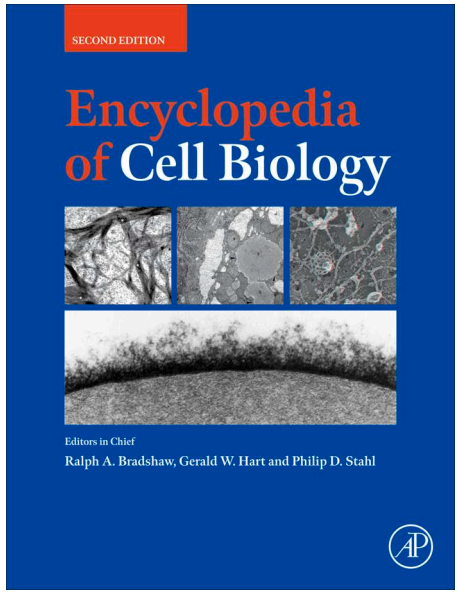

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The Endoplasmic Reticulum Signal Peptidase Complex

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Abstract

Proteins targeted for secretion contain an amino-terminus signal peptide that facilitates their recognition by a host of protein complexes that specialize in catalyzing protein export: chaperones, translocase, and a membrane bound hydrolase (signal peptidase) that cleaves off the signal peptide. In human cells the endoplasmic reticulum signal peptidase complex (hSPC) is a heterotetramer made up of the subunits SPCS1, SPCS2, SPCS3, with a catalytic subunit that can be either SEC11A or SEC11C. The SEC11 subunits utilize a serine nucleophile and a histidine general acid-base. Besides its critical role is cellular protein secretion, SPC is recruited by several RNA viruses to help in the viral protein maturation. There is also evidence to suggest that SPC plays a role in the progression of some cancers. This article summaries the progress on the structural and functional characterization of the eukaryotic signal peptidase complex.

Key Points

This article discusses.

- The chemical and biological background of signal peptides and their role in protein targeting and translocation.
- A brief description of the general protein secretion system.
- The discovery of signal peptidase activity and initial biochemical investigations into the processing of preproteins.
- Work on the canine signal peptidase complex
- Work on the yeast signal peptidase complex
- The cryo-electron microscopy structure of human signal peptidase complex at 4.9 Å resolution
- Work on the signal peptidase complex from other species
- The connection between signal peptidase complex and viral protein processing
- A natural product inhibitor of the signal peptidase complex
- The role for signal peptidase complex in gastric cancer and a potential preference for one of the Sec11 paralogs.

Introduction

Approximately 20% of all human proteins contain a targeting sequence (signal peptide) for secretion across the endoplasmic reticulum (Martoglio and Dobberstein, 1998; Nielsen *et al.*, 1997a). The ability to target and translocate proteins across membranes is essential for the viability of all living cells and this general cellular strategy of signal peptide guided protein section is conserved across all domains of life.

Signal peptides have very little sequence identity, but they do have consistent physical features (von Heijne, 1990; Izard and Kendall, 1994; Zheng and Gierasch, 1996). **Fig. 1** shows a schematic of a typical signal peptide that resides at the amino-terminus of secretory proteins. Signal peptides contain three regions of sequence, the N-region is approximately 1-5 residues in length and has a net positive charge , the hydrophobic H-region is approximately 7–15 residues in length, the C-region is approximately 3–7 residues in length and is thought to have an extended conformation. The C-region contains the specificity sequence A– X – A (alanine - any residue - alanine) immediately preceding the cleavage site (scissile bond). The −1 residue (also called the P1

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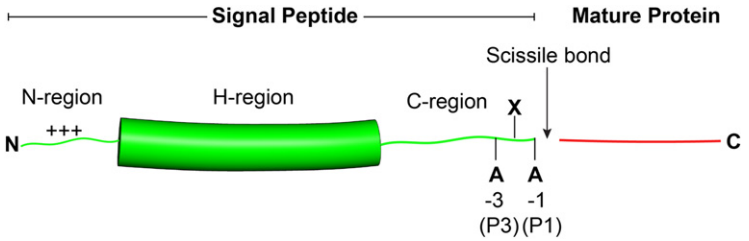


Fig. 1 The general features of a signal peptide. Signal peptides have common physical features: a positively charged region (N-region) followed by a stretch of hydrophobic residues (H-region), followed by a recognition sequence (C-region). The specificity residues are at the P1 (− 1) and P3 (− 3) positions relative to the scissile bond (peptide bond that is cleaved). The mature protein (red) follows the small aliphatic (most commonly alanine) – any residues (X) – small aliphatic (most commonly alanine) specificity cleavage sequence.

residue) is the first residue preceding the cleavage site (the new C-terminus of the released signal peptide – i.e., the N-terminal product). The + 1 residue (also called the P1′ residue) is the first residue after the cleavage site (the new N-terminus of the mature secretory protein – i.e., the C-terminal product). The general physical features of the signal peptide are conserved throughout evolutions except for the average length of the signal peptides. The average eukaryotic signal peptide is approximately 23 residues in length, the average Gram-negative bacterial signal peptide is approximately 25 residues in length, and the average Gram-positive bacterial signal peptide is approximately 32 residues in length (Nielsen *et al.*, 1997b,a). It is believed that the variations in the signal peptide lengths may reflect the differences in the thickness of the lipid bilayers in which the signal peptides reside. The signal peptides used to get across the endoplasmic reticulum have a higher content of leucine residues within the H-region than their bacterial counterparts (Nielsen *et al.*, 1996). Modern genomic sequencing methods (UniProt(Apweiler *et al.*, 2004; UniProt Consortium, 2013)) along with computational signal sequence prediction programs (SignalP(Bendtsen *et al.*, 2004; Petersen *et al.*, 2011; Emanuelsson *et al.*, 2007; Owji *et al.*, 2018; Almagro Armenteros *et al.*, 2019; Nielsen *et al.*, 2019; Teufel *et al.*, 2022)) have provided lists of potential SPC substrates for a large number of species whose genome has been sequenced. The number of experimentally verified secretory preproteins and therefore signal peptides is growing due to developments in tandem mass spectrometry proteomic techniques.

The Eukaryotic General Protein Secretion System

To catalyze the targeting and translocation of secretory preproteins across membranes, cells utilize a conserved molecular machine collectively known as the general secretion (Sec) system. The molecular machinery involved in eukaryotic protein targeting and translocation includes numerous gene products that reside on the cytoplasmic side and on the luminal side of the ER, as well as within the ER membrane. The complete assembly of these molecules is referred to as the "holo-translocase". The majority of the eukaryotic soluble secretory proteins are translocated co-translationally, i.e., as the protein is being synthesized on the ribosome. Conversely, most prokaryotic soluble secretory proteins are translocated post-translationally (after the protein has been fully synthesized and released from the ribosome). I will briefly describe the basic components of the eukaryotic general Sec-system and the steps in the targeting and translocation process. There are a number of reviews on the general secretions system (Liaci and Forster, 2021; Nyathi *et al.*, 2013; Voorhees and Hegde, 2016).

The N-terminal hydrophobic signal peptide of a nascent secretory preprotein, as it is being synthesized on the ribosome, is recognized by the signal recognition particle (SRP, which is made up of six different proteins and one RNA molecule). This interaction halts translation and guides the ribosome nascent chain complex (RNC) to the signal recognition particle receptor (SR, a heterodimeric membrane protein complex) on the ER membrane. The RNC is then transferred to the translocase (Sec61, a heterotrimeric membrane protein complex) where translation is allowed to continue. It is thought that translation (GTP hydrolysis) serves as the energy source for preprotein translocation through the Sec61 channel. The ER is the main intracellular calcium storage organelle; therefore, it is important that the Sec61 complex be tightly regulated in its opening and closing events. A HSP70-like (heat shock protein) chaperone called BiP, that resides on the luminal side of the ER, has been shown to play an essential role in this Sec61 regulation as well as contributing to the unidirectionality and efficiency of protein transport. After translocation, a pre-protein would be tethered (stuck) to the Sec61 complex and ER membrane via its hydrophobic signal peptide, if it were not for the essential membrane bound enzyme signal peptidase. The ER Signal Peptidase Complex (SPC) sometimes referred to as the microsomal signal peptidase (a hetero-multimeric protein complex) releases the mature secretory protein from the membrane by cleaving off the signal peptide.

In vitro investigations using peptidyl-tRNA suggests that SPC is able to interact with the nascent polypeptide during translocation and therefore SPC is in close proximity to the Sec61 translocase (Wollenberg and Simon, 2004). Besides the ribosome and the Sec61 translocase complex there are other important large membrane bound complexes such as the oligosaccharyltransferase complex (OST) that likely resides near SPC. The OST is the central enzyme in the N-linked glycosylation pathway that occurs at the ER membrane surface. OST catalyzes the transfer of the 14-sugar oligosaccharide glucose(x3)-mannose(x9)-N-acetylglucosamine (x2) to the sequence N-X-S or N-X-T (Wild *et al.*, 2018). SPC must cleave off the signal peptide before oligosaccharides can be

added (Chen *et al.*, 2001). The chaperone calnexin is also located nearby given its role in assisting the assembly of N-linked glycoproteins in the ER (Schrag *et al.*, 2001).

Signal Peptidase

The first evidence for the existence of a signal peptidase enzyme arrived with the discovery of the signal peptide in the early 1970s. The idea that proteins contain targeting postal codes originated in 1971, when Günter Blobel and David Sabatini postulated that the information needed to direct a nascent polypeptide to the ER is contained within the polypeptide itself (Blobel and Sabatini, 1971). A year later, César Milstein and colleagues provided experimental evidence for a transient signal sequence at the N-terminal end of a secretory protein (Milstein *et al.*, 1972). Milstein *et al.* using an in vitro translation system discovered IgG light chain from myeloma cells was synthesized as a higher molecular mass form and was converted to the mature form (lower molecular mass) with the addition of ER vesicles (microsomes). The bacterial enzyme responsible for cleaving off signal peptides was first detected in *Escherichia coli* in 1978 by Chang *et al.* (1978) and first purified from *E. coli* by Zwizinski and Wickner (1980). The bacterial monomeric enzyme that was purified has become known as type 1 signal peptidase (SPase I). It was shown that this enzyme is not inhibited by any of the standard protease inhibitors, and thus cannot be classified in any of the classical families of serine, aspartic, cysteine, or metallo proteases (Kuo *et al.*, 1993; Zwizinski *et al.*, 1981) based on its inhibition profile. Site-specific mutagenesis and chemical modification studies are consistent with bacterial SPase I utilizing a novel protease mechanism involving a conserved serine/lysine dyad at its catalytic site (Paetzel and Dalbey, 1997; Sung and Dalbey, 1992; Tschantz *et al.*, 1993; Paetzel *et al.*, 1997). The three-dimensional structure of *E. coli* SPase I provided direct evidence for the serine/lysine catalytic dyad and indicating a novel nucleophilic *si*-face attack on the substrate scissile carbonyl carbon and also explained the substrate preference for alanine and other small side chains at P1 and P3 residues (Paetzel, 1998). The structure of this enzyme has also been solved with a number of novel inhibitors bound (Luo *et al.*, 2009; Paetzel *et al.*, 2004; Liu *et al.*, 2011) and with a free active site (Paetzel *et al.*, 2002a). For a review of bacterial SPase see Paetzel *et al.* (2000). For a full review on all SPases see Paetzel *et al.* (2002b). For a detailed review on the *E. coli* SPase I see Paetzel (2014).

Initial studies on the SPC were performed using canine pancreas ER (cSPC) and the *Saccharomyces cerevisiae* ER (ySPC). These enzymes were purified and characterized leading to evidence for their oligomeric state, membrane topology, mechanism, and function.

The Canine Signal Peptidase Complex (cSPC)

The first characterization of a signal peptidase complex was that from canine pancreas cells (cSPC). A post-translational cleavage assay using radiolabeled presecretory proteins preprolactin and pregrowth hormone were used to monitor purification and characterize the activity of cSPC. It was shown that cSPC activity could be detergent solubilized from the canine pancreas rough endoplasmic reticulum but not from the smooth ER (Jackson and Blobel, 1977). Later it was shown that phospholipids are required for cSPC activity and that phosphatidylcholine was the most effective in restoring activity to delipidated cSPC (Jackson and White, 1981). Canine SPC was purified to near homogeneity using several chromatographic steps and sucrose gradient centrifugation and suspended in the detergent Nikkol (octaethylene glycol mono n-dodecyl ether) and a high salt buffer (Evans *et al.*, 1986). SDS-PAGE analysis of the purified stable cSPC revealed six bands with apparent molecular masses of 12, 18, 21, 22, 23, and 25 kDa. The 22 kDa and 23 kDa proteins were found to be glycosylated which allowed the complex to be bound to a lectin affinity column and elutes by α-D-mannoside. Later is was discovered that the 22 and 23 kDa proteins were different glycosylated forms of the same protein (SPC22/23 or SPCS3) (Shelness *et al.*, 1988). Sequencing of the cDNA encoding the 21-kDa subunit (SPC21 or Sec11C) of cSPC revealed that the corresponding gene product is 47% identical to the yeast SEC11 protein (Greenburg *et al.*, 1989), that was shown to be an essential protein that is required for signal peptide processing in *Saccharomyces cerevisiae* (Bohni *et al.*, 1988). Later the SPC 18-kDa subunit (SPC18 or Sec11A) was cloned and sequenced and was also shown to be a homolog of yeast SEC11 (Shelness and Blobel, 1990). The cloning and sequencing of the 25 kDa component of cSPC (SPC25 or SPCS2) was reported in 1994 (Greenburg and Blobel, 1994). The 12 kDa subunit (SPC12 or SPCS1) was cloned and sequenced and the membrane topology of SPC12 and SPC25 in rough ER were determined. Both proteins were shown experimentally, via proteolytic accessibility, to have two transmembrane segments with their amino- and carboxy-termini facing the cytosol (Kalies and Hartmann, 1996). SPC18, SPC21 and SPC22/23 were each shown previously to contain a single transmembrane segment (Shelness *et al.*, 1993).

The Yeast Signal Peptidase Complex (ySPC)

A collection of secretion defective mutants in the yeast *Saccharomyces cerevisiae* were used to discover cells called SEC11 that were defective in signal peptide cleavage (Bohni *et al.*, 1988). A DNA fragment that complemented the defect was discovered and the SEC11 gene was cloned and a null mutant at the SEC11 locus showed that the gene is essential. Sequencing of the gene predicts a 167-residue long protein with a molecular mass of 18.8 kDa and a calculated isoelectric point of 9.8. The optimized conditions for the solubilization of yeast signal peptidase complex (ySPC) from *S. cerevisiae* rough microsomes were significantly different from what was used for cSPC. A post-translational SPC activity assay using prepro-α-factor as the substrate was developed (YaDeau and Blobel, 1989). SDS-PAGE analysis was consistent with purified and stable ySPC included four polypeptides with apparent molecular

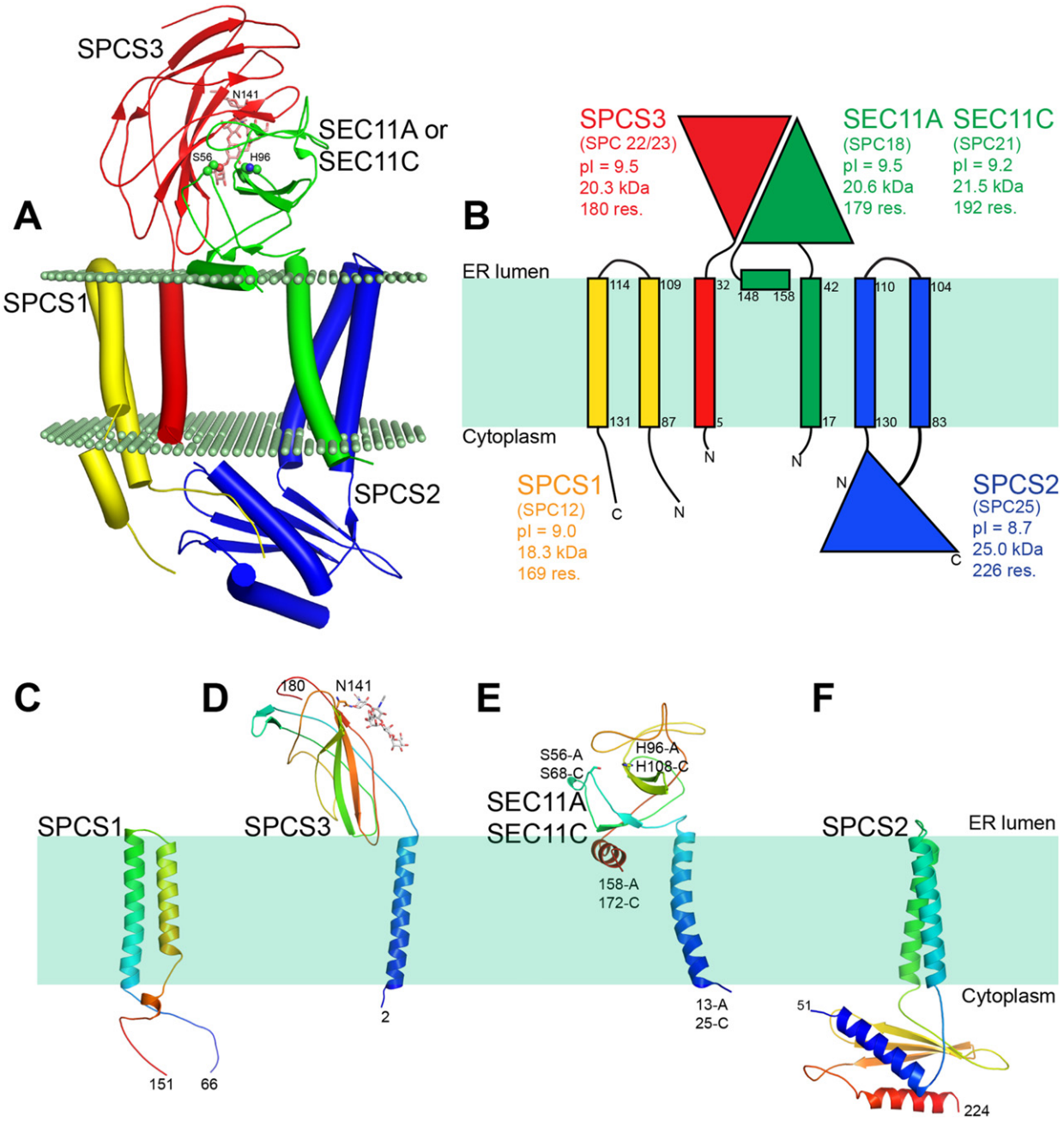
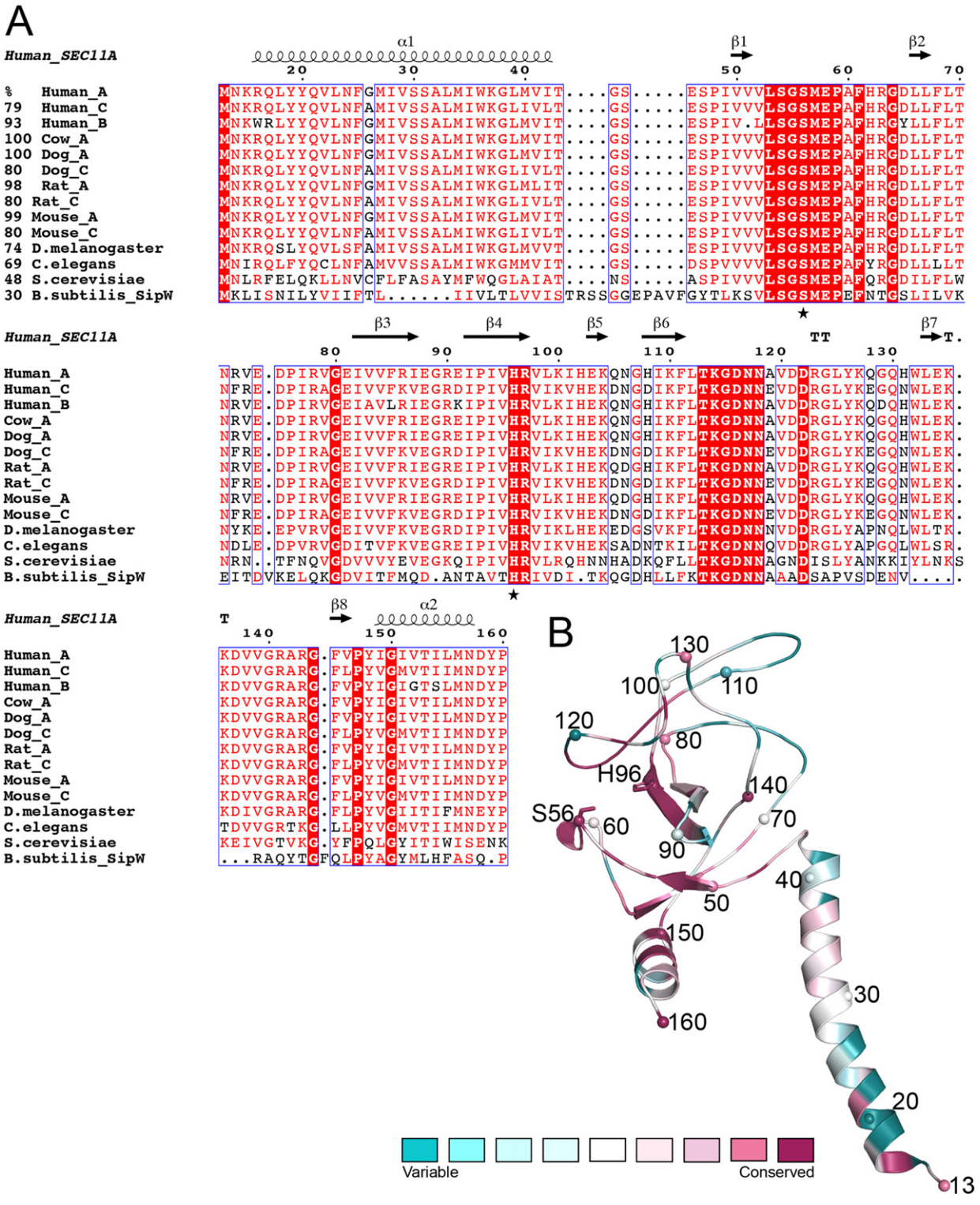


Fig. 2 The membrane topology, oligomeric nature, and protein architecture of hSPC. **A**. The path of the mainchain for hSPC-A (hSPC with SEC11A, PDB: 7p2p) is shown in cartoon. The transmembrane segments have been analyzed by the web server Positioning of Proteins in Membranes PPM 3.0. Layers of spheres represents the dimensions of the ER lipid bilayer thickness. The catalytic subunit SEC11A is in green. The nucleophilic Ser56 and general base His96 are shown as spheres. The glycoprotein SPCS3 is shown in red. The subunit SPCS2 is shown in blue and the subunit SPCS1 yellow. **B**. A schematic of the hSPC transmembrane segment topology. The light green rectangle depicts the ER membrane. The numbers correspond to the beginning and end of each transmembrane segment as determined by PPM 3.0. The triangles represent the globular domains in SPCS3, SEC11A and SPCS2. The vertical rectangles represent the alpha-helical transmembrane segments. SEC11 contains an amphipathic (monotopic) helix at its C-terminus that lays parallel to the lipid bilayer surface. The theoretical isoelectric point (pI), molecular mass and residue length for each hSPC subunit is listed, values we obtained from the UniProt database. The Uniprot accession numbers for the hSPC subunits are as follows: Sec11A P67812, Sec11C Q9BY50, SPCS1 Q9Y6A9, SPCS2 Q15005, SPCS3 P61009. **C**. hSPC subunit SPCS1 drawn in cartoon and colored spectrally from amino-terminus (blue) to carboxy-terminus (red). The first and last residues in the chain are labeled (PDB: 7p2p). **D**. hSPC subunit SPCS3 drawn in cartoon and colored spectrally from amino-terminus (blue) to carboxy-terminus (red). The first and last residues in the chain are labeled (PDB: 7p2p). The glycosylation at Asn141 is shown in stick and labeled. **E**. hSPC subunit SEC11A drawn in cartoon and colored spectrally from amino-terminus (blue) to carboxy-terminus (red). The first and last residues in the chain for Sec11A and Sec11C are labeled (PDB: 7p2p). The catalytic residues are shown as stick and labeled. **F**. hSPC subunit SPCS2 drawn in cartoon and colored spectrally from amino-terminus (blue) to carboxy-terminus (red). The first and last residue in the chain is labeled (PDB: 7p2p). Panels A, C-F were prepared with the program PyMol. Database and program references: Apweiler, R., Bairoch, A., Wu, C.H., *et al.*, 2004. UniProt: The universal protein knowledgebase. Nucleic Acids Res. 32, D115–D119. UniProt Consortium, 2013. Update on activities at the Universal Protein Resource (UniProt) in 2013. Nucleic Acids Res. D43–D47. Lomize, M.A., Pogozheva, I.D., Joo, H., Mosberg, H.I., Lomize, A.L., 2012. OPM database and PPM web server: Resources for positioning of proteins in membranes. Nucleic Acids Res. D370–D376. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol.

masses of 13, 18, 20, and 25 kDa. The 18 kDa protein was SEC11 and the 25 kDa was glycosylated (YaDeau *et al.*, 1991). It was demonstrated that SPC, specifically SEC11, is capable of digesting abnormal transmembrane proteins in *S. cerevisiae*, suggesting that SPC may have other functions in the ER membrane other than signal peptide cleavage (Mullins *et al.*, 1995). It was shown that the ySPC subunits SPC1 (SPC12 homolog) (Fang *et al.*, 1996) and SPC2 (SPC25 homolog) (Mullins *et al.*, 1996) are both non-essential, but are functionally distinct from each other despite having a similar predicted membrane topology. It was then discovered that the



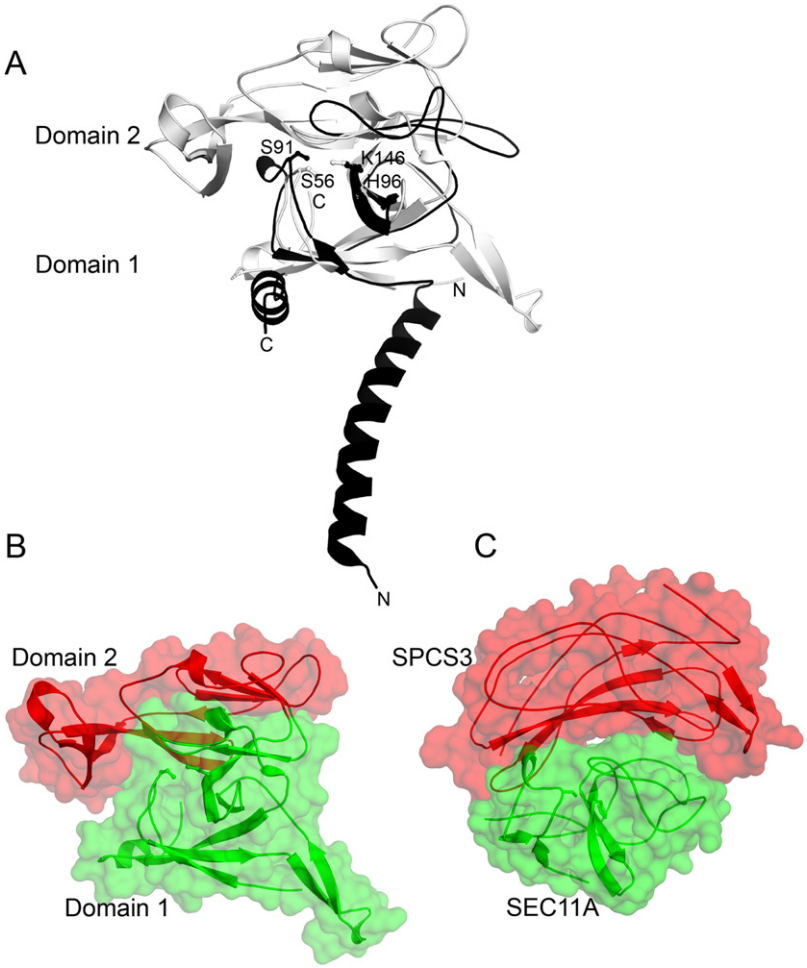
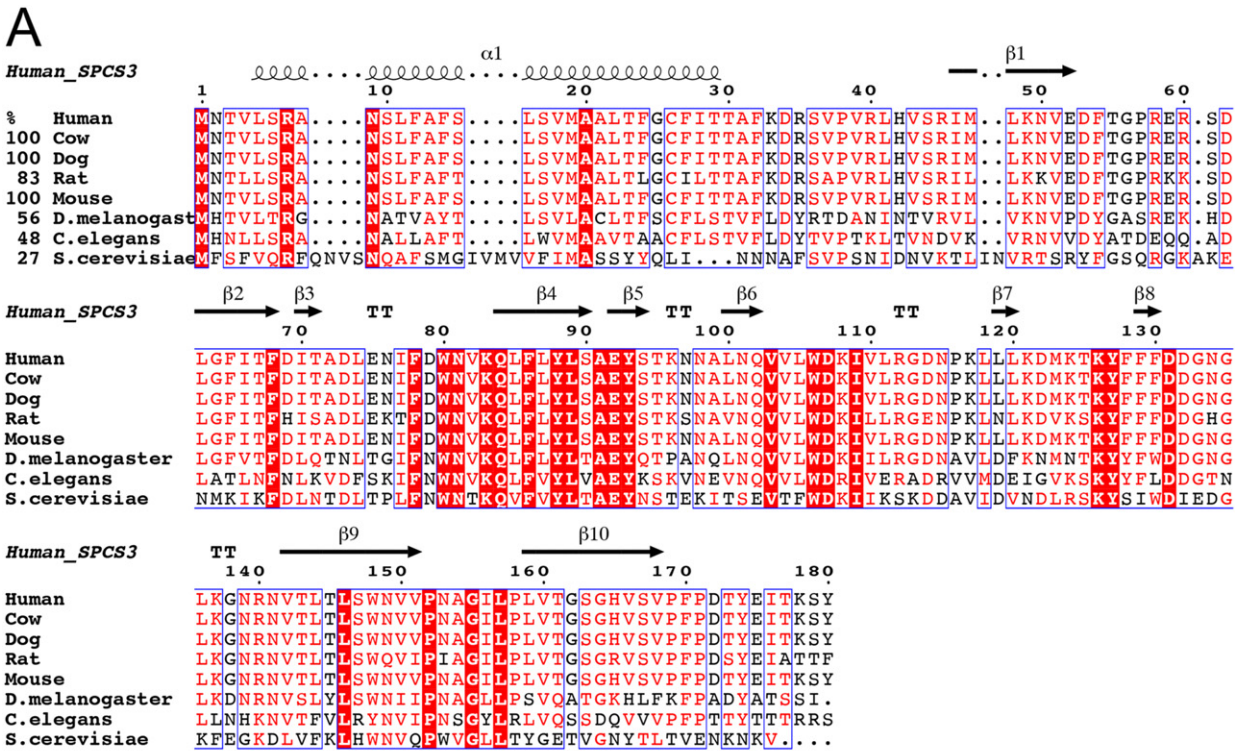
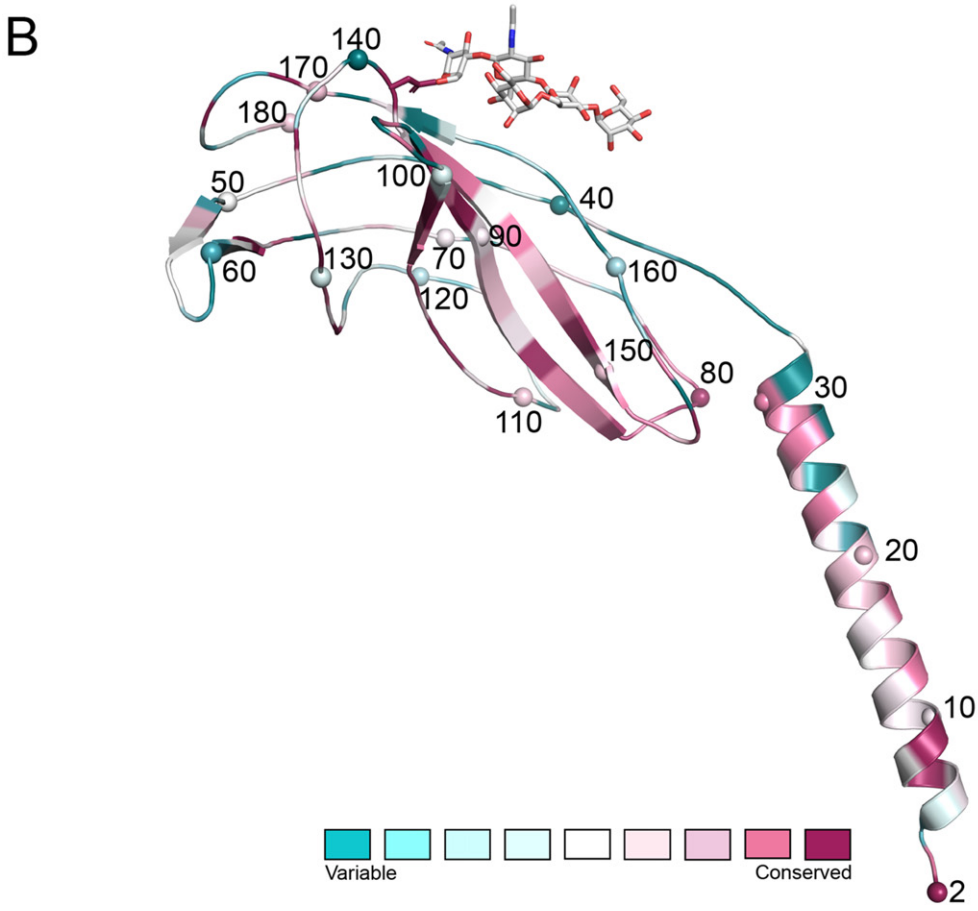


Fig. 4 The signal peptidase protein fold. **A**. a structural alignment of SEC11A (black, (pdb: 7p2p) and *E. coli* signal peptidase soluble domain (gray, pdb:1b12) – lacking the two amino-terminal transmembrane domains. The catalytic residues, termini and domain regions of E. *coli* signal peptidase are labeled. **B**. The catalytic domain 1 regions of *E. coli* signal peptidase is shown in green semitransparent surface with ribbon path for the mainchain shown behind the surface. Domain 2 is shown in red semitransparent surface (pdb:1b12). **C**. The interface between SEC11A (green) and SPCS3 (red). Only the globular domains are shown (pdb: 7p2p). The program PyMol was used to prepare this figure. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol.

Fig. 3 Sequence and structural conservation for Sec11. **A**. A sequence alignment that includes residues that are present in the cryo-electron microscopy structure (PDB: 7p2p). Many of the species have paralogs Sec11A and Sec11C, human has Sec11A, Sec11C and Sec11B. Identical residues are highlighted in red with white letters. Conserved residues have red letters. The nucleophilic serine and the general acid/base are marked with a black star. The secondary structure for human Sec11A (SC11A) is shown above the sequence. The percent identity to human Sec11A is shown to the left of the top row. The sequence numbers are those for human Sec11A. The UniProt accession numbers for the sequences are as follows: Human Sec11A P67812, Human Sec11C Q9BY50, Human Sec11B P0C7V7, Cow Sec11A P67810, Dog Sec11A P67811, Dog Sec11C P13679, Rat Sec11A P42667, Rat Sec11C Q9WTR7, Mouse Sec11A Q9R0P6, Mouse Sec11C Q9D8V7, *Drosophila melanogaster* (fruit fly) Sec11 O97066, *Caenorhabditis elegans* (nematode or roundworm) Sec11 Q9N3D0, *Saccharomyces cerevisiae* (yeast) Sec11 P15367, *Bacillus subtilis* (Gram-positive bacteria) sipW P54506 **B**. The mainchain path for human Sec11A using the coordinates from the cryo-electron microscopy structure (PDB: 7p2p). A sphere marks the location of the alpha-carbon for every tenth residue. The serine nucleophile (Ser56) and the histidine general acid/base (His96) are show in stick and labeled. The level of conservation is mapped onto the structure using the programs Consurf and PyMol. Sequence alignments were performed with Clustal-Omega and ESPript 3.0. Program references: Ashkenazy, H., Abadi, S., Martz, E., *et al.*, 2016. ConSurf 2016: An improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res, 44(W1), W344–50. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol. Sievers, F., Higgins, D. G. 2018. Clustal Omega for making accurate alignments of many protein sequences. Protein Sci, 27(1), 135–145. Gouet, P., Robert, X., Courcelle, E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res, 31 (13), 3320–3323.





gene SPC3, the homolog of the mammalian signal peptidase complex subunit SPC22/23, is essential for cell growth and SPC activity (Fang *et al.*, 1997; Meyer and Hartmann, 1997). Site-directed mutagenesis was performed on SEC11 and SPC3 to probe the conserved serine, histidine, lysine and aspartic acid residues for effect on SPC activity (VanValkenburgh *et al.*, 1999). It was discovered that Ser44, His83, Asp103 and Asp109 were essential, but no essential lysine was observed. Interestingly, Ser44 and His83 align well with the known catalytic serine nucleophile and lysine general base of bacterial signal peptidase. Therefore, it appears that the ER and bacterial signal peptidase have a conserved function with a similar substrate (cleaving of signal peptides from pre-proteins) with a conserved nucleophile but use a different general base residue to perform their catalysis. Interestingly ER-like signal peptidases that have a proposed histidine general base rather that a lysine have been observed in prokaryotes such as SipW of *Bacillus subtilius* (Tjalsma *et al.*, 1998, 2000). This histidine has been shown to be essential for activity but it can be replaced with a lysine (Tjalsma *et al.*, 2000). The same catalytic histidine in yeast Sec11 cannot be replaced with a lysine (VanValkenburgh *et al.*, 1999). Archaeal signal peptidases also appear to utilize a histidine general base (Ng *et al.*, 2007).

The Human Signal Peptidase Complex (hSPC)

Human SPC is made up of five different proteins. The proteins are called: SPCS1 (11.2 kDa), SPCS2 (25.0 kDa), SPCS3 (20.3 kDa), SC11A (20.6 kDa) and SC11C (21.5 kDa). The calculated physical features (length, calculated molecular mass, and theoretical isoelectric point) of the protein subunits along with their membrane topologies are shown in **Fig. 2**.

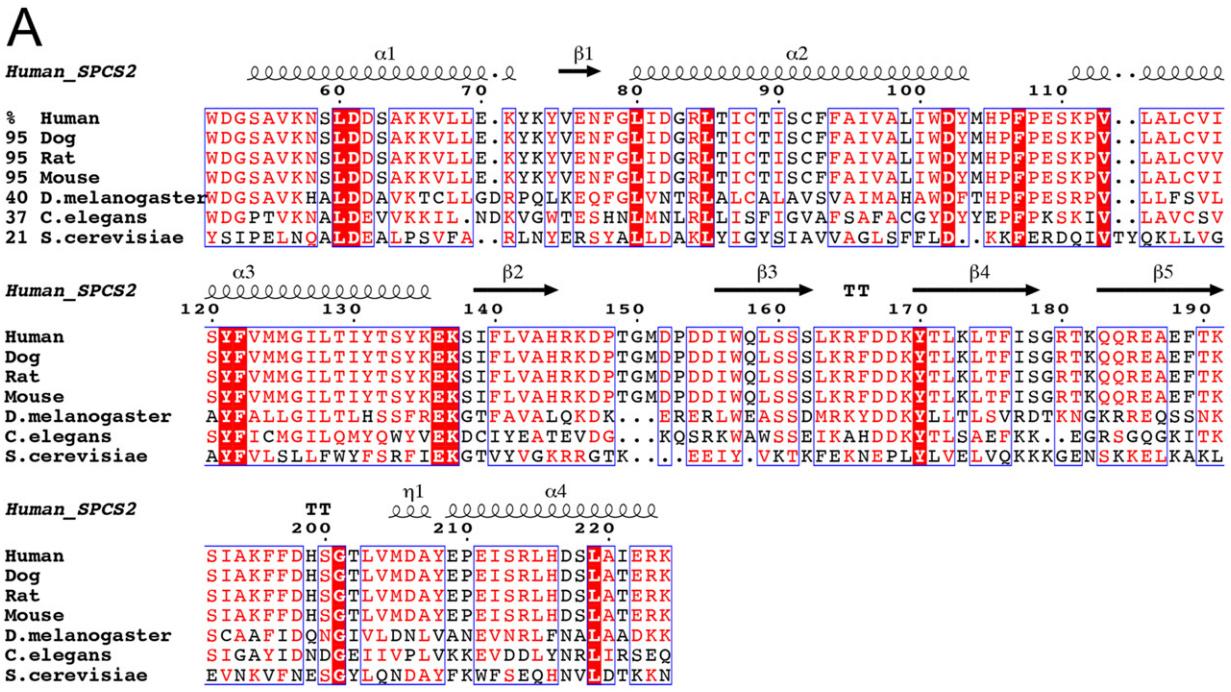
Recently the cryo-electron microscopy single-particle analysis at 4.9 Å resolution of hSPC was performed (Liaci *et al.*, 2021). The structural analysis, affinity chromatography and mass-spectrometry analysis are consistent with hSPC existing in two paralogous heterotetrameric structures, one with SEC11A and the other with SEC11C. Both paralogs were deposited in the protein database (PDB accession numbers: 7p2p for SPC-A that contains SEC11A and 7p2q for SPC-C that contains SEC11C). The structures show the membrane topology of the components, their stoichiometry, oligomeric juxtaposition as well as their general architecture.

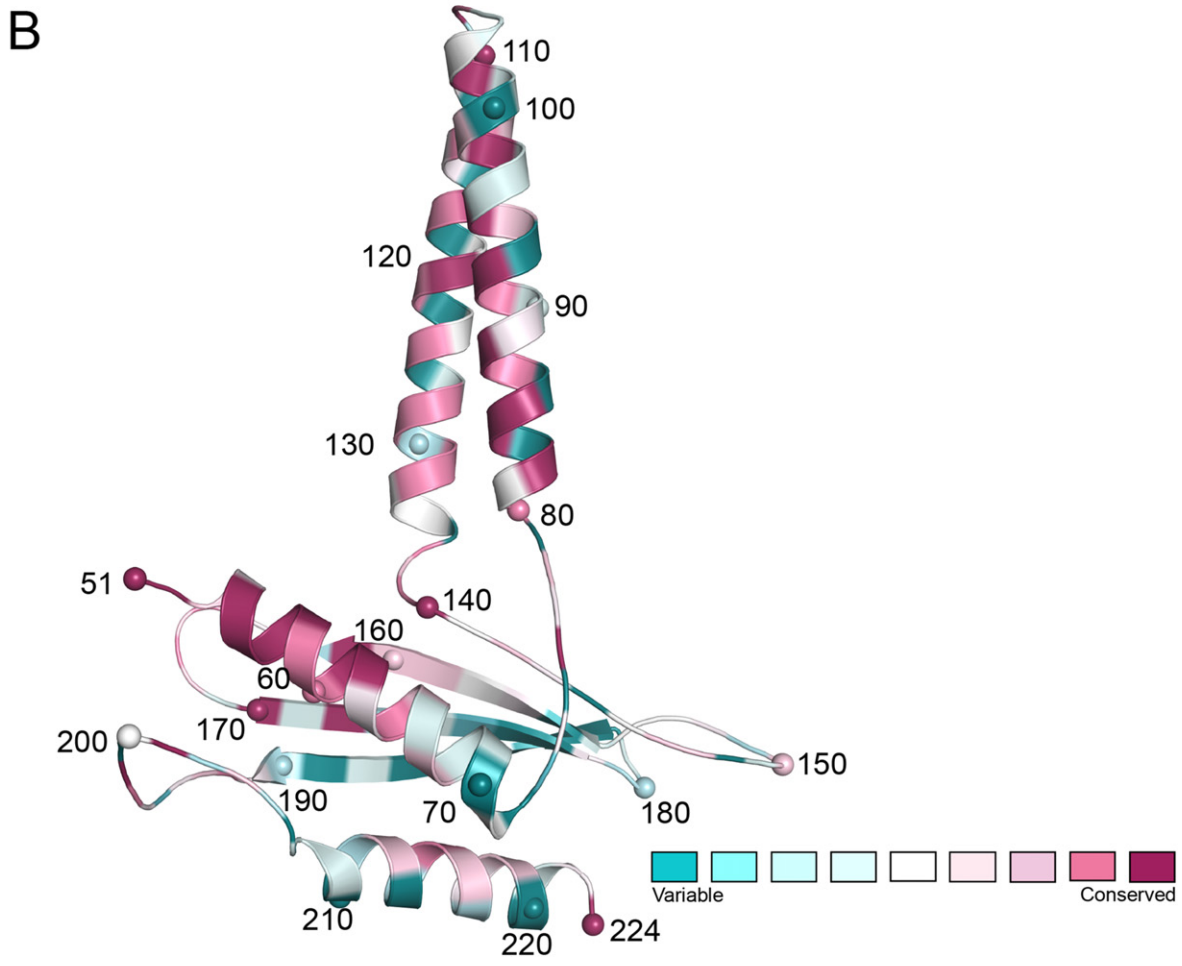
The structure reveals that the four integral membrane proteins (SEC11, SPCS1, SPCS2 and SPC3) contain a total of six transmembrane segments arrange in sets of three. One triplet consisting of the transmembrane segment from SEC11 (A or C) and the two transmembrane segments from SPCS2. The other triplet is assembled from the transmembrane segment of SPCS3 and the pair of transmembrane segments of SPCS1. SEC11 contains an amphipathic helix at its C-terminus that lays parallel to the lipid bilayer surface. The start and end of each transmembrane segment as analyzed by PPM 3.0 (Lomize *et al.*, 2012) is shown in **Fig. 2**.

Both amino- and carboxy- termini of SPCS1 are located within the cytoplasm. SPCS3 has its amino-terminus residing within the cytoplasm and its globular domain located within the ER lumen where it forms extensive interactions with the catalytic domain of SEC11. The amino-terminus of SEC11 is located within the cytoplasm. SPCS2 has both of its termini within the cytoplasm where it also contains a globular domain.

The globular catalytic domain of SEC11 (A/C) has a mostly β-sheet architecture like that of the prokaryotic signal peptidase (Paetzel, 1998; Paetzel and Strynadka, 1999; Paetzel *et al.*, 2002a). This ER Sec11 proteolytic domain has the enzyme classification number EC:3.4.21.89 and is classified by the protease database MEROPS to be a member of Clan SF, Family S26, and Subfamily B S26.010. The catalytic residues for Sec11 are Ser56/His96 in SEC11A, and Ser68/His108 in SEC11C. The conserved aspartate residues that have been shown to be critical for activity in yeast Sec11 correspond to Asp116 and Asp122 in human Sec11A, Asp128 and Asp134 in human Sec11C (**Fig. 3**). SEC11A-Asp116 and SEC11C-Asp128 point towards Arg97 and Arg109 and SPCS3. SEC11A-Asp122 and SEC11C-Asp134 are in the vicinity of the catalytic histidine. Structural alignment of *E. coli* SPase and Sec11A show that they have the same general protein fold (**Fig. 4**) despite having moderate sequence identity (20% for SEC11A and 17% for SEC11C). The alignment also confirms that the proposed catalytic histidine in SEC11 aligns with the lysine general base utilized in the bacterial type 1 signal peptidase (**Figs. 3** and **4**). SEC11 is missing the domain 2 region of *E. coli* SPase but SEC11 packs against the β-sandwich globular domain of SPCS3 in a similar way as domain 2 packs against domain 1 in *E. coli* SPase (**Fig. 4**). PISA analysis of this interaction shows that SPCS3 and Sec11A share an interface of 1132.7 $Å^2$ which is 10.4% of the total solvent accessible surface area of Sec11A and 8.7% of the total solvent accessible surface area SPCS3 (Krissinel and Henrick, 2007; Krissinel, 2010, 2015). The same analysis of the interface between Sec11C and SPC3 shows a slightly larger shared interface of 1406.3 $Å^2$ which is 12.4% of the total solvent accessible surface area of Sec11C and 11.0% of the total solvent accessible surface area SPCS3. The cryo-EM structure shows that SPCS3 is glycosylated at Asn141. Interestingly

Fig. 5 Sequence and structural conservation for SPCS3 (SPC22/23). **A**. A sequence alignment that includes residues that are present in the cryo-electron microscopy structure (PDB: 7p2p). Identical residues are highlighted in red with white letters. Conserved residues have red letters. The secondary structure for human SPCS3 is shown above the sequence. The percent identity to human SPCS3 is shown to the left of the top row. The sequence numbers are those for human SPCS3. The UniProt accession numbers for the sequences are as follows: Human P61009, Cow Q3SZU5, Dog P61008, Rat Q568Z4 and D3ZF12, Mouse Q6ZWQ7, *Drosophila melanogaster* (fruit fly) Q9VCA9, *Caenorhabditis elegans* (nematode or roundworm) P34525, *Saccharomyces cerevisiae* (yeast) Q12133 **B**. The mainchain path for human SPCS3 using the coordinates from the cryo-electron microscopy structure (PDB: 7p2p). A sphere marks the location of the alpha-carbon for every tenth residue. The glycosylation at Asn141 is shown in sticks (carbon-white, oxygen-red, nitrogen-blue). The level of conservation is mapped onto the structure using the programs Consurf and PyMol. Sequence alignments were performed with Clustal-Omega and ESPript 3.0. Program references: Ashkenazy, H., Abadi, S., Martz, E., *et al.*, 2016. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res, 44(W1), W344–50. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol. Sievers, F., Higgins, D. G. 2018. Clustal Omega for making accurate alignments of many protein sequences. Protein Sci, 27(1), 135–145. Gouet, P., Robert, X., Courcelle, E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res, 31(13), 3320–3323.





Asn141 is mostly conserved in SPCS3 from other species, but not in *Saccharomyces cerevisiae* (**Figs. 2** and **5**). Previous work suggested that *Saccharomyces cerevisiae* SPC3 maybe glycosylated at two different asparagine residues (Meyer and Hartmann, 1997).

SEC11A/C have a single amino-terminal transmembrane segment but also contain an amphiphilic monotopic helix located at their carboxy-terminus. Gram-positive bacterial signal peptidases typically have a signal amino-terminal transmembrane segment and gram-negative bacterial signal peptidases usually have two amino-terminal transmembrane segments. Extensive sequence alignment analysis reveals that some prokaryotic signal peptidases contain an additional predicted carboxy-terminal transmembrane segment (Paetzel *et al.*, 2000). For example three of the four signal peptidase from *Streptomyces lividans* TK21 have the predicted carboxy-terminal transmembrane segment (Parro and Mellado, 1998; Schacht *et al.*, 1998; Parro *et al.*, 1999; Geukens *et al.*, 2001).

SPCS2 has two transmembrane segments that pack against the transmembrane segment of Sec11(**Figs. 2** and **6**). The cytoplasmic globular domain of SPCS2 has a five-stranded β-sheet with two α-helices. This domain makes interactions with the termini of SPCS1 (**Figs. 2** and **7**). This is consistent with previously observations that SPCS2 is required for the association of SPCS1 with the full signal peptidase complex (Antonin *et al.*, 2000). Chemical crosslinking assays are consistent with SPCS2 being in close contact with the beta-subunit of the Sec61 translocase (Kalies *et al.*, 1998). Immunoprecipitation studies are also consistent with this finding (Antonin *et al.*, 2000).

The hSPC cryo-EM structure is missing regions from the subunit termini include residues 1–12 and 161–179 of SEC11A, residues 1–24 and 173–192 of SEC11C, residues 1–50 of SPCS2, and residues 1–65 and 152–169 of SPCS1. Alpha-Fold (Jumper *et al.*, 2021; Varadi *et al.*, 2022) predicts that most of the missing regions from the EM density are disordered.

SPC has mostly been isolated from the endoplasmic reticulum but interestingly it has been observed that the nuclear membranes from erythroleukemia cells contains signal peptidase activity. Detergent-solubilized membrane from isolated nuclei were able to correctly process the signal peptide from human preplacental lactogen (Puddington *et al.*, 1985). Future experiments are needed to see if this will be observed in all eukaryotic cells.

ER SPC From Other Species

Besides dog, yeast and human cells, the SPC has been investigated from a number of different species including hen oviduct (Lively and Walsh, 1983; Baker *et al.*, 1986; Baker and Lively, 1987; Newsome *et al.*, 1992), cow (Ashwell *et al.*, 2001), pig (Fujimoto *et al.*, 1984), *Drosophila melanogaster* – fruit fly (Brennan *et al.*, 1980; Haase Gilbert *et al.*, 2013), early metazoan such as the marine sponge *Amphimedon queenslandica* (Hammond *et al.*, 2019), insects (Zhang and Xia, 2014; Guan *et al.*, 2018), and plants (Yi *et al.*, 2021).

hSPC – Connection to Virology

Many viruses such as hepatitis C virus (Li *et al.*, 2014; Suzuki *et al.*, 2013; Oehler *et al.*, 2012; Pene *et al.*, 2009; Targett-Adams *et al.*, 2008; Okamoto *et al.*, 2008; Ma *et al.*, 2007; Hope *et al.*, 2006; Majeau *et al.*, 2005; Carrere-Kremer *et al.*, 2004; Lemberg and Martoglio, 2002; Wu, 2001; Blight *et al.*, 1998; Lin *et al.*, 1994), arenavirus (York and Nunberg, 2007), hantavirus (Lober *et al.*, 2001), flavivirus (Amberg and Rice, 1999; Stocks and Lobigs, 1998; Stocks and Lobigs, 1995), pestiviruses (Bintintan and Meyers, 2010), reubella virus(Law *et al.*, 2001), and influenza C virus(Hongo *et al.*, 1999; Pekosz and Lamb, 1998) have polyproteins and/or internal signal peptides that are processed by hSPC. Hepatitis C virus (HCV) is a major cause of chronic liver disease including steartosis, cirrhosis and liver cancer. Nearly 170 million people worldwide are chronically-infected with HCV, and therefore are at risk of developing cirrhosis and liver cancer (Suzuki *et al.*, 2013). HCV infections are also linked to insulin type 2 diabetes and kidney disease (Li *et al.*, 2014). HCV is an enveloped positive-stranded RNA virus that expresses a polyprotein that is cleaved (between residues 191 and 192) by hSPC to generate the Core_p21 protein (Pene *et al.*, 2009). It has been shown that hSPC interacts with the HCV structural protein E2 as well as the non-structural protein NS2 (the HCV encoded cysteine protease) (Suzuki *et al.*, 2013). Both proteins are critical integral membrane proteins directly involved in HCV assembly. The interactions between E2 and NS2 are directly mediated by hSPC subunit 1 (SPCS1). The knockdown of SPCS1 impairs the interaction between

Fig. 6 Sequence and structural conservation for SPCS2 (SPC25). **A**. A sequence alignment that includes residues that are present in the cryo-electron microscopy structure (PDB: 7p2p). Identical residues are highlighted in red with white letters. Conserved residues have red letters. The secondary structure for human SPCS2 is shown above the sequence. The percent identity to human SPCS2 is shown to the left of the top row. The sequence numbers are those for human SPCS2. The UniProt accession numbers for the sequences are as follows: Human Q15005, Dog Q28250, Rat D3ZD11, Mouse Q9CYN2, *Drosophila melanogaster* (fruit fly) Q9VYY2, *Caenorhabditis elegans* (nematode or roundworm) Q9XWW1, *Saccharomyces cerevisiae* (yeast) Q04969 **B**. The mainchain path for human SPCS2 using the coordinates from the cryo-electron microscopy structure (PDB: 7p2p). A sphere marks the location of the alpha-carbon for every tenth residue. The level of conservation is mapped onto the structure using the programs Consurf and PyMol. Sequence alignments were performed with Clustal-Omega and ESPript 3.0. Program references: Ashkenazy, H., Abadi, S., Martz, E., *et al.*, 2016. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res, 44(W1), W344–50. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol. Sievers, F., Higgins, D. G. 2018. Clustal Omega for making accurate alignments of many protein sequences. Protein Sci, 27(1), 135–145. Gouet, P., Robert, X., Courcelle, E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res, 31(13), 3320–3323.

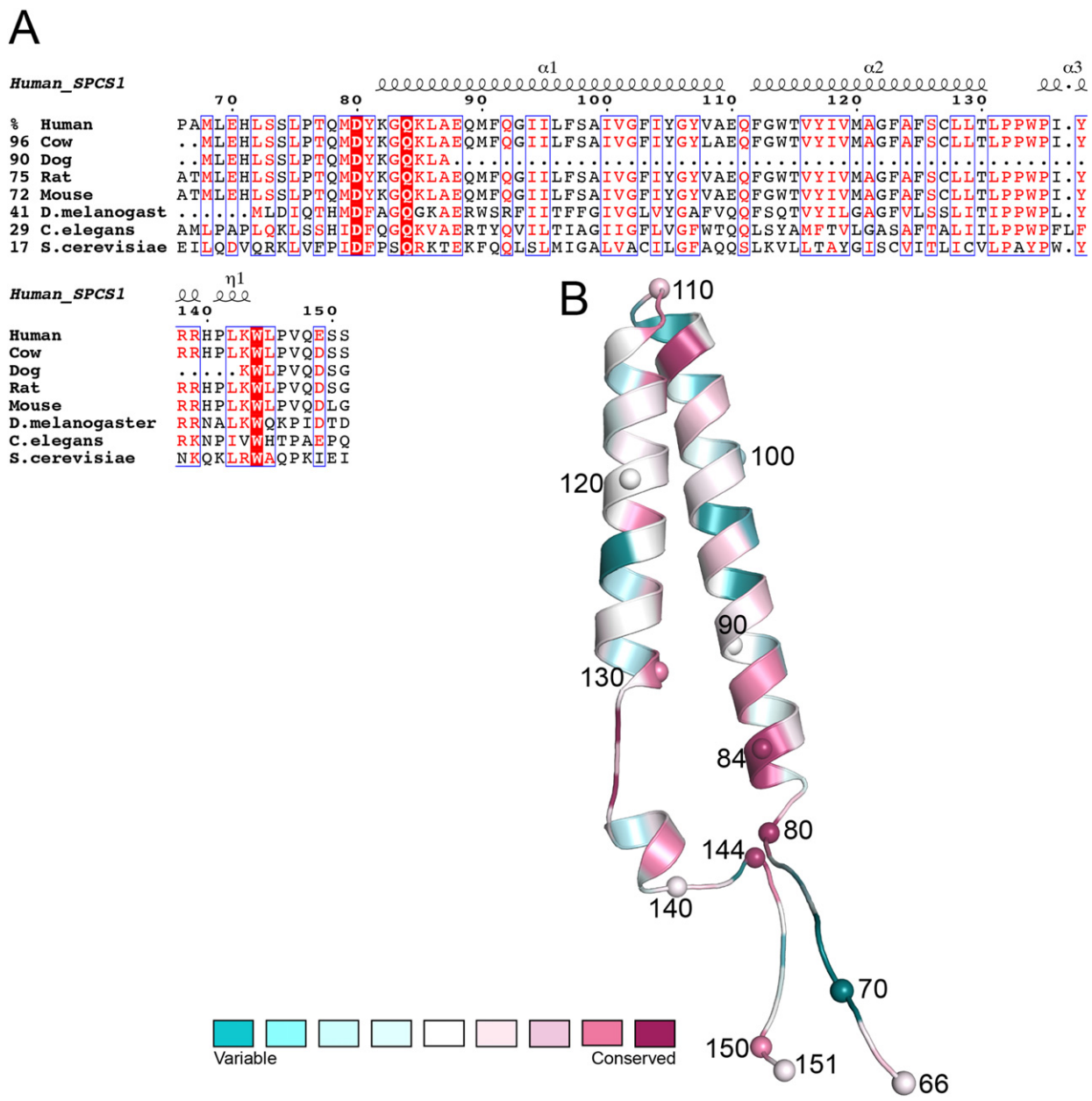


Fig. 7 Sequence and structural conservation for SPCS1 (SPC12). **A**. A sequence alignment that includes residues that are present in the cryo-electron microscopy structure (PDB: 7p2p). Identical residues are highlighted in red with white letters. Conserved residues have red letters. The secondary structure for human SPCS1 is shown above the sequence. The percent identity to human SPCS1 is shown to the left of the top row. The sequence numbers are those for human SPCS1. The UniProt accession numbers for the sequences are as follows: Human Q9Y6A9, Cow Q3T134, Dog P83362, Rat D3ZFK5, Mouse Q9D958, *Drosophila melanogaster* (fruit fly) Q9VAL0, *Caenorhabditis elegans* (nematode or roundworm) O44953, *Saccharomyces cerevisiae* (yeast) P46965 **B**. The mainchain path for human SPCS1 using the coordinates from the cryo-electron microscopy structure (PDB: 7p2p). A sphere marks the location of the alpha-carbon for every tenth residue. The conserved residues Asp80, Gln84 and Trp144 are labeled. The level of conservation is mapped onto the structure using the programs Consurf and PyMol. Sequence alignments were performed with Clustal-Omega and ESPript 3.0. Program references: Ashkenazy, H., Abadi, S., Martz, E., *et al.*, 2016. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res, 44(W1), W344–50. Schrödinger, L., DeLano, W., 2020. PyMOL, Available at: http://www.pymol.org/pymol. Sievers, F., Higgins, D. G. 2018. Clustal Omega for making accurate alignments of many protein sequences. Protein Sci, 27(1), 135–145. Gouet, P., Robert, X., Courcelle, E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res, 31(13), 3320–3323.

Table 1 SPC cleavage of proteins from single stranded RNA viruses

Virus (Family)	*Sense*	*Segments*	*Envelop*	*Proteins cleaved*	*References*
Arenaviruses (Family: *Arenaviridae*)	+/−	2	yes	glycoprotein (GP-C) signal peptide	(York and Nunberg, 2007)
Bunyamwera orthobunyavirus (Family: *Peribunyaviridae)*	−	3	yes	Glycoprotein precursor (GPC)	(Shi *et al.*, 2016)
Hantaviruses (Family: *Hantaviridae*)	−	3	yes	Glycoprotein precursor (GPC)	(Lober *et al.*, 2001)
influenza C virus (Family: *Orthomyxoviridae*)	+	7	yes	Glycoprotein precursor p42	(Hongo *et al.*, 1999)
Mouse mammary tumor virus (Family: *Retroviridae*)	+	1	yes	Signal peptide-Rem-CT	(Byun *et al.*, 2010)
Rubella virus (Family: *Matonaviridae*)	+	1	yes	Signal peptide-E2 glycoprotein	(Law *et al.*, 2001)
Tomato Ringspot Virus (Family: *Secoviridae)*	+	2	no	cNTB-VPg	(Wei *et al.*, 2016)
Flavivirus (Family: *Flaviviridae*)	+	1	yes	C-prM-E-NS1	(Alzahrani *et al.*, 2020)
Zika virus (Family: *Flaviviridae*)	+	1	yes	SP/pr, M/E, E/NS1, NS4A/NS4B	(Rother and Naumann, 2021)
Hepatitis C virus (Family: *Flaviviridae*)	+	1	yes	E2/p7/NS2 precursor	(Alzahrani *et al.*, 2022)
Pestiviruses (Family: *Flaviviridae*)	+	1	yes	^{Erns}-E1-E2-p7 preglycoprotein	(Mu *et al.*, 2021)

these two viral proteins and subsequent assembly of the infectious HCV virion (Suzuki *et al.*, 2013). SPCS1 has also been shown to be essential for Zika virus replication in trophoblasts (Rother and Naumann, 2021). This observation is interesting given that SPCS1 has little if any interaction with the catalytic machinery of SPC, but likely associated with the substrate within the lipid bilayer or within the cytoplasmic region. N- and C-terminal cytoplasmic regions of SPCS1 are missing from the hSPC cryo-EM structure (residues 1–65 and 152–169), likely dues to disorder, which may play a role in molecular recognition. There has been a large amount of investigation in to SPC catalyzed processing of viral proteins from single stranded RNA viruses (**Table 1**). It will be interesting to see if this extends to other classes of viruses as well.

A Natural Product SPC Inhibitor

A natural product lipopentapeptide aldehyde called Cavinaflungin has been discovered (Ortiz-Lopez *et al.*, 2015) and shown to interfere with the replication of the flaviviruses Zika virus and Dengue virus by inhibiting the host SPC (Estoppey *et al.*, 2017). This peptide has a typical signal peptidase specificity sequence P1 (alaninal) P3 (valine) (**Fig. 8**). Genome wide CRISPR/Cas9 studies in human cells show that SPC is the target of Cavinaflungin. Selection of resistant mutants in *S. cerevisiae* are consistent with Sec11 being the conserved target. Natural product lipopeptides such as Arylomycin have been shown to be inhibitors of prokaryotic signal peptides (Paetzel *et al.*, 2004; Holtzel *et al.*, 2002).

Role of SPC in Gastric Cancer

It has been shown that only one of the two proposed active site containing subunits within hSPC (SEC11A) is responsible for processing Transforming Growth Factor (TGF)-α which is known to promote malignant progression in gastric cancer – one of the most common human cancers (Oue *et al.*, 2014). These results provide the first hints that the catalytic subunits within hSPC (SEC11A and SEC11C) may have separate substrate specificities. This suggests that inhibitors developed to one of the subunits (SEC11A) may not necessarily spell doom for the whole essential workings of the hSPC and therefore SEC11A maybe a suitable target for the development of novel compounds that could be a treatment for gastric cancer and potentially other such diseases.

Summary

Bacterial signal peptidase (SPase) has been considered as a potential antibiotic target because it is an essential and accessible enzyme on the bacterial membrane surface. SPase also plays a key role in secreting antibiotic resistance enzymes such as

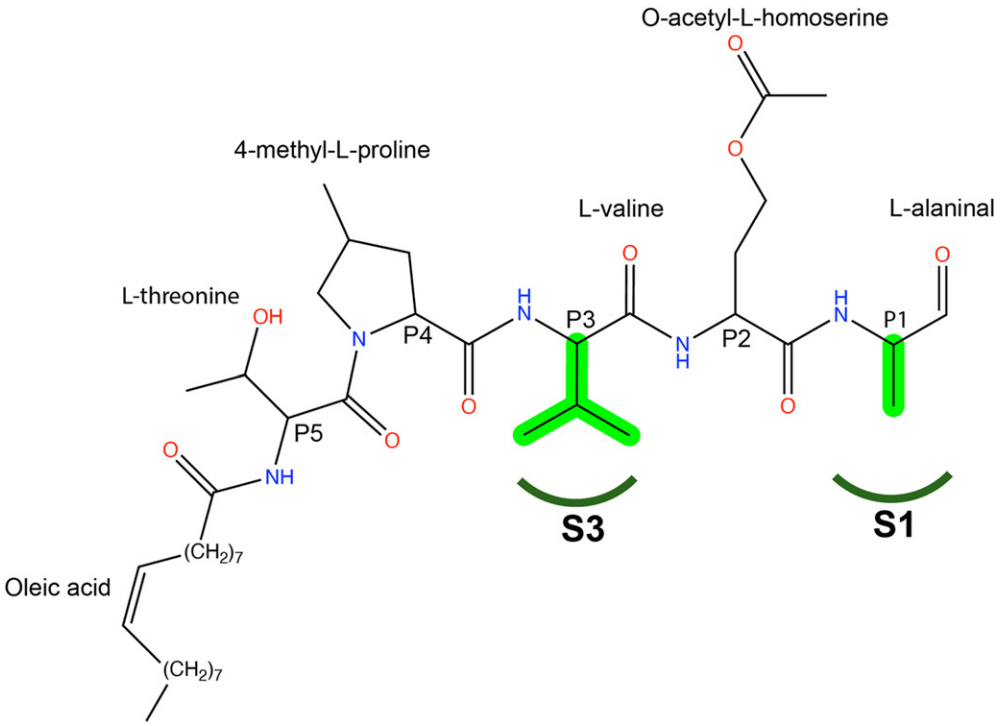


Fig. 8 The molecular structure of Cavinaflungin, the natural product lipopentapeptide aldehyde inhibitor of SPC. The P1-P5 residues are labeled. The P1 and P3 substrate specificity residues sidechains are highlighted in green. These residues bind within the substrate specificity binding pockets S1 and S3 of the Sec11 subunit of SPC.

β-lactamase and in the assembly of specialized secretion systems, export toxins and adhesins used in pathogenic mechanisms. Continued work on structures of hSPC with substrates and inhibitors bound will be helpful in the rational design of inhibitors that react with the bacterial SPase but not with the human SPC. The discovery of hSPC specific inhibitors that interferes with viral polyprotein processing will be a useful tool for investigations into the Flaviviridae family of viruses and other viruses.

It is important to investigate whether there are proproteins, preproteins or viral polyproteins that bind and cleave preferentially by either Sec11A or Sec11C. It is also interesting that human cells have a third Sec11 paralog (SEC11B, **Fig. 3**).

Investigations into the role of the so-called non-essential subunits SPCS1 and SPCS2 may provide insights into SPC substrate preferences.

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