). This suggests that mitochondrial targeting may be a result of relaxed specificity for ER and that residues essential for ER targeting are not found at the extreme carboxyl ter-

![Figure 2](image2.png)

**FIG. 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 h 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 × g) for 10 min at 4 °C in an A100/30 rotor in an Airfuge (Beckman Instruments). After SDS-polyacrylamide gel electrophoresis, 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.

![Figure 3](image3.png)

**FIG. 3.** Vamp does not bind to other membranes. A, phospholipid vesicles were added to translation reactions. After incubation at 24 °C for 1 h 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 of translation buffer were sequentially layered on top of the sample. After centrifugation in a TL-100 (Beckman Instruments) for 2 h at 55,000 rpm (120,000 × g), gradients were fractionated from the top (T) into five fractions (50 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 total protein) for 1 h. Membranes were separated from the reaction by centrifugation over sucrose step gradients. Gradients were divided into three fractions top (T), middle (M), and bottom (B) or a pelleted fraction. Proteins are identified below the panels. C, in vitro translated Vamp2 mutants and fusion proteins were incubated with 1 equivalent of microsomes for 1 h at 24 °C. The microsomes were separated from the reaction by centrifugation over sucrose step gradients as above. gPAVM8, gPA fused to the minimal binding domain of Vamp2. Molecules indicated by mito contain the Vamp mitochondrial targeting sequence. (+) indicates that the six positively charged amino acids amino-terminal of the hydrophobic core of the insertion sequence have been replaced with noncharged hydrophilic amino acids.

16 Amino Acids Preceding the Transmembrane Domain Are Required for Microsomal Binding—The requirement for a receptor on the ER membrane to target Vamps suggests the presence of a specific signal that is common in the amino acid sequence of Vamp proteins but is not shared by proteins with promiscuous insertion. A candidate sequence for targeting Vamps to ER membranes is the helix 1 sequence previously shown to be important for targeting to synaptic vesicles (2). Sequence comparisons of the eight known Vamps revealed

nus of the Vamps.
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Several other highly conserved regions among those with well-defined insertion sequences. Because it is possible that there may also be more than one sequence that contributes to ER-specific binding, we screened a series of Vamp2 deletion mutants fused to gPA to identify those sequences sufficient for binding to microsomes in vitro (Fig. 4A). Fusion proteins containing residues 80–116 of Vamp fused to gPA bound to microsomes as efficiently as Vamp2. The fusion protein gPAVMB includes residues 80–116 of Vamp2 that contain the endoplasmic reticulum minimal binding region. Binding assays for gPA-Vamp2 fusion proteins; averages from three independent experiments. The error (one standard deviation) ranged from 1–1.6% for the different mutants. The resulting fusion protein, gPAVMB (0+), did not bind to membranes above background (Fig. 4A). The resulting fusion protein, gPAVMB (0+) did not bind to membranes above background (Fig. 4A). To determine the importance of the individual lysines in the same face of the helix a series of mutants were constructed containing various combinations of these four residues but not the lysine or arginine from the other side of the helix. No single residue was found to be critical for membrane binding. Instead, it appears that the four residues together form the binding signal. Therefore, membrane binding was averaged for mutants with the same number of positively charged amino acids. When presented in this format (Fig. 6A), the direct relationship between the number of positive charges and membrane binding is obvious. As expected from this result, the corresponding region from Vamp8 fused to gPA were also sufficient to confer membrane binding equivalent to the full-length protein (Fig. 6B, lanes 1 and 2). When the four conserved lysine residues of Vamp8 are replaced by noncharged hydrophilic residues (Fig. 5A), membrane binding was greatly diminished (Fig. 6B, lanes 2 and 3).

Thus the hydrophobic carboxyl-terminal sequence and the 16 amino acids directly amino-terminal of this sequence constitutes a Vamp targeting signal for ER membranes. Because this sequence is present in the Vamp1B isoform that targets to mitochondria, we tested to see if the changes at the extreme carboxyl terminus of the protein would alter the mechanism of targeting, rendering targeting to the ER insensitive to the lysines in the amphipathic helix. The signal that permits mitochondrial binding in Vamps is unable to confer ER binding when added to the gPAVMB (0+), the mutant that lacks the six positively charged residues (Fig. 3C, lanes 4 and 5). Together these experiments confirm that the four lysines on one face of the helix are essential for binding to ER microsomes and suggest that the changes in the carboxyl terminus that permit targeting to mitochondria do not alter the mechanism of binding to ER. They further suggest that the “mitochondrial” isoform of Vamp is likely to be found at both the ER and mitochondria.

To determine if the two conserved tryptophans and asparagine also play a role in binding of Vamps to membranes, these residues were changed to a variety of other amino acids by cassette mutagenesis using doped oligonucleotides. These oligonucleotides were designed to encode 1 of 10 amino acids at
the sites of tryptophan and asparagine. Substitution of the asparagine residue (with Y, T, P, H, or D) significantly reduced membrane binding (Fig. 7). In contrast, with only one exception, membrane binding was insensitive to mutation of either of the tryptophan residues. The only mutant with decreased binding compared with wild type resulted in a marginal decrease ($p = 0.02$), observed when one of the tryptophans was changed to a proline (Fig. 7) likely by disrupting local secondary structure.

**DISCUSSION**

Most Vamp proteins are initially targeted to the ER membrane and then sorted to their ultimate destinations within the secretory pathway. The membrane targeting of distantly related Vamps exhibit identical binding curves (Fig. 2) and require both ATP and a trypsin sensitive membrane component (Fig. 1 and Ref. 14). These data strongly suggest that Vamps are targeted to pancreatic ER microsomes by a single targeting mechanism. Consistent with Vamp proteins containing a unique targeting signal within and around the hydrophobic transmembrane sequence this region is 50% identical between Vamp1 and Vamp8, two molecules with an overall identity of only 29%.

Deletion and point mutations generated in Vamps as well as fusions to gPA were used to identify sequences other than the insertion sequence that are important for binding Vamps to ER. Taken together, our data suggest that the signal for ER targeting includes the 16 residues amino-terminal to the hydrophobic transmembrane domain. This region is predicted to form an amphipathic helix that contains four conserved lysine residues on one face. These lysines were shown to contribute to binding for these Vamps requires a membrane-bound receptor that is not found on liposomes, lysosomes, or mitochondria (Fig. 3, A and B, and data not shown).

Other proteins have been shown to use polar or charged residues to mediate the initial step in membrane insertion. Several myristoylated proteins such as Src and myristoylated alanine-rich protein kinase C substrate require both the hydrophobic acyl chain and a sequence of positively charged residues for membrane binding (31–33). In this case the cluster of basic residues is required for nonspecific interaction with acidic phospholipids, whereas the myristate is inserted into the lipid bilayer. A synaptic vesicle-associated protein, SNAP-25, requires an interhelical Gly-Pro-Xaa-Arg motif for membrane targeting (34). Although this process is protein-mediated, unlike Vamp, the subsequent membrane insertion of SNAP-25 requires the palmitoylation of cysteine residues. Because membrane binding of Vamps requires ATP and a trypsin-sensitive, saturable component of the ER membrane, the positive charged residues are more likely to mediate a protein interaction than bind nonspecifically to acidic phospholipids.

Sequence comparison with other Vamp proteins revealed that the 16-residue predicted amphipathic helix is conserved only in those Vamp proteins with a hydrophobic putative transmembrane sequence. Comparison of the sequences of the 16-residue amphipathic targeting sequences for Vamps 1, 2, and 8 (Fig. 5A) revealed five additional conserved amino acids (K, R, N, and two Ws). Of these only asparagine contributes to binding to microsomes in vitro, suggesting that it plays an important but as yet undefined role in membrane binding.

The evidence for separate motifs that lead to either mitochondrial or ER membrane binding suggests a novel model for the targeting of insertion sequence proteins to the appropriate membrane. If Vamp isoforms with a mitochondrial targeting sequence are targeted exclusively to the mitochondria, our results indicate that there must not be a cytoplasmic receptor present in reticulocyte lysate that prevents binding of these proteins to ER membranes. It is also likely that a protein is needed to insert the mitochondrial-targeted Vamps into the outer mitochondrial membrane. Our results demonstrate that the changes at the carboxyl terminus that lead to mitochondrial targeting do not lead to spontaneous insertion into ER membranes (the four lysines are still required (Fig. 3D)) or into liposomes (data not shown). It remains to be determined if a single protein serves both functions of targeting and insertion for the mitochondrial isofrom. In principle a cytoplasmic receptor is not essential for ER-specific targeting as membrane binding for these Vamps requires a membrane-bound receptor that is not found on liposomes, lysosomes, or mitochondria (Fig. 3, A and B, and data not shown).

Our data suggesting a common receptor-mediated, ATP-de-
FIG. 6. 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 noncharged hydrophilic residues as K83S, K85S, R86T, K87T, K91Q, K94S, and K94S, where the first amino acid indicates the original residue, the number is 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 Fig. 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), (K85S,K91Q), (K88S,K94S), (K87T,K91Q), (K87T,K94S), (K91Q,K94S); 3K, three mutants (K83S,K87T,K91Q), (K85S,K87T,K94S), (K85S,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 four 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 transmembrane domain from Vamp8 (gPAV8MB) in single and the transmembrane sequence. Identification of this sequence will be useful in predicting the membrane binding mechanism used by other proteins with insertion sequences that are targeted to ER membranes. Examples include SS1p, Sec61γ, and Sec61β, proteins that all contain a positively charged region predicted to form an amphipathic helix amino-terminal of an insertion sequence. Our results will also be useful for identifying the molecules that mediate membrane targeting and insertion of these proteins.

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