

Membrane Topology of the 12- and the 25-kDa Subunits of the Mammalian Signal Peptidase Complex*

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The cleavage of signal sequences of secretory and membrane proteins by the signal peptidase complex occurs in the lumen of the endoplasmic reticulum. Mammalian signal peptidase consists of five subunits. Four have been cloned, SPC18, SPC21, SPC22/23, and SPC25, of which all but SPC25 have been demonstrated to be single-spanning membrane proteins exposed to the lumen of the endoplasmic reticulum.

We have determined the cDNA sequence of the remaining 12-kDa subunit (SPC12) as well as the membrane topologies of SPC12 and SPC25 in rough microsomes. Both polypeptides span the membrane twice with their N and C termini facing the cytosol and contain only very small, if any, luminal domains. Therefore, SPC12 and SPC25 are likely to be involved in processes other than the enzymatic cleavage of the signal sequence.

Translocation of polypeptide chains across the endoplasmic reticulum (ER)¹ membrane is triggered by signal sequences (Blobel and Dobberstein, 1975a). In the cotranslational mode of translocation the signal sequence is initially recognized in the cytosol by the signal recognition particle (Walter *et al.*, 1981). Subsequently, signal recognition particle interacts with its membrane receptor (Gilmore *et al.*, 1982; Meyer *et al.*, 1982), and the ribosome-bound nascent chain is targeted to the ER where it is transferred into a protein-conducting channel (for review, see Walter and Johnson (1994)). At some point, a second signal sequence recognition event takes place in the membrane (Jungnickel and Rapoport, 1995). How the signal sequence is recognized during the posttranslational mode of translocation is presently unknown. However, it has been speculated that Sec72p (Feldheim and Schekman, 1994) or Sec62p (Johnsson and Varshavsky, 1994) are somehow involved in this process.

During the next step, translocation of the nascent chain through the membrane, the signal sequence of most secretory and membrane proteins is cleaved off. Cleavage occurs by the signal peptidase complex (SPC) as soon as the luminal domain

of the translocating polypeptide is large enough to expose its cleavage site to the enzyme (Blobel and Dobberstein, 1975b). The signal peptidase complex is possibly also involved in proteolytic events in the ER membrane other than the processing of the signal sequence, for example the further digestion of the cleaved signal peptide (Lyko *et al.*, 1995) or the degradation of membrane proteins (Mullins *et al.*, 1995).

Mammalian signal peptidase has been purified from dog pancreas microsomes as a complex of five different polypeptide chains (Evans *et al.*, 1986). The cDNAs of four of the subunits (SPC18, SPC21, SPC22/23, SPC25) have been cloned and sequenced (Shelness *et al.*, 1988; Greenburg *et al.*, 1989; Greenburg and Blobel, 1994). SPC18 and SPC21 are single-spanning membrane proteins; the majority of both proteins is located within the lumen of the ER and contains a second only moderately hydrophobic sequence (Shelness *et al.*, 1993). Both subunits show high homology to each other (Shelness *et al.*, 1988). Moreover, they are related in sequence to leader peptidase (van Dijk *et al.*, 1992), the enzyme responsible for signal sequence cleavage during translocation of proteins across the plasma membrane of bacteria (Zwizinski and Wickner, 1980). These polypeptides may therefore function as catalytic subunits. SPC22/23 has an identical topology to SPC18 and SPC21. It is also a single-spanning membrane protein with a second only somewhat hydrophobic segment located in the lumen of the ER (Shelness *et al.*, 1993). SPC22/23 contains an *N*-glycosylation site, and its migration in SDS-gels as two bands of 22 and 23 kDa, respectively, is likely to arise from differential trimming of its oligosaccharide moiety (Evans *et al.*, 1986). SPC25 has recently been cloned. It again contains two hydrophobic sequences and was proposed to be a single-spanning membrane protein as well, with a large N-terminal domain in the lumen of the ER (Greenburg and Blobel, 1994). However, its membrane topology has not yet been directly determined.

In this paper, we present the sequence of the remaining subunit of the mammalian signal peptidase complex (SPC12) as well as the membrane topologies of SPC12 and SPC25. Surprisingly, both polypeptides are largely exposed to the cytosolic compartment and have very few, if any, amino acid residues in the lumen of the ER. Since this is where the active site of the enzyme must be located, SPC12 and SPC25 are not likely to be directly involved in signal sequence cleavage.

EXPERIMENTAL PROCEDURES

Materials—DNA purification kit was purchased from QIAGEN, proteinase K from Boehringer Mannheim, *Taq* DNA polymerase and DNA sequencing system from Promega, and DNA modifying enzymes from New England Biolabs or Boehringer Mannheim. [γ -³²P]ATP was from Amersham. Keyhole limpet hemocyanine and sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) were used from Pierce.

Antibodies—Antibodies against the N terminus of TRAP β and the C terminus of SPC22/23 have been described (Görlich *et al.*, 1990; Vogel *et al.*, 1990). Anti SPC12 antibodies were made against an N-terminal

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¹ The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PK-RMs, ribosome-stripped microsomes; SPC, signal peptidase complex; sulfo-SMCC, sulfo-succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate.

	1	TGGGCTTAGAAGGCCCGGCTACTGACGCGCAGTGCCAGACCTTACCCCTCACGGTCCTTA
	61	AGTCTCGGTGCGCCTCGCCTCGCAGCCTGCCACCCGCGCTCAGCTGCCCGCCTCCTCAGC

SPC12, dog		M--L--E--H--L--S--S--L--P--T--Q--M--D--Y--K--G--Q--K--
SPC12, human	1	M L E H L S S L P T Q M D Y K G Q K
	121	CAGCCATGCTGGAGCATCTGAGCTCGCTGCCACCGCAGATGGATTACAAGGGCCAGAAGC
SPC12, dog		L--A
SPC12, human	19	L A E Q M F Q <u>G I I L F S A I V G F I Y</u>
	181	TAGCTGAACAGATGTTTCAGGAATTATCTTTTCTGCAATAGTTGGATTATCTACG
SPC12, dog		
SPC12, human	39	<u>G Y V A E Q F G W T V Y I V M A G F A F</u>
	241	GGTACGTGGCTGAACAGTTCCGGGTGGACTGTCTATATAGTTATGGCCGGATTGCTTTTT
SPC12, dog		
SPC12, human	59	<u>S C L L T L P P W P I Y R R H P L K W L</u>
	301	CATGTTTGCTGACACTTCTCCATGGCCCATCTATCGCCGGCATCTCTCAAGTGGTTAC
SPC12, dog		P--V--Q--ASP-S-GlySerGlu-D--K K--P--G--E--R--K
SPC12, human	79	P V Q E S S T D D K K P G E R K I K R H
	361	CTGTTCAAGAATCAAGCACAGACGACAAGAAACAGGGGAAAGAAAATTAAGAGGCATG
SPC12, dog		
SPC12, human	99	A K N N -
	421	CTAAAAATAATGAGGTTTTCATGATTTCAGCACCTGCTTTTGTCTGTGAGATGAGCTA
	481	AATTGCTTTCATACCCAGATAAGAGCTAAAACACCTAATGCTCTTATGGCACAGCTGT
	541	GTATAGATTTAGTTCTCTTTATACCTTCATTTCTAGCCAGTTGGGTTTT

FIG. 1. **Sequence of human SPC12.** The human nucleotide and deduced amino acid sequences are shown. Peptides obtained from purified dog SPC12 are aligned above in one-letter codes; amino acid residues that differ from the human sequence are indicated in three-letter codes. Predicted membrane-spanning segments are *double underlined*. The peptide used to raise antibodies is indicated with *asterisks* above.

peptide (MLEHLSSLPTQMDYKC) and anti-SPC25 antibodies against a C-terminal peptide (CHDSLATERKIK), respectively. The peptides were coupled to keyhole limpet hemocyanine that had been activated with sulfo-SMCC. The immunization of rabbits as well as the affinity purification were performed as described (Görlich *et al.*, 1992).

Purification of SPC12 and Amino Acid Sequencing—Purification of the signal peptidase complex from dog pancreatic microsomes was carried out as described previously (Görlich and Rapoport, 1993). The polypeptides of the purified enzyme complex were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Partial amino acid sequences were determined either from the N terminus of the protein or from internal peptides obtained after its cleavage with endopeptidase Lys-C.

cDNA Cloning and DNA Sequencing—All procedures were done as described earlier (Görlich *et al.*, 1992). A degenerate oligonucleotide (ACNCA(A/G)ATGGA(T/C)TA(T/C)AA(A/G)GG) corresponding to the N-terminal peptide TQMDYKG was used to isolate a polymerase chain reaction fragment with 3'-rapid amplification of cDNA ends reverse transcriptase polymerase chain reaction using fetal human brain mRNA as a template. The obtained cDNA fragment was cloned into a pGEMT vector and used to isolate a corresponding cDNA from a unidirectional cloned human fetal brain library. From the positive clones obtained, one containing the entire coding region was sequenced on both strands. The extreme 5'-end was determined by sequencing a polymerase chain reaction fragment obtained with an internal antisense primer of hssp12 and a 5'-end primer of the cloning vector of the library. Sequence analysis was done using BLAST Program of NCBI E-mail server and the PCGENE program system.

Proteolysis of Ribosome-stripped Microsomes—Microsomes stripped of ribosomes by a treatment with puromycin and high salt (PK-RMs) were prepared as described previously. PK-RMs were diluted to a concentration of 0.2 eq/ μ l (see Walter and Blobel (1983)) in 50 mM Hepes/KOH (pH 7.6), 500 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 100 mM sucrose, and 50 mM dithiothreitol. The microsomes were incubated at different

temperatures for 15 min and then immediately cooled down to 0 °C. Proteinase K was added to a final concentration of 250 μ g/ml, and proteolysis was carried out for 1 h on ice. As indicated, 1% Triton X-100 was added before starting proteolysis. The samples were subsequently precipitated with 20% trichloroacetic acid. Pellets were washed with acetone and analyzed by SDS-PAGE and immunoblotting as described previously (Görlich *et al.*, 1992).

RESULTS

Sequence of SPC12—The signal peptidase complex was purified from canine microsomes as described. The 12-kDa polypeptide was isolated, and partial amino acid sequences were obtained. Degenerate oligonucleotides were designed and used to clone the entire coding region of the gene from a human fetal brain cDNA library. The nucleotide sequence and the derived amino acid sequence, which contains all the directly determined peptide sequences, are shown in Fig. 1. The correct assignment of the N terminus of the protein is supported by N-terminal peptide sequence data and by the fact that the upstream region contains no other start codons. Mismatches between the canine peptides and the human protein are probably due to species differences. The predicted size of the protein (11,810 Da) corresponds very well to that determined by SDS-PAGE. The sequence shows no similarity to any of the other four signal peptidase subunits cloned previously and does not share any obvious homology with other protein sequences in the data base except for several "unidentified" sequence tags from human cDNA libraries and for a sequence tag from rat (accession number H35785). Like the other signal peptidase subunits, SPC12 does not have a cleavable signal sequence.

The polypeptide contains two hydrophobic segments between residues 26–47 and 49–69 (Fig. 1). Sequence analysis with computer programs using different algorithms (SOAP, Klein *et al.* (1985); RAOARGOS, Rao and Argos (1986); HELIXMEM, Eisenberg *et al.* (1984)) predicts that both hydrophobic regions have a high probability of functioning as transmembrane segments. Moreover, according to the charge distribution between the flanking regions of the membrane anchors, it is likely that the N and C termini of the protein face the cytoplasm (Hartmann *et al.*, 1989).

Membrane Topology of SPC12—To determine the membrane topology of SPC12, ribosome-stripped canine pancreatic microsomes were treated with proteinase K, and digestion of the protein was followed by immunoblot analysis. The latter was carried out with affinity-purified antibodies raised against the N terminus of SPC12 (Fig. 1). These antibodies recognized a single protein of the expected size in dog pancreatic microsomes (Fig. 2, lane 1).

If microsomes were treated in the cold with proteinase K, SPC12 migrated in SDS gels with an apparent molecular mass of 11 kDa, in contrast to 12 kDa for the undigested protein, and showed an unchanged reactivity toward the antibody (Fig. 2, lane 2). The same results were observed when trypsin was used instead of proteinase K (data not shown). The N-terminal antigenic peptide of SPC12 does not contain a trypsin cleavage site. For all of these reasons, it is clear that a portion of the C terminus was cut off, and thus this region must be located in the cytosol, accessible to the protease. Surprisingly, if the digestion was carried out after solubilization of the microsomes in Triton X-100, no further degradation was observed (Fig. 2, lane 3). A control protein, TRAP β (originally called SSR β), a

single-spanning membrane protein with only few C-terminal residues located in the cytosol (Hartmann *et al.*, 1993), did not change significantly in size after proteinase K treatment but was completely digested if Triton X-100 was also added (lanes 2 and 3). On the other hand, SPC22/23 behaved similarly to SPC12 in that its accessibility to proteases was the same before and after solubilization of the membranes. In both cases, the mobility of SPC22/23 was unchanged after protease digestion (lanes 2 and 3). The remarkable protease resistance of SPC18, SPC21, and SPC22/23 has been reported before (Shelness *et al.*, 1993). Therefore, it seems that the signal peptidase is a stable complex of polypeptide chains remaining almost resistant to protease treatment even after solubilization of the membranes.

We reasoned whether this unusual behavior could be exploited to determine the localization of the N terminus of SPC12, which is recognized by the antibody. We pretreated microsomes at elevated temperatures with the hope that the tight interaction between the signal peptidase subunits can be broken by heat. The subsequent protease digestion was carried out on ice in the absence or presence of Triton X-100. As shown in Fig. 2 (lanes 6–9), pretreatment at 50 or 60 °C allowed the complete degradation of the N terminus of SPC12, even in the absence of detergent. In contrast, TRAP β and SPC22/23 were only digested in the presence of Triton X-100, demonstrating that these proteins have maintained their membrane orientation with a major luminal domain and that the membrane integrity was not compromised by the heat treatment. These data confirm the membrane topologies of TRAP β and SPC22/23 and show that both the N and C termini of SPC12 are located in the cytoplasmic compartment. Such a model is in good agreement with the predicted membrane topology of SPC12 having two adjacent membrane-spanning segments.

SPC25 Shows the Same Membrane Topology as SPC12—We next asked whether other signal peptidase subunits might have the same membrane topology as SPC12. In this respect, it is interesting that SPC18, SPC21, and SPC22/23 contain a second somewhat hydrophobic domain, although they have all been shown experimentally to be single-spanning membrane proteins (Shelness *et al.*, 1993). SPC25 also has two hydrophobic domains. Based on the observation that the native protein contains at least one disulfide bridge, it was suggested that a major portion of the polypeptide is likely to be in the lumen of the ER. Thus, SPC25 was proposed to span the membrane once (Greenburg and Blobel, 1994). However, in contrast to the other three subunits mentioned above, both hydrophobic segments of SPC25 are predicted to function as membrane anchors (Fig. 3). To examine the membrane topology of SPC25, we treated PK-RMs with proteinase K as described above and followed the degradation of the protein with affinity-purified antibodies raised against a C-terminal peptide (Fig. 3). After protease treatment on ice, SPC25 was still recognized by the antibody with the same intensity but changed in size from 25 to about 22 kDa, suggesting that a part of the N terminus was cut off (Fig. 2, lane 2). If the membranes were solubilized before starting proteolysis, no further digestion of the polypeptide could be detected (Fig. 2, lane 3), again demonstrating that the

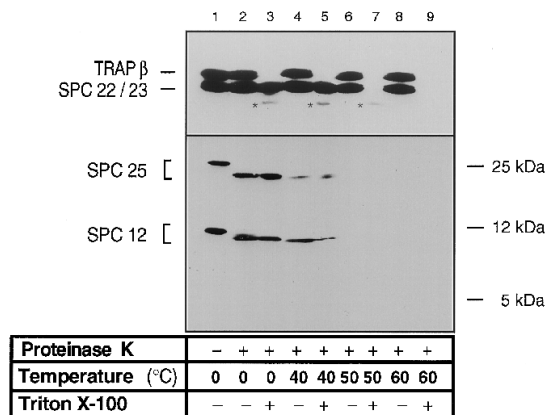


FIG. 2. **Accessibility of SPC12 and SPC25 to proteases.** Ribosome-stripped microsomes were incubated at the indicated temperatures for 15 min and then subsequently cooled down to 0 °C. Proteolysis was carried out with 250 μ g/ml proteinase K for 1 h on ice. As indicated, 1% Triton X-100 was added before starting proteolysis. The samples were precipitated with 20% trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting. The following peptide-specific antibodies were used: TRAP β , N terminus; SPC22/23, C terminus; SPC25, C terminus; SPC12, N terminus. The stars indicate degradation products of TRAP β .

FIG. 3. **Sequence of canine SPC25.** The dog amino acid sequence is shown (Greenburg and Blobel, 1994). Proposed membrane anchors are double underlined. Peptides used to raise antibodies are indicated with asterisks above.

SPC25, dog	MAAASAQGGRTGGGGSSGPGGGPTCGSGSGRSLDKWKIDDKPKIDKWDGS
SPC25, dog	AVKNSLDDSAKKVLEKYKYVENFGLIDGR <u>LTICTISCF</u> FAIVALIWDMHPPFESK
SPC25, dog	<u>PVAL</u> LCVISYFVMMGILTIYTSYKEKSIFLV AHRKDPTGMDPDIDWQLSSSLKRFDD
SPC25, dog	KYTLKLTIFISGRTKQREAEFTKSIAKFFDHSGTLVMDAYEPEISRLHDSLATERKIK

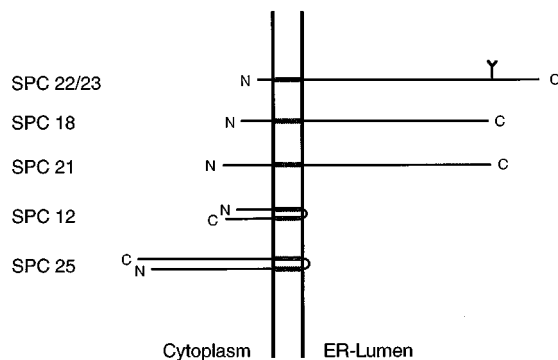


FIG. 4. **Membrane topology of mammalian signal peptidase subunits.** The topologies of all five signal peptidase subunits are schematically illustrated. The gray boxes indicate the membrane-spanning domains of the polypeptides. SPC 22/23 is drawn in the glycosylated form.

signal peptidase complex is almost protease resistant even after solubilization of the membrane. Only by preincubating the membranes at temperatures above 40 °C could the C terminus of SPC25 be degraded, even without solubilization of the microsomes (Fig. 2, lanes 4–9). As in the case of SPC12, these findings strongly suggest that SPC25 spans the membrane twice and that the N terminus as well as the C terminus of the protein are located in the cytosol. Only a very small loop of about 10 amino acids may be exposed to the lumen of the ER (Fig. 3). Thus, SPC12 and SPC25 have the same membrane topology.

DISCUSSION

The mammalian signal peptidase complex consists of five subunits (Evans *et al.*, 1986), and, with the data on SPC12 and SPC25 presented here, the sequences and membrane topologies of all subunits are now known (Fig. 4). The primary structure of SPC12 is not related to any of the other four signal peptidase subunits (SPC18, SPC21, SPC22/23, and SPC25) and shows no homology to any known protein in the data bases. SPC12 contains two hydrophobic segments close to each other. As in the case of SPC25, the hydrophobicity of either segment is substantially higher than that of the second somewhat hydrophobic regions found in SPC18, SPC21, and SPC22/23. This suggested to us that SPC12 and SPC25 must span the membrane twice. To test this, dog pancreatic microsomes were treated with proteinase K, and the degradation of both polypeptides was followed using affinity-purified peptide-specific antibodies. Indeed, SPC12 and SPC25 each contain two adjacent membrane anchors with both their N and C termini oriented toward the cytosolic compartment (Fig. 2). Surprisingly, both subunits expose only very few, if any, amino acids to the lumen of the ER where the catalytic site of the signal peptidase complex must be positioned. Our data clearly contradict the recent assumption that SPC25 is a single-spanning membrane protein with an extended N-terminal segment inside the ER lumen (Greenburg and Blobel, 1994). The reported disulfide bridge in the native protein (Greenburg and Blobel, 1994) could be a linkage between two of the three cysteines present in the membrane anchors as has been found in the leader peptidase of the *Escherichia coli* plasma membrane (Whitley *et al.*, 1993).

Interestingly, SPC12 and SPC25 remained almost protease resistant even when the membranes were solubilized with detergent before adding the protease. The same phenomenon has previously been described for SPC18, SPC21, and SPC22/23 (Shelness *et al.*, 1993). Thus, the five polypeptides of the mammalian signal peptidase seem to form a very tight complex whose core is resistant to protease treatment even after solubilization of the membrane. Our results further indicate that

the compact structure of the signal peptidase complex can be broken by heat treatment. After incubation of the microsomes at 50 or 60 °C, additional regions of SPC12, SPC22/23, and SPC25 became accessible to the added protease. Heat pretreatment of the microsomes did not influence the membrane orientation of the two control proteins, SPC22/23 and TRAP β . These data are in good agreement with results of other groups. Fujimoto *et al.* (1984) had found that an incubation of partially purified SPC at 40 °C slightly decreased its enzyme activity and that further increase of the temperature inactivated the enzyme. Shelness *et al.* (1993) observed that SPC18, SPC20, and SPC22/23 were more accessible to proteases after treating a detergent extract of microsomes at 75 °C.

What might be the functions of the different signal sequence subunits? Because of their homology to the leader peptidase of bacteria, SPC18 and SPC21 may function as catalytic subunits and are believed to represent members of a novel protease family, which have a serine as a key amino acid in the active site (van Dijk *et al.*, 1992; Dalbey and von Heijne, 1992; van Dijk *et al.*, 1995). The membrane topologies of SPC18 and SPC21 fit with this hypothesis as the majority of both polypeptides, including the proposed catalytic center, are exposed to the lumen of the ER (Shelness *et al.*, 1993). Even though SPC22/23 has the same membrane orientation, a direct contribution to signal sequence cleavage seems unlikely as it has no homology to known proteases. However, it is possible that this polypeptide represents a new protease with an unknown substrate specificity. Alternatively, it has been speculated that SPC22/23 may be involved in substrate binding (Shelness *et al.*, 1988).

The membrane topology of SPC12 and SPC25 is quite unexpected as neither protein exposes a substantial domain to the lumen of the ER. Therefore, SPC12 and SPC25 are likely to be involved in processes other than substrate binding or the actual enzymatic reaction. What then could be the functions of SPC12 and SPC25? The extended cytosolic regions of SPC12 and SPC25 may interact with soluble factors or with domains of membrane proteins facing the cytosolic compartment. One could speculate that both polypeptides may be involved in fixing the signal peptidase in a distinct position to the translocation channel or in recruiting the entire enzyme complex to the translocation site when the translating ribosome becomes membrane bound. Moreover, it is also possible that the four membrane-spanning segments of SPC12 and SPC25 are involved in the transfer of the signal sequence from its insertion site to the active center of the signal peptidase.

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