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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 13, Issue of March 29, pp. 7583–7586, 1996

Printed in U.S.A.

A 12-Residue-long Polyleucine Tail Is Sufficient to Anchor Synaptobrevin to the Endoplasmic Reticulum Membrane*

(Received for publication, December 12, 1995, and in revised form, January 18, 1996)

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Synaptobrevin is a tail-anchored protein with a hydrophobic C-terminal transmembrane segment that inserts into the endoplasmic reticulum membrane independently of the SRP/Sec61p pathway. Here, we show that idealized hydrophobic segments composed of 11–17 leucines and 1 valine function as insertion signals *in vitro*, whereas shorter segments do not. These results suggest that there are no specific requirements beyond overall hydrophobicity for C-terminal endoplasmic reticulum insertion signals.

A class of cytoplasmically exposed membrane proteins with a C-terminal membrane anchor (tail-anchored proteins) has recently attracted attention since it encompasses members of the family of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Other tail-anchored proteins are ER[1]-bound enzymes such as cytochrome b_5, heme oxygenase, and microsomal aldehyde dehydrogenase, and the class also includes certain viral proteins such as the middle T antigen (1). In contrast to the majority of integral membrane proteins, tail-anchored proteins have no N-terminal signal sequence and do not utilize the SRP/Sec61p pathway for targeting and membrane insertion (2, 3).

Synaptobrevin/vesicle-associated membrane protein (VAMP) is a tail-anchored SNARE protein functioning in the fusion of synaptic vesicles to the plasma membrane in neuronal and neuroendocrine cells (4–7). Synaptobrevin first inserts into the ER membrane and is then transported through the secretory pathway to synaptic vesicles (2). ER insertion is post-translational and requires ATP and as yet unidentified protein components of the ER membrane distinct from the components of the SRP/Sec61p pathway (2). Membrane anchoring is provided by a membrane-spanning, C-terminal hydrophobic tail (2, 3), and further transport to synaptic vesicles may be determined by a predicted amphiphilic α-helix in the cytoplasmic domain (8).

While the subcellular sorting of tail-anchored proteins has thus been studied to some extent, the initial membrane insertion event is poorly characterized. Here, we show that targeting and insertion of human synaptobrevin 2 (Syb2) into dog pancreas microsomes *in vitro* can be mediated by a C-terminal tail containing as little as 12 hydrophobic residues, and that the precise amino acid sequence of the hydrophobic anchor is unimportant for this step.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless otherwise stated, all enzymes were from Promega. T7 DNA polymerase was from Pharmacia Biotech Inc. Proteinase K was from Merck. [^{35}S]Met was from Amersham. Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analog m^{7}G(5′)ppp(5′)G were from Pharmacia. Plasmid pGEM1 and reticulocyte lysate were from Promega. Spermidine, phenylmethylsulfonyl fluoride, bovine serum albumin, creatine phosphate, and creatine phosphokinase were from Sigma. The glycosylation acceptor peptide *N*-benzoyl-Asn-Leu-Thr-*N*-methylamide and the non-acceptor peptide *N*-benzoyl-Asn-Leu-(allo)Thr-*N*-methylamide were synthesized according to Ref. 9.

DNA Techniques—Site-specific mutagenesis used to introduce stop codons at various positions of synaptobrevin (positions 99, 105, 111) was performed using the *In Vitro* Mutagenesis Kit from Promega. The resulting mutants were sequenced and recloned into pGEM3βGl behind the T7 promoter.

To make the Syb2-*n*L series of constructs, the *Pst*I-*Bam*HI fragment from Syb2-G13 in pGEM3βGl (2) was cloned into phage M13mp18 (Pharmacia). An *Nde*I restriction site was introduced by site-specific mutagenesis according to the method of Kunkel (10, 11) around amino acid Val^{112} of Syb2-G13, changing the DNA sequence from GTTTACTTC to GCATATGTC (*Nde*I site underlined). This changes the amino acid sequence from Val^{112}-Tyr-Phe to Ala^{112}-Tyr-Val. In order to introduce the polyleucine tails, double-stranded oligonucleotides were cloned between the *Bcl*I site and the new *Nde*I site of Syb2-G13 in pGEM3βGl. Thus, the natural transmembrane segment M^{95}IILGVICAIILIIIIAYV was replaced with M^{95}MIKKKKL$_n$VQQQPYV. All mutants were confirmed by DNA sequencing of M13 or plasmid DNA using T7 DNA polymerase.

In Vitro Transcription and Translation in Reticulocyte Lysate—Synthesis of RNA by T7 RNA polymerase was done according to the manufacturer's (Promega) protocol, and translation in reticulocyte lysate in the presence of dog pancreas microsomes was performed as described (12). Translocation of polypeptides to the lumenal side of the microsomes was assayed by resistance to exogenously added proteinase K and by prevention of *N*-linked glycosylation through competitive inhibition by addition of a glycosylation acceptor tripeptide but not by a non-acceptor tripeptide (13).

Alkaline Extraction of Microsomes—Alkaline flotation assays (Fig. 1) were performed as described (2). For the alkaline extraction experiments (Fig. 2*C*), 10 μl of the *in vitro* translation mixture was added to 100 μl of 100 mM Na_2CO_3 (pH 11.5) and incubated on ice for 20 min. This mixture was layered onto a 50-μl cushion containing 200 mM sucrose, 100 mM Na_2CO_3 (pH 11.5) and centrifuged at 100,000 × *g* for 10 min (Beckman TL 100 centrifuge). The supernatant was carefully removed and placed in an Eppendorf centrifuge tube. The microsomal pellet was resuspended in 50 μl of SDS-PAGE sample buffer and heated to 95 °C for 5 min prior to analysis by SDS-PAGE. The supernatant fraction was acid-precipitated by the addition of trichloroacetic acid to 10% and incubated at 4 °C for 10 min. The precipitate was pelleted by centrifugation in a Microfuge, washed with acetone, and dried. The pellet corresponding to the supernatant fraction from the alkali wash was resuspended in 50 μl of SDS-PAGE sample buffer and heated to 95 °C for 5 min prior to analysis by SDS-PAGE. Gels were scanned on

* This work was supported by grants from the Swedish Natural Sciences Research Council, the Swedish Cancer Foundation, and the Göran Gustafsson Foundation (to G. vH.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; SRP, signal recognition particle.

TABLE I
C-terminal regions of tail-anchored proteins
Hydrophobic segments are underlined. Sequences are identified by their Swiss-Prot (23) or PIR (24) codes.

BC2A_HUMAN	FSWLSLKTLLSLALVGACITLGAYLGHK
BET1_YEAST	RSGISIKTWLIIFFMVGVLFFWVWIT
BOS1_YEAST	KRVFKDKLVFWIALILLIIGIYYVLKWLR
CYB5_RAT	TTVESNSSWWTNWVIPAISALVVALMYRLYMAED
DHA4_RAT	LKQFNKGRLQLLLLVCLVAVAAVIVKDQL
DPM1_YEAST	VFKFGANNLILFITFWSILFFYVCYQLYHLVF
EPMO_HUMAN	QSKARRKKWIIIAVSVVLVVIIVLIIGLSVGK
EPMO_RAT	QSKARRKKWIIAAVAVAVIAVLALIIGLSVGK
HO1_HUMAN	RSQAPLLRWVLTLSFLVATVAVGLYAM
KAR1_YEAST	NIMKKYREYFLWTICILILLYCNIYVYYRF
PE12_YEAST	QKRTSRWRVYLLIVLLVMLLFIFLIMKL
PPLA_PIG	QARQNLQNLFINFCLILICLLLICIIVMLL
PTN1_HUMAN	HALSYWKPFLVNMCVATVLTAGAYLCYRFLFNSNT
PTN2_HUMAN	YWQPILTKMGFMSVILVGAFVGWRLFFQQNAL
S43101(dSED5)	FQSVSKNRWLMIKIFGVLIFFFLFFVVFMS
SC22_YEAST	NFDLLISQYAPIVIVAFFFVFLFWWIFLK
SED5_YEAST	SNRWLAAKVFFIIFVFFVIWVLVN
SNC1_YEAST	WYKDLKMKMCLALVIIILLVVIIVPIAVHFSR
SNC2_YEAST	WWKDLKMRMCLFLVVIILLVVIIVPIVVHFT
SSO1_YEAST	KARKNKIRCWLIVFAIIVVVVVVVVVPAVVKTR
SSO2_YEAST	KARKNKIRCLIICFIIFAIVVVVVVVPSVVETRK
SYB1_HUMAN	KYWWKNCKMMIMLGAICAIIVVVIVIYFFT
SYB2_HUMAN	KYWWKNLKMMIILGVICAIILIIIIVYFSS
SYB_APLCA	KYWWKNCKMMLILGAIIGVIVIIIIVWVVTSQDSGGDDSGSKTPATAGTSPKPVESGVQGGGGRQQR PHSQLVERRNVLRRTEDHIGCRPHIHSFIHIFMICLV
SYB_DROME	KQWWANMKMMIILGVIAVVLLIIVLVSVWPSSSDSGSGGGNKAITQAPPH
SYB_TORCA	KYWWKNCKMMIMLGGIGAIIVIVIIIYFFT
SYNA_BOVIN	QSKARRKKIMIVICCVVLGIVIASTFGGIFG
SYNA_RAT	QSKARRKKIMIIICCVILGIIIASTIGGIFG
SYNB_RAT	QSKARRKKIMIIICCVVLGVVLASSIGGTLGL
TAMI_POVM3	HLRRLGRTLLLVTFLAALLGICLMLFILIKRSRHF
UBC6_YEAST	KEPNDSSSMVYIGIAIFLFLVGLFMK
YSY6_YEAST	TAPVISKTWLGILLFLLVGGGVLQLISYIL

a Fuji BAS1000 phosphoimager and analyzed using the MacBAS (version 2.1) software.

RESULTS

The Hydrophobic Segments in Tail-anchored Proteins Contain Less Leu Than Transmembrane Segments from Proteins Targeted to the SRP/Sec61p Pathway—A collection of tail-anchored proteins is shown in Table I. In general, the hydrophobic, presumably transmembrane, segments are located very near the C terminus and are 15–22 residues long. The average length is slightly shorter than that of membrane-spanning segments from single-spanning (bitopic) proteins targeted to the SRP/Sec61p pathway, which are typically 19–27 residues long (14), and the overall amino acid composition is different with much less leucine (17% *versus* 28%) and alanine (7% *versus* 10%) than found in the latter segments (15).

To ascertain whether these compositional differences relate to different requirements for ER targeting and membrane integration, a number of mutants were made in the Syb2 C-terminal tail.

16 but Not 10 Hydrophobic Residues in the Synaptobrevin Tail Are Sufficient for Anchoring in the ER Membrane—In a preliminary study, deletion mutants were made in the Syb2 C-terminal region and their association with microsomal membranes was analyzed by a flotation assay (Fig. 1). Both Syb2 wild-type and the Syb2-110 deletion mutant were quantitatively recovered with the microsomes, whereas only background levels were found in the membrane fraction for the Syb2-104 and Syb2-98 mutants. A C-terminal hydrophobic stretch of 16 residues is thus sufficient for anchoring, whereas a stretch of only 10 hydrophobic residues is not.

Idealized Synaptobrevin Tails: 12 Hydrophobic Residues Suffice for Transmembrane Integration—In order to determine (i) whether there are any strict sequence requirements on the C-terminal segment beyond overall hydrophobicity for anchoring of Syb2 to the ER membrane and (ii) the minimum length of the hydrophobic segment required for transmembrane integration, the wild-type C-terminal region of Syb2 was replaced by an idealized transmembrane segment composed of contiguous leucines and one valine plus a C-terminal extension (Fig. 1*A*). The number of leucines in the tail was varied from 5 to 17 in steps of 3, and an acceptor site for Asn-linked glycosylation was included near the C terminus in all constructs (*cf.* Ref. 2).

As shown in Fig. 2*A*, the construct with 17 leucines in the tail (Syb2-17L) gave rise to a higher molecular weight form in the presence of microsomes (compare *lanes 1* and *2*). The formation of this species was inhibited by a competing glycosylation acceptor tripeptide (*N*-benzoyl-Asn-Leu-Thr-*N*-methylamide; *lane 3*) but not by a related non-acceptor peptide (*N*-benzoyl-Asn-Leu-(allo)Thr-*N*-methylamide; *lane 4*). Finally, proteinase K digested Syb2-17L both in the absence and presence of detergent (*lanes 7* and *8*), demonstrating that the large N-terminal domain is exposed toward the cytoplasmic side of the microsomes. Also, removal of the C-terminal glycosylation site by an Asn → Gln mutation resulted in a nonglycosylated but membrane-inserted molecule (data not shown). Thus, the shift in molecular weight observed in the presence of microsomes can be attributed to Asn-linked glycosylation of the acceptor site present near the C terminus and demonstrates that Syb2-17L is inserted in the correct transmembrane orientation. We conclude that a C-terminal hydrophobic stretch composed of 17

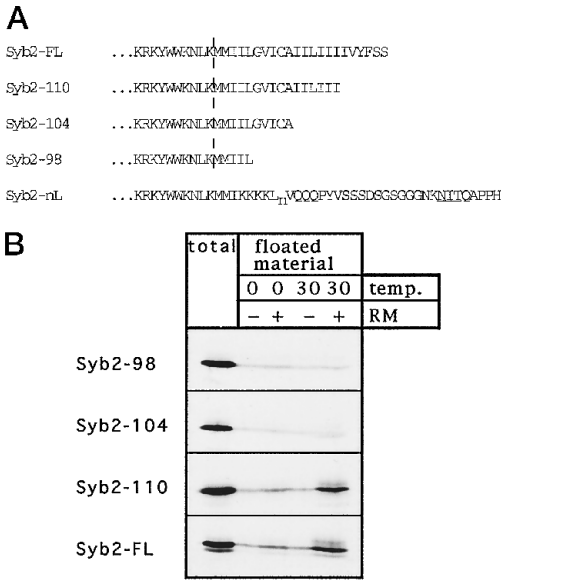

FIG. 1. **Deletion analysis of the Syb2 C-terminal insertion signal.** *A*, amino acid sequences for full-length Syb2 and deletions mutants. The general sequence of the Syb2-*n*L constructs is also shown (*n* denotes the number of leucine residues in the hydrophobic segment; the C-terminal glycosylation acceptor site is *underlined*). *B*, full-length Syb2 and three C-terminal deletion mutants, Syb2-98, -104, -110, were synthesized *in vitro* and post-translationally incubated at 0 °C or 30 °C in the absence or presence of microsomes. To test for membrane insertion, samples were submitted to flotation in an alkaline sucrose gradient. The floated material was analyzed by SDS-PAGE and fluorography.

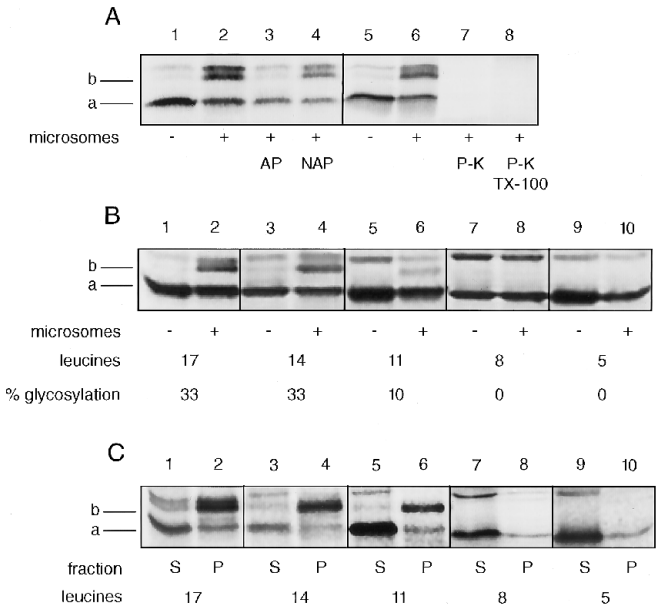

FIG. 2. **Twelve but not nine hydrophobic residues are sufficient to anchor Syb2 in the ER membrane.** *A*, Syb2-17L (which has 17 leucines and 1 valine in the hydrophobic segment) was translated *in vitro* either in the absence (*lanes 1* and *5*) or presence (*lanes 2–4* and *6–8*) of microsomes. Acceptor peptide (*AP*), a competitive inhibitor of *N*-linked glycosylation, was present during the incubation in *lane 3*, and a related, noninhibiting peptide (*NAP*) was present in *lane 4*. After translation, microsomes were digested with proteinase K (*lane 7*) or with proteinase K after solubilization with Triton X-100 (*lane 8*). The weak background bands just above and in the position of *band b* in *lanes 1* and *5* are not related to Syb2 (data not shown). The *upper band* purifies with the supernatant (*C*) and thus represents a cytoplasmic protein made from an mRNA contaminating the reticulocyte lysate. *B*, Syb2-17L (*lanes 1* and *2*), Syb2-14L (*lanes 3* and *4*), Syb2-11L (*lanes 5* and *6*), Syb2-8L (*lanes 7* and *8*), and Syb2-5L (*lanes 9* and *10*) were translated *in vitro* either in the absence (*odd-numbered lanes*) or presence (*even-numbered lanes*) of microsomes. *Band a* is nonglycosylated Syb2, *band b* is glycosylated Syb2. *C*, microsomes incubated with the various constructs were washed in Na_2CO_3 (pH 11.5) and sedimented. *S* = supernatant, *P* = pellet.

leucines and 1 valine is sufficient for targeting to and transmembrane integration of Syb2 in the microsomal membrane.

Similar experiments were carried out for the other constructs with progressively shorter hydrophobic segments. Syb2-17L and Syb2-14L were glycosylated to about 33%, Syb2-11L to about 10%, and Syb2-8L and Syb2-5L not at all (Fig. 2*B*). Membrane anchoring was also assayed by alkaline extraction (16). Only the glycosylated forms of Syb2-17L, Syb2-14L, and Syb2-11L were found in the membrane pellet (Fig. 2*C*). Thus, a 12-residue-long hydrophobic stretch (11 leucines and one valine) is just enough to provide proper targeting and transmembrane anchoring of Syb2.

DISCUSSION

Synaptobrevin belongs to a small group of tail-anchored integral membrane proteins and inserts into the ER membrane independently of the SRP/Sec61p machinery (2). Compared to membrane-spanning hydrophobic segments in proteins that use the SRP/Sec61p machinery for membrane insertion, transmembrane segments in tail-anchored proteins are on the average shorter and have a distinct overall amino acid composition with less leucine and alanine (Table I), suggesting specific sequence constraints that may relate to ER targeting and integration, to sorting events later in the secretory pathway, or to other functional requirements.

We have studied the first step in the synaptobrevin assembly pathway, *i.e.* targeting and insertion into the ER membrane. Our results show that there are no specific sequence requirements for this step: hydrophobic segments composed almost exclusively of leucine residues function as insertion sequences and form transmembrane anchors, provided that they are longer than about 12 residues. Interestingly, this apparent "minimum length" is significantly longer than what has been shown to be required for signal peptide and stop-transfer functions in the SRP/Sec61p pathway, where in both cases only 7–8 consecutive leucines are necessary (17, 18).

Although the atypical amino acid composition of tail-anchored proteins thus cannot be explained by the requirements of ER integration *per se*, it may nevertheless reflect the fact that tail-anchored proteins need to avoid early mis-sorting to other abundant membrane systems such as mitochondria (19, 20). It is also possible that some property of the transmembrane anchor may be important for retention in the proper compartment of the secretory pathway (21, 22), although a signal for targeting to synaptic vesicles has recently been proposed to be located in the cytoplasmic domain of synaptobrevin (8).

Acknowledgments—Dog pancreas microsomes were a kind gift of Dr. Masao Sakaguchi (Fukuoka). Glycosylation acceptor and non-acceptor tripeptides were kindly provided by Dr. Henrik Garoff (Huddinge).

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J. Biol. Chem. 1996, 271:7583-7586.
doi: 10.1074/jbc.271.13.7583