Doctoral Thesis

Signal Peptide Peptidase (SPP)
identification of a presenilin-type aspartic protease

Author(s):
Weihofen, Andreas

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Signal Peptide Peptidase (SPP)
Identification of a Presenilin-type Aspartic Protease

A dissertation submitted to the
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for the degree of
Doctor of Natural Sciences

presented by

Andreas Weihofen
Dipl. phil II, University of Basel
born on April 28th, 1973
citizen of Giffers (FR)

accepted on the recommendation of

Prof. Dr. Ari Helenius, examiner
Prof. Dr. Ulrike Kutay, co-examiner
Dr. Bruno Martoglio, co-examiner

2003
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Summary

Proteases are key players in many regulated biological pathways. The discovery of intramembrane proteolysis revealed the existence of intramembrane cleaving proteases (I-CliP's) that catalyse the apparently paradox event of hydrolysis in a hydrophobic environment. Moreover, these new proteases are involved in alternative pathways in cell signalling, cell regulation and protein processing.

Signal sequences, which are essential to direct nascent membrane and secretory proteins to translocation sites at the endoplasmatic reticulum (ER), are liberated from their precursor protein co-translationally. Recent work led to the speculation that liberated signal sequences undergo intramembrane proteolysis catalysed by an activity named signal peptide peptidase (SPP), resulting in the release of bioactive peptides from the ER membrane. The aim of the present thesis was the identification of this putative I-CliP SPP.

Using affinity-labelling, protein purification and sequencing, SPP was identified as a polytopic aspartic I-CliP with sequence similarities to presenilin that is implicated in the cause of Alzheimer's Disease (AD). Database search revealed SPP as member of a so far uncharacterised conserved family of putative aspartic I-CliP's with five representatives in human. Proteolytic activity of human SPP was demonstrated by recombinant expression in yeast. Furthermore, inhibitor studies showing that γ-secretase inhibitors target presenilin and SPP gave further evidence that presenilin is a protease.

Taken together with the recent finding that SPP cleaves selected signal peptides, signal peptide processing by SPP is considered as a novel pathway for the intracellular generation of bioactive peptides.

Signal Sequenzen werden benötigt, um Membranproteine und sekretorische Proteine an die Translokationsstelle am endoplasmatischen Reticulum (ER) zu dirigieren. Es wurde spekuliert, dass Signal Sequenzen, nachdem sie co-translational von ihrem Vorläufer abgespalten wurden, intramembran gespalten werden, so dass sie dann von der ER Membrane als bioaktive Peptide freigesetzt werden. Die Aktivität, welche diese Reaktion katalysiert, wurde als Signal Peptide Peptidase (SPP) bezeichnet. Das Ziel der vorliegenden Doktorarbeit war die Identifizierung dieser mutmaßlichen I-CliP SPP.


In Anbetracht der Entdeckung, dass nur selektierte Signal Sequenzen von SPP gespalten gespalten werden, wird die Signal Sequenz Prozessierung durch SPP nun als neue Art der Produktion von bioaktiven Peptiden betrachtet.
Chapter 1

1. Introduction
1.1. Proteases – a short overview

Proteases play an important role in the regulation of many biological pathways in all living organisms and are implicated in many diseases (Barret, 1998; Neurath, 1986; Sternlicht and Werb, 2001). They hydrolyse peptide bonds in proteins by which activity and localisation of many enzymes, hormones and growth factors are controlled. Protein degradation, the complete breakdown of proteins, provides a free pool of amino acids for protein biosynthesis and prevents accumulation of dispensable proteins in the cell. Proteolytic processing, limited substrate cleavage, tailors proteins to appropriate function. Not surprisingly, protease activity must be tightly controlled to prevent damage to the cell by random proteolysis as illustrated by many diseases such as Alzheimer's disease (AD), cancer or neuroinflammatory diseases that have been in part associated with uncontrolled protease activity (Rosenberg, 2002; Selkoe, 2001; Sternlicht and Werb, 2001).

Two sets of proteases, which are nowadays preferable named peptidases\(^1\), are recognized based on the position of the peptide bond they are able to cleave: These are the exopeptidases, which act only near the N- or C-terminal end of polypeptide chains and the endopeptidases, which cleave within polypeptide chains. Both types are further allocated to five classes according to the primary catalytic group catalysing the hydrolysis of the peptide bond. Those classes are the serine-, threonine-, cysteine-, aspartic- and metallo-type peptidases (Barret, 1998) (Table 1.1). The classes are further subdivided into clans and families based on structural and functional similarities (Rawlings and Barrett, 1993, Rawlings et al., 2002, MEROPS protease database\(^2\)).

\(^{1}\) Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC34/.

\(^{2}\) http://merops.sanger.ac.uk/
Table 1.1 Classes of proteases (modified from Barret, 2001)

The completion of many large-scale genome-sequencing projects revealed the enormous number of proteases in nature. For example the human genome encodes according to the most recent release of the MEROPS database 508 proteases and homologues. But likely there are genes encoding proteins that are not yet recognised as proteases because of their lack of homology to known proteases. Examples of such “hidden” proteases are intramembrane-cleaving proteases (I-ClIP’s).
1.2. Intramembrane proteolysis

According to textbooks proteolysis is limited to aqueous environments and thus excluded from a hydrophobic environment such as biological lipid bilayers. Over the past years however, it has become clear that membrane proteins can be cleaved within their membrane-spanning regions. These reactions are referred to as "intramembrane proteolysis". Interestingly, intramembrane proteolysis releases membrane-tethered proteins in a regulated manner revealing alternative pathways in cell signalling, cell regulation and protein processing. This mode of pathways has been termed regulated intramembrane proteolysis (RIP) and is conserved from bacteria to humans (Brown et al., 2000). In eukaryotes RIP plays a regulatory role, for instance, in embryonic development, cholesterol homeostasis and the unfolded protein response. An example of RIP in bacteria is the regulation of spore formation in *Bacillus subtilis* (for review see chapter 3, Brown et al., 2000; Urban and Freeman, 2002). RIP gained more attraction as soon as it emerged that it plays a pivotal role in Alzheimer's disease (AD). In the subsequent section 1.2.1 RIP will be explicated by means of the two most prominent examples.

Intramembrane proteolysis claims the existence of ICliP's (Wolfe et al., 1999a). Presently, three types of proteins with putative intramembrane cleaving protease activity have been identified. They show no homology to proteases, but seem to apply the classical mechanistic principles (see Table 1.1). These candidates, namely site-2 protease, rhomboid and presenilin, will be discussed separately in section 1.2.2.
1.2.1. Regulated intramembrane proteolysis (RIP)

In nature survival depends on the ability to respond quickly and adequately to changes in the environment. On the cellular level such response often includes the alteration of gene expression to provide the required proteome. The strategy mostly employed is the activation of previously synthesised transcription factors. Many variations on this strategy are applied in the cell. Transcription factors are activated by phosphorylation, ligand binding or limited proteolysis.

Recently, a new role of proteolysis has been recognized in the activation of dormant membrane-tethered transcription factors or nuclear signalling factors. The dormant factor is activated by two-step proteolytic processing resulting in its release from the membrane and subsequent translocation into the nucleus. The two cleavages are carried out sequentially by separated proteases, namely a "classical" protease and an I-CliP. The latter does not cleave until the first protease has removed the bulk of the dormant factor on the extracytosolic site. The first cleavage is tightly controlled. In light of these observations, this phenomenon of activation has been termed regulated intramembrane proteolysis (Brown et al., 2000).

To date, it is generally accepted that RIP activates not only transcription factors or nuclear signalling factors, but also signalling factors such as epidermal growth factor (EGF) ligands and pheromones. Thus RIP can promote intercellular and intracellular events (Urban and Freemann (2002)). Furthermore, intramembrane proteolysis does not necessarily require a primary cleavage. In this case, RIP is likely controlled solely by the transcription of the I-CliP substrate. Thus common to all RIP processes is that dormant membrane-tethered "biological information" is released from a membrane by an I-CliP (for a current overview see chapter 3).
1.2.1.1. RIP of sterol regulatory element binding protein (SREBP)

The mammalian cell continuously adjusts its sterol content by regulating levels of key enzymes of sterol biosynthesis and levels of receptors mediating sterol uptake (reviewed in Hampton, 2002). One form of regulating sterol biosynthesis, the transcriptional control of enzymes engaged in sterol synthesis, comprises RIP.

SREBP's are ~1150 amino acids (aa) long proteins and are inserted in a helical hairpin fashion in the ER membrane with a short hydrophilic lumenal loop. Their N-terminal domains facing the cytosol are transcription factors of the basic-loop-helix-leucine zipper family (bHLH-Zip). They activate genes involved in cholesterol biosynthesis (Smith et al., 1988).

The cholesterol levels of the cell control the transport of SREBP from the ER to the Golgi where it gets activated. In sterol-depleted cells, the polytopic SREBP cleavage-activating protein (SCAP) escorts SREBP in budding vesicles to a post-ER compartment, likely the cis-Golgi (Nohturfft et al., 2000). In sterol rich cells, a yet not fully understood mechanism prevents budding of the SREBP-SCAP complex. Because SCAP changes its conformation under high cholesterol levels (Steck and Lange, 2002), it has been suggested that SCAP plays a role in sensing the cellular cholesterol levels and thereby regulates budding of SREBP from the ER. The recently identified ER membrane protein INSIG binds to SCAP and has been implicated in retention of the SREBP/SCAP complex in the ER (Yabe et al., 2002; Yang et al., 2002).

Once reached the cis-Golgi, SREBP undergoes a two-step proteolytic processing (Figure 1.1): First, the membrane-attached serine protease Site-1 (S1P) (Sakai et al., 1998) cleaves SREBP in its lumenal loop. The resulting type II membrane attached bHLH-Zip transcription factor is then released from the membrane after intramembrane proteolysis by the putative I-CliP S2P.
(see 1.2.2.1) from the membrane and translocates into the nucleus to activate genes involved in cholesterol biosynthesis.

The proteases can only act when the SREBP/SCAP complex has moved to the cleavage compartment. Hence, even the activity of the I-ClIP S2P is not directly controlled by sterols, it is under cholesterol control since it requires the sterol-mediated transport and the first cleavage to proceed. Thus in this case, RIP allows cholesterol to inhibit its own synthesis in a classical feedback fashion.
Figure 1.1 Classical RIP - Activation of SREBP The sterol regulatory-element binding protein (SREBP) is a hairpin-like membrane protein residing in the endoplasmic reticulum (ER) with its N- and C- termini facing the cytosol. The N-terminal portion has transcriptional activity (bHLH-Zip) and is liberated by two sequential cleavages before it can travel to the nucleus. The SREBP cleavage activating protein (SCAP) complexes and mediates SREBP out of the ER to the Golgi, where the responsible proteases are located. The export is tightly regulated by the cellular sterol content, which is likely sensed by SCAP. In the Golgi the site-1 protease (S1P) cleaves SREBP in its luminal loop domain. The metallo-CII site-2 protease (S2P) then cleaves the transmembrane domain (TMD), releasing the N-terminal transcription factor domain that translocates to the nucleus and activates genes involved in cholesterol biosynthesis.
1.2.1.2. RIP of β-amyloid precursor protein (APP) – A key process in Alzheimer’s disease

Alzheimer’s disease (AD), described first by the Bavarian psychiatrist Alois Alzheimer in 1906, is the most common form of dementia worldwide. The histological hallmarks of AD patients are neuritic amyloid plaques that are proteinaceous deposits constituted mainly of ~4 kDa amyloid β peptides (Aβ) in the brain. Around 10 years ago it has been hypothesised that the deposition of Aβ is the primary influence driving AD pathogenesis and that preventing Aβ accumulation is a promising therapeutical approach against AD ("Amyloid cascade hypothesis", Hardy and Higgins, 1992, for review see Hardy and Selkoe, 2002; Selkoe, 2001; Sommer, 2002). The finding that intramembrane proteolysis plays a key role in Aβ generation, attracted academia as well the pharmaceutical industry to RIP and the I-CliP γ-secretase (see below).

Aβ results from proteolytic cleavage of the ubiquitously expressed type I transmembrane glycoprotein β-amyloid precursor protein (APP), which consists of a large external domain, a single transmembrane domain and a short cytoplasmic tail (Kang et al., 1987). Four types of proteases, which are named according to their processing sites α-, β-, γ- and ε-secretase, cleave APP (see Figure 1.2). Cleavage by β-secretase sheds the large N-terminal domain of APP resulting in a membrane associated C-terminal fragment (CTFβ). Subsequently, the I-CliP γ-secretase cleaves CTFβ in the middle of its transmembrane domain releasing a 40 residue variant Aβ40 or a 42 residue variant Aβ42. Alternative processing of APP by α- and γ-secretase results in formation of the non-amyloidogenic p3 peptide. Secretase cleavages likely occur all distal the Golgi in the late secretory pathway (Annaert et al., 1999). Interestingly, β-secretase and α-secretase are classical proteases that remove the large part of the ectodomain and γ-secretase performs intramembranous cleavage resulting in the release of the peptides. Consequently, APP processing displays analogy to RIP.
So far only α- and β-secretases have been convincingly identified. Several ADAM metalloproteases can perform α-secretase processing (Schlondorff and Blobel, 1999), whereas β-secretase has been recently identified as aspartic proteases with similarities to the pepsin family (Hussain et al., 2000; Vassar et al., 1999). The identities of the l-CliPs γ-secretase and ε-secretase (see below) are still not clear, but as described later in detail (see 1.2.2.3), presenilin is the main candidate for both activities.

Aβ peptide is a normal product of cells (Haass et al., 1992; Shoji et al., 1992). It is normally degraded by numerous proteases (for review see Suh and Checler, 2002). Under normal conditions 90% of total Aβ appears as the 40 residue variant form, and only 10% as the 42 variant. Aβ42 that is less soluble than Aβ40 can form Aβ fibrils leading to amyloid plaques (Burdick et al., 1992; Jarrett et al., 1993). Thus the disordered degradation of Aβ42 peptides or overproduction of Aβ42 may lead to AD. The latter is exemplified by several familial AD associated mutations that all result in increased Aβ42 production. These mutations were found close to the processing sites in APP and mainly in presenilins. The physiological role of Aβ peptides is not known, but as described below, recent data suggests that it is just a harmful by-product of a "classical" RIP process.

Recently the C-terminal proteolytic fragment resulting from CTFβ processing, which is the counterpart of the N-terminal Aβ peptide, has been analysed in detail. This cleavage product was initially overlooked because it is very unstable and difficult to detect. Interestingly, the C-terminus of this fragment, which is referred to as APP intracellular domain (AICD), does not correspond to the γ-secretase cleavage site, indicating a further intramembranous cleavage close to the cytosolic interface (Sastre et al., 2001; Weidemann et al., 2002). This cleavage site has been termed ε-site (Fig 1.2) (Weidemann et al., 2002). Importantly, there are other substrates that undergo RIP involving γ-secretase (Chapter 3 and for review see Fortini, 2002). Strikingly, almost all of these γ-secretase substrates undergo additional cleavages at sites
corresponding to ε-site (Lammich et al., 2002; Okochi et al., 2002) and in the
known cases the physiological relevant proteolytic product is always the C-
terminal fragment generated by "ε-secretase", which is likely the same protein
as γ-secretase (see 1.2.2.3). It was proposed recently that AICD could also be
the relevant product of APP processing. AICD has been found in the nucleus
to mediate the assembly of a transcriptionally active complex (Cao and
Sudhof, 2001; Kimberly et al., 2001) and thereby regulates phosphoinositide-
mediated calcium signalling, which maintains calcium levels in the ER
(Leissring et al., 2002). Although further work is needed to substantiate this
finding, it seems that proteolytic processing of APP serves to activate the
nuclear signalling factor AICD and Aβ peptides are only by-products.
Figure 1.2 RIP of APP

The type I membrane protein APP can undergo several proteolytic processings distal to the Golgi in the late secretory pathway and plasma membrane. Proteolytic processing that results in the Alzheimer disease causing fibrillogenic Aβ42 is initiated with ectodomain shedding by β-secretase yielding membrane-tethered CTFβ. This fragment is then further processed by the I-CLIP γ-secretase generating Aβ peptides. Cleavage by γ-secretase can occur at two distinct sites that lead to the physiological Aβ40 and/or the pathological Aβ42 that induces amyloid plaque formation. Alternative proteolytic processing of APP by α-secretase and subsequent γ-secretase cleavage results in p3. Simultaneous with γ-secretase cleavage, ε-processing yields AICD that acts as nuclear signalling factor.
1.2.2. Intramembrane cleaving proteases (I-CliP’s)

I-CliPs are the key players in RIP par excellence (Brown et al., 2000; Urban and Freeman, 2002). They promote by intramembrane proteolysis release of the dormant membrane-tethered functional domains, thereby activating distinct biological pathways. Proteolysis in the hydrophobic environment of biological lipid bilayers is indeed astonishing because it means in principle mixing oil and water. Nevertheless, there are now three candidate proteins that are believed to carry out this seemingly paradoxical process of intramembrane proteolysis.

There are four characteristic features of candidate I-CliPs: First, they are all hydrophobic polytopic membrane proteins with potential catalytic residues located within predicted transmembrane regions. Second, they show no overall sequence homology to other proteases, but they have short sequence motifs that are typical for proteases. This indicates that I-CliPs apply the same mechanistic principles as the established proteases (see Table 1.1). Third, they do not recognize a specific consensus sequence within their substrates but rather less well-defined parameters such as length, flexibility or mobility. Last, it seems that they can cleave either type I or type II orientated membrane proteins.

All putative I-CliPs have initially been identified by genetical approaches and their putative active site residues have been examined by site-directed mutagenesis. Although evidence for their proteolytic activity is strong, no formal proof for their proteolytic activity has been provided so far and thus their role as protease remains to some extent controversial.
1.2.2.1. Site-2 Protease (S2-P)

Brown and Goldstein reported in 1997 the first identification of a putative I-CLiP. They cloned a gene that complements mutant CHO cells (M19), which cannot cleave SREBP at its intramembranous site-2. This gene, now termed site-2 protease, encodes a hydrophobic polytopic membrane protein that contains a consensus metallo protease zinc-binding site, HExxH, but no further homology to proteases (Rawson et al., 1997). Replacement of either of the two histidines or the glutamic acid destroys the ability of S2 cDNA to restore SREBP cleavage in M19 cells (Rawson et al., 1997). In classical metallo proteases histidines in HExxH motifs coordinate zinc binding at the active site and the glutamic acid acts together with a water molecule as nucleophile in peptide bond cleavage. Most HExxH-containing zinc metallo proteases contain a third residue that also coordinates the active site zinc. Such a putative residue, an aspartic residue within a LDG motif, was also found to be essential for S2P activity (Zelenski et al., 1999).

The putative active site residues, HExxH and LDG, are embedded within transmembrane regions close to the interface with the cytosol (Zelenski et al., 1999). Altogether, mutational analysis and topology analysis convincingly demonstrate that S2P is a metallo protease with active site residues within transmembrane regions, i.e. S2P is a metallo I-CLiP. Topology analysis revealed more that S2P is a membrane protein with several membrane-associated separated by hydrophilic loops accessible to the glycosylation machinery of the ER and Golgi. The hydrophobic regions separating the hydrophilic domains do not span the membrane but appear to be embedded within it (Zelenski et al., 1999).

To date, two substrates of S2P have been identified. Both are type II orientated membrane-tethered dormant transcription factors, namely SREBP (see above) and ATF6 (Ye et al., 2000b). The latter controls transcription of genes encoding folding enzymes and chaperones proteins (Ellgaard and
Helenius, 2001), when unfolded proteins accumulate in the ER, described in more detail in chapter 3. Based on the membrane orientation of the known substrates, it thus seems that S2P cleavage is restricted to type II membrane proteins.

Is there a sequence motif required for S2P cleavage? Cysteine panning revealed that the site-2 cleavage site (Leu-Cys) is located just three residues within the first predicted transmembrane region of SREBP (Duncan et al., 1998). Replacement of this Leu-Cys motif by alanines as well as single replacement of any other residue in the predicted transmembrane region by alanines, does not abolish site-2 cleavage (Ye et al., 2000a). This suggests that there are no strictly defined consensus cleavage site residues.

Nevertheless, a conserved Asn-Pro motif in the middle of the scissile transmembrane region of SREBP plays a key role for cleavage by S2P (Ye et al., 2000a). Single alanine replacement partially and double replacement of this motif totally abolishes cleavage. But the Asn-Pro motif can be positioned anywhere within the transmembrane segment without affecting processing and the site of cleavage. A similar motif, Asn-X-X-Pro, is also present in ATF6 and is essential for cleavage by S2P (Ye et al., 2000b). Thus, a model was proposed that the Asn-Pro and Asn-X-X-Pro motifs permit partial unfolding of the helical transmembrane region to a more extended conformation that allows S2P to attack a peptide bond.

S2P-like proteases are widely conserved across most species. All prokaryotic and metazoan genomes sequenced so far encode S2P homologues, but only higher eukaryotes contain S2P orthologues that are supposed to be involved in SREBP activation. The similarities within the homologues are rather low, but the putative catalytic motifs HExxH and LDG are conserved. Three distant S2P homologues have been investigated yet, namely SpoIVFB of Bacillus subtilis (Rudner et al., 1999), Eep of Enterococcus faecalis (An et al., 1999) and YaeL of Escherichia coli (Alba et al., 2002; Kanehara et al., 2001)(see Chapter 3). In these proteins the motifs HExxH and LDG are also required for
proper function, supporting the view that these motifs are involved in proteolysis.

All published data are in agreement with the notion that S2P and its homologues are metallo l-CliPs. But because they are mainly obtained from genetical work it will be important to confirm this notion with other approaches. For example, reconstitution of S2P activity in vitro would be a powerful tool to further explore this unusual metalloprotease. So far all in vitro attempts were not fruitful, examplifying the difficulties of working with l-CliPs.

1.2.2.2. Rhomboid family

Rhomboid-1 is a recent discovered putative l-CliP. In Drosophila, it promotes intramembranous cleavage of the membrane-anchored TGFα-like epidermal growth factors (EGF) Spitz, Keren and Gurken. Cleavage releases the EGF ligand domains for subsequent secretion, allowing them to activate EGF receptors on neighbouring cells (Lee et al., 2001; Urban et al., 2001; Urban et al., 2002a).

In 1990 Rhomboid-1 was initially described as a membrane protein required for dorsoventral axis establishment and peripheral nervous system development in Drosophila. Rhomboid-1 is located in the Golgi and has a predicted seven transmembrane topology (Bier et al., 1990). It required more than 10 years of intensive research to identify its molecular function as a proteolytic enzyme (Urban et al., 2001). Its direct part in proteolytic activation of the EGF ligand Spitz was verified by genetic analysis (Golembo et al., 1996; Wasserman and Freeman, 1997), but Rhomboid-1 itself was not considered as a protease because it shows no obvious homology to known proteases.

Freeman and co-workers have recently provided strong evidence that Rhomboid-1 is an l-CLiP (Urban et al., 2001): In mammalian cells the
irreversible specific serine protease inhibitors TPCK and 3,4-Dichlorisocoumarin inhibited reconstituted Spitz processing, whereas inhibitors for metallo-, cysteine and aspartic-type proteases have no effect, suggesting that Spitz is processed by a serine protease. In Rhomboid-1 four of six amino acids, which are completely conserved in all rhomboid homologues (Wasserman et al., 2000) and are essential for Spitz processing, match the residues required for a serine catalytic triad charge-relay system (S217, H281 and N169) and an oxyanion stabilization site (G215 and S217). As expected for catalytic residues, even conservative mutations such as S217T and S217C abolished processing. Furthermore, S217 and G215 are localised in a motif GASGG, which is similar to a conserved motif GDSGG found in more than 200 different serine proteases.

From the analysis of the size of Spitz cleavage products by SDS-PAGE Freeman and colleagues have concluded that processing occurs within the transmembrane region close to the lumenal face. Consistent with the cleavage site, the positions of the putative catalytic residues in Rhomboid-1 are positioned within predicted transmembrane regions close to the luminal side. Thus Rhomboid-1 fulfils the criteria of an l-CliP and is the archetype of a serine l-CliP (Urban et al., 2001).

Rhomboids are conserved throughout evolution (Gallio and Kylsten, 2000; Wasserman et al., 2000). Their overall similarity is generally low, but they share all the conserved set of amino acids for the putative catalytic site. Apart from Drosophila, rhomboid homologues have only been investigated in two other organisms. In the gram-negative human pathogen *Providencia stuartii*, the homologue AarA controls secretion of a virulence factor (Rather et al., 1999; Rather et al., 1997). In the fungi *Saccharomyces cerevisiae* the homologue PCP1 processes a mitochondrial leader sequence (Esser et al., 2002). Thus it is likely that there are many more pathways involving rhomboids.
The substrate specificity of different rhomboids is rather low, indicated by the finding that several distantly related prokaryotic rhomboids can process Spitz (Urban et al., 2002b). The requirements for a rhomboid substrate have not yet been explored in detail, but in fact a clear consensus sequence can be excluded, since all substrates so far explored show no sequence similarities around their proposed cleavage site.

1.2.2.3. Presenilins (γ-ε-secretase)

The γ-secretase is the most prominent I-CLIP to date, because of its direct role in generating the AD causing Aβ42. Protease inhibitor studies strongly suggest that γ-secretase is an aspartic protease (Wolfe et al., 1999b). γ-Secretase is a multicomponent high molecular weight complex (Li et al., 2000b; Yu et al., 1998) and presenilin (PS) is the prime and exclusive candidate for the catalytic subunit of the γ-secretase complex. Nevertheless, there is speculation that PS plays a more indirect role during γ-secretase cleavage (Sisodia et al., 2001; Sisodia and St George-Hyslop, 2002).

PS is a polytopic membrane protein with a proposed eight transmembrane topology forming a ring structure (Annaert et al., 2001; Doan et al., 1996). It is mainly located in the ER, but a small proportion was also found in the Golgi, endosomes and at the plasma membrane (Kaether et al., 2002). After their biosynthesis, PS undergoes endoproteolysis catalysed by an unknown aspartic protease termed presenilase (Campbell et al., 2002). Endoproteolysis occurs in the large cytoplasmic loop between TM6 and TM7 generating a stable PS heterodimer consisting of an N-terminal (NTF) and a C-terminal fragment (CTF) (Thinakaran et al., 1997). PS, which has so far been identified only in animals and plants, shares no homology to any known proteins. It is therefore difficult to predict its molecular function.

In 1992, geneticists identified PS by genome-wide linkage studies and positional cloning, searching for mutations that result in familial AD (FAD)
(Levy-Lahad et al., 1995; Schellenberg et al., 1992; Sherrington et al., 1995). About 5-10% of all AD cases are associated with FAD mutations (Selkoe, 2001). In human two PSs, PS1 and PS2, have been identified. They are highly homologous proteins and functionally redundant (Steiner and Haass, 2000). To date, more than 100 missense mutations in PS1 have been associated with FAD. The molecular phenotype of most of these FAD-associated mutations is characterised by an increased Aβ42 production (Scheuner et al., 1996). In presenilin-deficient cells Aβ production is completely abolished and the γ-secretase substrates β-CTF and α-CTF accumulate (De Strooper et al., 1998; Herreman et al., 2000). Thus these findings show a direct physical participation of PS in γ-secretase activity (Steiner and Haass, 2000; Wolfe et al., 1999a).

There is now evidence suggesting that PS is the catalytic subunit of the aspartic protease γ-secretase, even though at first glance, PS does not look like a protease: Two conserved aspartic residues within adjacent transmembrane segments (TM6/TM7) of PS are essential for γ-secretase activity, indicating their potential catalytic role (Steiner et al., 1999; Wolfe et al., 1999c). The C-terminal aspartic residue is located in a motif GxGD (Steiner et al., 2000) that is conserved and essential in the recently identified unusual polytopic bacterial aspartic proteases type IV prepilin (TFPP) (LaPointe and Taylor, 2000). Compared to PS this putative catalytic motif is not embedded within a transmembrane region. Furthermore PS1 is co-purified and enriched as one purifies γ-secretase activity (Esler et al., 2002). These arguments are consistent with PS being a co-factor for γ-secretase activity. Another line of evidence, supporting the notion PS being an aspartic protease, is pharmacologic in nature. γ-secretase inhibitors based on aspartic transition state analogues directly bind to the heterodimeric form of PS (Esler et al., 2000; Li et al., 2000c). Taken together, these arguments are indirect and not a formal proof that PS is a protease.

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3 see http://www.alzforum.org
Different observations question the role of PS as protease (reviewed in Sisodia et al., 2001; Sisodia and St George-Hyslop, 2002). First of all, Checler and colleagues have reported endogenous Aβ production in PS1/PS2-deficient embryonic mouse fibroblasts, indicating that PS is not required for γ-secretase activity (Armogida et al., 2001). Remarkably, other groups could not reproduce this indeed exceptional result (Grimm et al., 2002; Nyabi et al., 2002). Furthermore, co-expression of distinct PS1 aspartic mutants (D257A or D257A/D385A) in mouse neuroplastoma cells resulted in the accumulation of γ-secretase substrates, but not in abrogation of Aβ production (Kim et al., 2001a), arguing that PS plays a role in γ-secretase substrate stability and trafficking, but not cleavage. Another argument, the subcellular distribution of PS to the ER apparently does not correlate with the cellular localisation of γ-secretase activity in the late secretory compartments (Annaert et al., 1999; Cupers et al., 2001). However, approximately 1/33 of total PS was recently found at the plasma membrane (Kaether et al., 2002).

Assuming that PS is a protease, it likely mediates a simultaneous dual intramembrane cleavage, namely at the γ- and ε-processing site (Okochi et al., 2002)(Fig 1.2). Both cleavages have been shown to be strictly dependent on PS (De Strooper et al., 1999; De Strooper et al., 1998) and to be equally affected by inhibitors (Kimberly et al., 2003; Okochi et al., 2002). Most importantly, no intermediate product that is processed only at one of both sites has ever been detected (Kimberly et al., 2003; Moehlmann et al., 2002). How do PS achieve to cleave at two topologically distinct sites has been proposed by multiple active site conformations of PS (Haass and Steiner, 2002). It was also proposed that these two cleavages might be catalysed by distinct catalytic activities that both require PS as a co-factor.

PS does not recognize a specific consensus cleavage site and the substrates show almost no similarities apart that they are all type I transmembrane proteins (Struhl and Adachi, 2000). Solely, a conserved valine close to the ε-processing site attracted attention. Mutagenesis of this valine abolishes ε-cleavage of the PS substrate NOTCH but not in APP (Huppert et al., 2000;
Murphy et al., 1999). A recent report however showed that mutation of the valine only decreases the stability of the cleavage product, which thereby escapd detection (Blat et al., 2002). Thus, the suggestion that PS may cleave type I transmembrane proteins, provided that the extracellular domain is relatively short (> 50aa), is still valid (Struhl and Adachi, 2000). It is also speculated that PS might play a general role in removal of transmembrane domains (Haass and Steiner, 2002).

Biochemical reconstitution of γ-/ε-secretase activity using purified components would proof whether or not PS catalyses one or both of these proteolytic activities. PS is part of a high molecular weight complex including several essential components for γ- and ε-secretase activity (Francis et al., 2002; Li et al., 2000b; Yu et al., 2000). Apart from PS, the essential components of this complex are the membrane proteins Nicastrin, PEN-2 and APH-1 (Chen et al., 2001; Francis et al., 2002; Hu et al., 2002; Yu et al., 2000). It has been suggested that these proteins are required for coordinated complex assembly, maturation and stability (Kaether et al., 2002; Luo et al., 2003). So far, it cannot be excluded that the complex contains even more essential components. Considering the inherent difficulties of working with the γ-secretase complex membrane proteins and limited knowledge about the components and the stoichiometry, the biochemical reconstitution will hardly be achieved in a short time. Collecting evidence for the different suggestions is the current way to explore the secrets of PS.
1.3. Signal peptide processing – Liberation of bioactive peptides by I-ClIP SPP?

1.3.1. Protein targeting by signal sequences

The pathway of secretory proteins from their synthesis to their extrusion from the cell, the so-called “secretory pathway”, was initially described mainly by Palade and co-workers in the sixties (for review see Palade, 1975). They found that secretory proteins cross the ER membrane during or shortly after synthesis, raising the question how secretory proteins are sorted to the ER, while cytosolic proteins are not.

Addressing this question, Blobel formulated his first version of the “signal hypothesis” in 1971 (Blobel and Sabatini, 1971). He proposed a sequence “X” in the nascent polypeptide chain that is recognized by a “binding factor” that mediates binding to the ER membrane. At that time there was no supporting evidence for this proposal, but later findings confirmed the “signal hypothesis” (for review see Blobel, 2000). In brief, secretory and also most membrane proteins contain an N-terminal signal sequence (“X”) that is essential for targeting to the ER protein-conducting channel, referred to as translocon, and their subsequent translocation across the ER membrane (for review see Johnson and van Waes, 1999; Rapoport et al., 1996). Similar principles are also applied for targeting proteins to mitochondria, chloroplasts, peroxisomes or bacterial plasmamembrane (reviewed in Schnell and Hebert, 2003; Paetzel et al., 2002; Schatz and Dobberstein, 1996).

The characteristic feature of ER signal sequences is a tripartite structure: a positively charged N-terminal region (n-region); a central hydrophobic region (h-region) of 7-15 amino acid residues; and a more polar C-terminal region (c-region). The h-region is thought to span the ER membrane after insertion (Briggs et al., 1986). Signal sequences display almost no sequence similarity
and have substantial variability in their overall length (15-50 amino acids) (von Heijne, 1985).

In the co-translational mode of targeting, a signal recognition particle (SRP, "Binding factor"; for review see Keenan et al., 2001; Stroud and Walter, 1999) subunit binds via hydrophobic interaction to the signal sequence and to the ribosome as soon as the signal sequence emerges from the ribosome during protein biosynthesis. Then, the entire complex consisting of the ribosome, the nascent chain and SRP is directed via the SRP receptor (SR) to the ER membrane. There, a GTP-dependent interaction of SRP with SR triggers a series of events that includes the release of the signal sequence from SRP, the tight binding of the ribosome to the translocon and the release of SRP and SR from the ribosome/translocon complex. After signal sequence dependent channel opening, protein synthesis continues and the nascent chain is moved through the sec61 translocation machinery into the ER (for review see Johnson and van Waes, 1999).

After targeting, the membrane inserted signal sequence is usually cleaved co-translationally from the precursor by the membrane-associated serine protease signal peptidase complex (SPC) (Dalbey et al., 1997; Paetzel et al., 2002). Cleavage occurs at the end of the c-region close to the membrane spanning region in the lumen of the ER and requires small uncharged residues in positions -3 and -1 relative to the cleavage site (von Heijne, 1990). After removal from the precursor, the former signal sequence becomes a type II orientated membrane protein, here termed signal peptide (SP).
1.3.2. Signal peptide processing

The role of signal sequences in protein targeting and translocation is well established (see above), but their fate after they have been cleaved from the precursor protein is largely unknown. It has been shown that synthetic signal peptides can enter lipid bilayers and lyse biological membranes (Killian et al., 1990) and that they inhibit protein translocation. Therefore, an efficient mechanism to remove signal peptides from the membrane may be necessary.

In *E. coli* signal peptides, which are similar to the ER signal peptides, are cleaved by the cytosolic membrane anchored serine protease IV (Ichihara et al., 1986; Suzuki et al., 1987) and further hydrolyzed by the cytoplasmic metallo oligopeptidase A (Novak and Dev, 1988). Interestingly, in strains with a deletion of the gene sppA encoding protease IV, signal peptides are still degraded, showing that other proteases also function in signal peptide degradation (Suzuki et al., 1987). Since homologues of protease IV and oligopeptidase A are only found in bacteria, another pathway to remove signal peptides must be applied in eukaryotes.

Recent studies addressed the fate of signal peptides in the ER. Using a synchronized *in vitro* translation/translocation system it has been shown that the signal peptide of bovine pre-prolactin (p-Prl) is cleaved after SPC cleavage by an unknown activity resulting in the release of a N-terminal signal peptide fragment (SPF) from the ER membrane into the cytosol (Lyko et al., 1995; Klappa et al., 1996). This unknown enzyme activity has been termed signal peptide peptidase (SPP or SPPase).

Most standard protease inhibitors do not affect SPP activity (Klappa et al., 1996). Interestingly, the addition of the immunosuppressant cyclosporin A inhibits signal peptide processing, suggesting that SPP might be cyclophilin dependent (Klappa et al., 1996). Recent data have shown that two cysteine
protease inhibitors, namely calpain inhibitor I and (ZLL)$_2$-ketone$^4$, inhibit SPP activity, but not signal sequence cleavage, indicating that SPP and SPC are distinct activities.

The estimated size of the SPP cleavage product proposes that processing occurs most likely within the transmembrane spanning h-region of the signal peptide (Lyko et al., 1995). This led to the speculation that SPP could be an I-CliP (Martoglio, 1999). Whether signal peptide processing by SPP mainly removes dispensable signal peptides from the ER membrane to the cytosol where they are degraded or whether it represents limited processing that results in a functional change of the substrate, is subject of speculations.

1.3.3. Post-targeting functions of signal peptides

The main function of signal sequences is addressing proteins for secretion. But the hydrophobic interaction between the h-region and SRP does not require a conserved primary sequence (von Heijne, 1985), allowing signal sequences to have a high degree of sequence variability. In principle, signal sequences can therefore comprise more information than required for SRP binding. This idea is supported by the recent finding that signal sequences control gating of the translocon in a substrate-specific manner (Kim et al., 2002), influence topology of its precursor protein (Kim et al., 2001b) and can control SPC cleavage timely and in efficiency (Rutkowski et al., 2001).

The diversity of signal sequence may pinpoint to post-targeting function of some signal peptides as bioactive peptides. In this line, ER membrane released p-Prl and HIV gp160 signal peptide fragments (SPF) associate in vitro with calmodulin (CaM) in a Ca$^{2+}$ dependent manner (Martoglio et al., 1997). These two signal peptides have the propensity to form basic amphiphilic a(bba) helixes characteristic for CaM binding domains (O'Neil and

$^4$ see Chapter 2.1 (Result Part I), data not provided by thesis author.
DeGrado, 1990). Whether this interaction influences Ca2+/CaM dependent signal-transduction pathways and has a physiological role remains to be established. It has been speculated that p-Prl SPF might regulate cAMP–dependent Prl secretion via modulating Ca2+/CaM dependent cAMP levels. Evidence for the relevance of the CaM binding domain of p-Prl SPF is also implicated from the genomic arrangement of the Prl signal sequence. Normally signal sequences are encoded by a single exon, but in the case of p-Prl distinct exons encode the CaM binding domain (n-region) and the SRP binding domain (h-region) indicating that they could be two functionally distinct domains (Gilbert, 1985).

Furthermore signal peptides of the classical histocompatibility complex (MHC) class I molecules HLA-A, -B, C and -G HLA-C are implicated in post-targeting functions. The signal peptides contain epitopes that are presented by Non-classical MHC Class I molecules (HLA-E) on most nucleated cells (Braud et al., 1997). At the cell surface the HLA-E-epitope complex binds to the antigen-specific CD94/NKG2A receptor on natural killer (NK) cells and thereby prevents NK cells mediated lysis (Borrego et al., 1998; Braud et al., 1998a; Lanier, 1998). HLA-E is only transported from the ER toward the plasma membrane when it is correctly assembled and peptide loaded or differently only when classical MHC Class I molecules are synthesized in the cell. Thus in this case SPFs are used to monitor the synthesis of their precursor protein.

It is not known how signal peptide derived epitopes presented by HLA-E are generated. Usually, epitopes derived from pathogens and cellular proteins are mainly generated by proteosomal degradation. Interestingly, some analysed signal peptide derived epitopes contain a hydrophobic stub of the h-region and their presentation is dependent of the transporter associated with antigen presentation (TAP) (Braud et al., 1998b; Lee et al., 1998), suggesting that after targeting the peptides are released into the cytosol and then transported by TAP back into the ER. Therefore, it has been speculated that HLA-E signal sequence derived epitope generation could be another example of signal
peptide processing involving the potential I-CliP SPP and likely additional trimming peptidases (Martoglio and Dobberstein, 1998).

In summary, it emerges that some signal peptides are required for processes beyond targeting. It is tempting to speculate that two-step signal peptide processing by SPC and the putative I-CliP SPP is used to promote the release of bioactive peptides from the ER membrane, in analogy to RIP (see Figure 1.3 for illustration).

**Figure 1.3 Signal peptide processing – Intramembrane proteolysis of signal peptides**

After targeting membrane and secretory proteins to the ER Sec61 translocation machinery, signal peptides are co-translationally liberated from their precursor in the ER lumen by the signal peptidase complex (SPC). It is hypothesised that liberated signal peptides undergo intramembrane proteolysis catalysed by the putative I-CliP Signal peptide peptidase (SPP). Cleavage products are thereby released from the ER membrane into the cytosol and the ER lumen, respectively, to perform functions beyond targeting.
1.4. Aim of thesis

The aim of this thesis was the molecular identification of the putative I-CLiP SPP that is implicated in the activation of signal sequence-derived bioactive peptides. So far all putative I-CLiPs have been identified based on genetical approaches. This was mainly due the inherent difficulties of working with polytopic membrane proteins or the lack of suitable biochemical in vitro assays for intramembrane proteolysis. Nevertheless, genetic studies provided strong evidence for the proteolytic activity of the respective I-CLiPs, but it cannot be excluded that the identified proteins are just essential co-factors of the elusive I-CLiPs. Indeed, most genetical approaches cannot discriminate between essential factors and the enzyme itself; a beneficial effect to reveal factors involved in a biological pathway, but not to discover a targeted enzyme.

This thesis aimed to identify SPP by biochemical purification and subsequent sequencing, initiating biochemistry in I-CLiP research. The basis for purification of the unknown protein was an assay that allowed monitoring SPP activity during protein fractionation. In order to purify the native protein, the assay is preferably an activity assay, indicating the native structure. Alternatively, the protein of interest may be selectively labelled by an affinity-label, and subsequently purified under denaturing conditions. For the purpose of SPP identification, the former strategy was initially intended, but it turned out that a combination of both strategies was eventually successful.

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5 In 1999, when the author started his thesis, indeed no biochemical data about any I-CLiPs was published. Today, γ-secretase has been investigated to some extent by biochemical methods.
1.5. Outline of thesis

In the first part of the following result section, the development of tools is described that were later used to identify SPP, confirm its proteolytic activity and perform inhibitor studies. On the one hand, this is an assay that allows monitoring intramembrane proteolysis by SPP with detergent solubilised proteins. On the other hand the preparation of detergent solubilised dog pancreas derived rough ER membrane proteins was established, providing the source for SPP purification. And last, a specific SPP inhibitor was designed and synthesised (not contributed by thesis author).

The second result part - the core of this thesis - reports the identification of SPP using an affinity labelling strategy, including protein purification and sequencing. Subsequently, bioinformatical sequence analysis, recombinant protein expression, and mutational analysis are described.

The last result part addresses the specificity of γ-secretase and SPP inhibitors, revealing that several γ-secretase inhibitors that are considered therapeutical molecules against AD also direct SPP.

The discussion then starts with a general review about l-CNPs and RIP that includes results provided by this thesis and related work in our laboratory (Chapter 3). The review is followed by a thesis specific discussion and concluding remarks.
Chapter 2

2. Results
The results section contains three publications with the contributions of myself and additional people. Therefore, a short appendix ("Thesis author's work") after each article highlights the work performed by myself.

The publications contain only short descriptions of "Materials and Methods". Therefore, an independent chapter of this thesis will describe the methods established and improved by myself in more detail.

While this thesis was ongoing, it has been shown that intramembrane proteolysis by SPP is required for HLA-E epitope generation (Lemberg et al., 2001) and Hepatitis C virus core processing (McLauchlan et al., 2002). Furthermore, the requirements for cleavage by SPP have been investigated (Lemberg and Martoglio, 2002). These reports took advantage of results provided by this thesis and are therefore discussed later in chapter 4 and 5.
2.1. Result Part I:
Release of Signal Peptide Fragments into the Cytosol Requires Cleavage in the Transmembrane Region by a Protease Activity That Is Specifically Blocked by a Novel Cysteine Protease Inhibitor

Andreas Weihofen\textsuperscript{1}, Marius K. Lemberg\textsuperscript{1}, Hidde L. Ploegh\textsuperscript{2}, Matthew Bogio\textsuperscript{3} and Bruno Martoglio\textsuperscript{1}

\textsuperscript{1} Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
\textsuperscript{2} Dept. of Pathology, Harvard Medical School, 200 Longwood Ave., Boston, MA02115
\textsuperscript{3} Dept. of Biochemistry and Biophysics, University of California, 513 Parnassus Ave., San Francisco, CA 94143-0448

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2.1.1. Abstract

Signal peptides of secretory and membrane proteins are generated by proteolytic processing of precursor proteins after insertion into the endoplasmic reticulum membrane. Liberated signal peptides can be further processed, and the resulting N-terminal fragments are released toward the cytosol, where they may interact with target proteins like calmodulin. We show here that the processing of signal peptides requires a protease activity distinct from signal peptidase. This activity is inhibited specifically with a newly developed cysteine protease inhibitor, 1,3-di-(N-carboxybenzoyl-L-leucyl-L-leucyl)amino acetone ((Z-LL)₂ ketone). Inhibitor studies revealed that the final, (Z-LL)₂ ketone-sensitive cleavage event occurs within the hydrophobic transmembrane region of the signal peptide, thus promoting the release of an N-terminal fragment into the cytosol.
2.1.2. Introduction

Secretory proteins and most membrane proteins of eukaryotic cells are expressed as a pre-protein with an N-terminal signal sequence that is essential for protein targeting to the endoplasmic reticulum (ER) membrane and entry into the translocon (Walter and Johnson, 1994). Signal sequences are usually released from the precursor protein by signal peptidase during passage of the growing polypeptide chain through the ER membrane. Little is known about the fate of the liberated signal peptides. However, they have been postulated to have important biological functions both in the lumen of the ER as well as in the cytosol (Martoglio and Dobberstein, 1998).

Signal peptides, liberated from the precursor protein, can be processed further, resulting in fragments that are released from the membrane (Klappa et al., 1996; Lyko et al., 1995). In the case of the hormone preprolactin (p-Prl) and the human immunodeficiency virus-1 gp160, the N-terminal portion of the respective signal peptide is released into the cytosol and binds to Ca\(^{2+}\)/calmodulin \textit{in vitro} (Martoglio et al., 1997). These findings imply that processing of liberated signal peptides releases functional peptides that may influence signal transduction pathways in the cell.

More recently, a distinct peptide derived from an N-terminal portion of a signal sequence was found to play a crucial role in immune surveillance of healthy cells. The signal peptides of polymorphic major histocompatibility complex class I molecules contain a highly conserved sequence that is capable of binding to so-called non-polymorphic major histocompatibility complex class I molecules (HLA-E in human) (Braud et al., 1997). At the cell surface, this peptide-HLA-E complex specifically interacts with an inhibitory receptor on natural killer (NK) cells, thereby monitoring indirectly the level of class I molecule expression (Borrego et al., 1998; Braud et al., 1998a). Presentation of the signal peptide-derived epitope is dependent on the transporter associated with antigen presentation (TAP) and is independent of the proteasome (Bai and Forman, 1997). These results suggest that the signal
peptide of class I molecules is processed in a manner analogous to the p-Prl signal sequence, leading to release of the epitope-containing portion toward the cytosol.

In eukaryotes, proteases involved in the processing of signal peptides have yet to be characterized or identified. Protease IV and oligopeptidase A process signal peptides in *Escherichia coli*, but homologous proteases have not been found in eukaryotes (Novak and Dev, 1988). A new class of metalloproteases that catalyze so-called intramembrane proteolysis has recently been discovered (Rudner et al., 1999). These proteases cleave their substrate proteins within transmembrane regions and promote release of protein domains toward the cytoplasmic or exoplasmic side of the membrane. Signal peptides may be processed by a similar type of protease that cleaves the peptide within the transmembrane region and facilitates release of peptide fragments from the membrane.

In the present study, we characterize the processing of the p-Prl signal peptide. By using different types of protease inhibitors, we ascribe signal sequence cleavage and signal peptide processing to distinct proteolytic activities. A new inhibitor is introduced that prevents signal peptide processing efficiently without affecting signal peptidase and other proteases such as the lysosomal cathepsins and the proteasome. We show that processing promotes release of the Ca\(^{2+}\)/calmodulin binding signal peptide portion from the membrane into the cytosol. Furthermore, we have determined the cleavage site of the final processing reaction to lie in the center of transmembrane region of the signal peptide.
2.1.3. Results

Synthesis of Peptide Ketone Inhibitors—Analysis of the amino acid sequence surrounding the putative intra-membrane cut site of the signal peptide from preprolactin and human immunodeficiency virus-1 gp160 indicated that both contain a cluster of hydrophobic amino acids on either side of the scissile amide bond (Fig. 2.1.1A). Based on this information, we designed protease inhibitors that contain a central electrophile surrounded by aliphatic amino acid residues. Previously, Veber and co-workers (Marquis et al., 1999; Marquis et al., 1998) found that symmetrical ketone derivatives served as potent inhibitors of the cysteine protease cathepsin K. These compounds bind to the enzyme such that the peptide portions of the molecules on either side of the ketone electrophile occupy both the prime and non-prime binding sites. We reasoned that analogs of these compounds might be well suited for mimicking the hydrophobic core of the signal peptide and, therefore, might serve as potent inhibitors of the putative signal peptide peptidase activity. We first synthesized the simple symmetrical di-leucine-containing derivative in which both N termini were capped with the hydrophobic carboxybenzoyl group ((Z-LL)$_2$ ketone; Fig. 2.1.1B). The related compound in which the Z capping group was replaced with a $t$-butoxycarbonyl group was also synthesized. This derivative was de-protected with acid, resulting in a di-free amino derivative that was then alkylated by biotin ((Bio-LL)$_2$ ketone; Fig. 2.1.1B).
Figure 2.1.1 Synthesis of symmetrical di-leucine-containing ketones. A, sequence of the signal peptide of preprolactin and human immunodeficiency virus-1 gp160. The transmembrane regions are shown shaded, and brackets are used to indicate the clusters of long hydrophobic amino acid residues on either side of the predicted signal peptide peptidase cleavage region (arrow). B, synthesis of di-leucine ketone derivatives from di-amino acetone. (i) HATU, collidine in N,N-dimethylformamide; (ii) 50% trifluoroacetic acid in CH₂Cl₂; (iii) biotin-p-nitrophenyl-ester, N,N-diisopropylethylamine in N,N- dimethylformamide.
Analysis of Specificity of Ketone Inhibitors-- To initially determine the specificity of both the Z and biotin ketone derivatives, we performed competition experiments in crude cellular extracts. Lysates from the dendritic cell line DC2.4 were incubated with increasing concentrations of both (Z-LL)$_2$ ketone and (Bio-LL)$_2$ ketone for 30 min at room temperature (Fig. 2.1.2). After pre-incubation, a radiolabeled general cysteine protease inhibitor $^{125}$I-JPM-565 was added to the extracts. This compound covalently modifies the active site of most of the papain family of cysteine proteases. Intensity of labeling of protease targets serves as readout of enzymatic activity. Thus, prior modification of the active site of the enzyme by either (Z-LL)$_2$ ketone or (Bio-LL)$_2$ ketone is observed as a loss of labeling by $^{125}$I-JPM-565. The results from DC2.4 extracts indicated that at low concentrations (0.1-10 µM) neither the Z nor biotin-capped derivatives are reactive toward any of the multiple lysosomal cysteine proteases targeted by the general cysteine protease label $^{125}$I-JPM-565. The Z compound at high concentrations specifically blocks labeling of a single 25-kDa polypeptide. The general cysteine protease inhibitor leupeptin blocked labeling of most of the JPM-565-reactive proteases, indicating that the assay was an effective readout of lysosomal cysteine protease activity. Therefore, biological effects observed from treatment of samples with low concentrations of the inhibitors (Z-LL)$_2$ ketone and (Bio-LL)$_2$ ketone are not likely due to inhibition of lysosomal proteases.

To further establish the reactivity of these ketone derivatives, we performed a similar set of competition experiments in NIH-3T3 extracts using the proteasome label $^{125}$I-NLVS as readout. These experiments provided complementary information about the reactivity of the two compounds toward the proteasome. Both the (Z-LL)$_2$ ketone and the (Bio-LL)$_2$ ketone showed no modification of the proteasomal active site at concentrations as high as 100 µM (data not shown). Thus, these compounds do not block the action of the multicatalytic proteasome complex.
Figure 2.1.2 Di-leucine-containing ketones do not inhibit the lysosomal cysteine proteases. The ketone derivatives (Z-LL)$_2$ ketone, (Bio-LL)$_2$ ketone, and the peptide aldehyde leupeptin were added to extracts from the dendritic cell line DC2.4 at the concentrations indicated. Lysates were incubated for 30 min at room temperature, and then the cysteine protease affinity label $^{125}$I-JPM-565 was added. Cat, cathepsin.
Distinct Proteases Catalyze Signal Sequence Cleavage and Signal Peptide Processing—To investigate processing of the p-Prl signal peptide, we used a previously established in vitro assay that includes synchronized entry of short p-Prl chains into ER-derived rough microsomes (Martoglio et al., 1997). Truncated mRNA coding for the 86 N-terminal residues of p-Prl was translated in the presence of rough microsomes. The resulting p-Prl/86 chains were bound to the ribosomes at their C terminus and inserted into the translocons via their N-terminal signal sequence. Signal sequence cleavage did not occur because the p-Prl/86 chains were too short (Fig. 2.1.3 A, lane 1). Microsomes were isolated and resuspended in buffer, and p-Prl/86 chains were released from the ribosome by the addition of puromycin. p-Prl/86 chains were translocated, and the signal sequence was cleaved and processed. The liberated, 30-residue signal peptide was seen after a short incubation with puromycin (Fig. 2.1.3 A, lane 2), whereas the processed peptide was obtained after longer incubation (Fig. 2.1.3 A, lane 3).

To distinguish the individual proteolytic steps of signal sequence cleavage and signal peptide processing, we tested several different classes of protease inhibitors. The newly developed cysteine protease inhibitors (Z-LL)₂ ketone and (Bio-LL)₂ ketone inhibited signal peptide processing without affecting signal peptidase activity (Fig. 2.1.3 B, lanes 2 and 3). The apparent IC₅₀ value determined by our assay was ~50 nM for (Z-LL)₂ ketone (Fig. 2.1.3 C) and 1-2 μM for (Bio-LL)₂ ketone (not shown). Similarly, signal peptide processing was inhibited by calpain inhibitor I, another cysteine protease inhibitor, albeit at much higher concentration (Fig. 2.1.3 B, lane 4). In contrast, the serine protease inhibitor dichloroisocoumarin inhibited signal peptidase, thereby blocking release of the signal sequence from the precursor protein (Fig. 2.1.3 B, lane 5). These results indicate that signal peptide processing requires at least one protease that is distinct from signal peptidase.
Figure 2.1.3 (Z-LL)$_2$ ketone inhibits processing of the p-Prl signal peptide. A, cleavage and processing of the p-Prl signal sequence. ER-derived rough microsomes were loaded with ribosome-bound p-Prl/86 chains. Microsomes were next isolated and resuspended in buffer (lane 1). To induce synchronized signal peptide cleavage and processing, the p-Prl/86 chains were released from the ribosome by the addition of puromycin. Samples were incubated for 2 min and 10 min, respectively, and subsequently analyzed by SDS-PAGE (lanes 2 and 3). In vitro translated reference signal peptide is shown in lane 4. SP, 30-residue-long signal peptide; SPF, N-terminal signal peptide fragment. B, inhibition of signal peptide processing. p-Prl/86 chains were released from the ribosome as in A upon the addition of 1 μM (Z-LL)$_2$ ketone (lane 2), 10 μM (Bio-LL)$_2$ ketone (lane 3), 500 μM calpain inhibitor I (lane 4), and 500 μM dichloroisocoumarin (lane 5). C, the apparent IC$_{50}$ value for (Z-LL)$_2$ ketone was determined by releasing p-Prl/86 chains upon the addition of increasing amounts of (Z-LL)$_2$ ketone. Half-maximal activation is observed at ~50 nM inhibitor.
**Processing Promotes Release of the N-terminal Signal Peptide Fragment toward the Cytosol**—We have reported that the N-terminal portion of the p-Prl signal peptide is released into the cytosol in vitro, where it binds to Ca\(^{2+}\)/calmodulin upon cleavage and processing (Martoglio et al., 1997). To determine whether processing is required for release of the N-terminal peptide into the cytosol, we treated microsomes with (Z-LL)\(_2\) ketone before release of p-Prl/86 chains with puromycin. After treatment with puromycin, we separated microsomes from the release buffer by centrifugation and analyzed the microsomes and buffer fraction separately. In the presence of (Z-LL)\(_2\) ketone, the unprocessed signal peptide is found exclusively in the membrane fraction (Fig. 2.1.4 A, lane 5). In the control reaction where the inhibitor is omitted, the signal peptide is processed, and the N-terminal fragment is released from the membrane toward the cytosolic side and, hence, found in the supernatant fraction (Fig. 2.1.4 A, lane 4).

Cytosol facilitates the release of the processed p-Prl signal peptide from the membrane, most likely due to the presence of its target, calmodulin (Martoglio et al., 1997). To test whether signal peptide processing is required for efficient release of the N-terminal portion, we translated full-length p-Prl in reticulocyte lysate in the presence of microsomes and (Z-LL)\(_2\) ketone. Microsomes were extracted with 500 mM salt and sedimented through a sucrose cushion to remove excess cytosolic proteins that interfere with the analysis of small peptides. When (Z-LL)\(_2\) ketone was added to the translation mixture, the unprocessed signal peptide was found in the membrane fraction (Fig. 2.1.4 B, lane 3). In contrast, no corresponding peptide was obtained in the control reaction without the inhibitor (Fig. 2.1.4 B, lane 2). These results show that processing of the p-Prl signal peptide is required for efficient release of the N-terminal peptide portion, even in the presence of cytosol.
Figure 2.1.4 Inhibition of processing prevents release of the signal peptide from the membrane. A, microsomes loaded with ribosome-bound p-Prl/86 chains were isolated and resuspended in buffer, and where indicated, 1 μM (Z-LL)2 ketone was added (lanes 5 and 6). p-Prl/86 chains were released from the ribosome by the addition of puromycin (lanes 3-6). Samples were incubated for 10 min, and microsomes were subsequently separated from the buffer by centrifugation. Membrane pellets (Pel) and buffer fractions (Sup) were analyzed separately. The N-terminal portion of the processed signal peptide was released from the membrane (lane 4), whereas inhibition of processing retained the signal peptide in the membrane fraction (lane 5). B, full-length p-Prl chains were synthesized in reticulocyte lysate in the presence of rough microsomes (lanes 2 and 3) and (Z-LL)2 ketone (lane 3). Microsomes were extracted with 500 mM salt and recovered by centrifugation. The unprocessed signal peptide (SP) was retained in the membrane fraction, even in the presence of cytosol (lane 3). Lane 4 shows in vitro translated reference signal peptide.
The Signal Peptide Is Processed in the Center of the Transmembrane Region-- The p-Prl signal peptide is thought to be processed in the region between the two leucine clusters of its hydrophobic region (Lyko et al., 1995). To determine the cleavage site, we compared the electrophoretic mobility of the cleavage product with reference peptides. The cleavage product detected must represent the N-terminal fragment, because the p-Prl signal peptide is radioactively labeled at a single methionine residue at its N terminus. Reference peptides were thus easily obtained by in vitro translating mRNAs coding for the N-terminal 18-, 20-, 25-, and 30-amino acid residues of the p-Prl signal sequence (Fig. 2.1.5 A).

The cleavage product had mobility identical to that of the 20-residue reference peptide (Fig. 2.1.5A, lanes 3 and 4), indicating that the p-Prl signal peptide is cleaved in the center of its transmembrane region, where the polar residues serine and asparagine interrupt the hydrophobic segment. Interestingly, a common feature of the transmembrane region of signal peptides is the acquisition of a helix-break-helix structure in an apolar environment (van Klompenburg and de Kruijff, 1998). The break in the helix structure is thought to facilitate membrane entry at the initial phase of protein translocation and may also make the scissile peptide bond accessible to proteolysis during signal peptide processing.

The investigation of signal peptide processing requires ER-derived microsomes that are functional in protein targeting, translocation, and signal sequence cleavage. To analyze processing independently of preceding reactions, we simplified the assay by using the p-Prl signal peptide (p-Prl/30) as the immediate substrate for the cleavage reaction. Because synthetic signal peptides are known to enter the translocon without the aid of cytosolic components (Simon and Blobel, 1992), p-Prl/30 is expected to enter the translocon and be processed similarly to the signal peptide that enters the translocon by the conventional protein targeting pathway. Indeed, when in vitro translated p-Prl/30 was incubated together with microsomes, we obtained a cleavage identical to the one obtained with the previous assay,
where p-Prl/86 chains were inserted into the translocons and cleaved by signal peptidase before the liberated signal peptide could be processed (Fig. 2.1.5 B, lanes 2 and 3).

In an initial attempt to characterize the protease that catalyzes processing of the p-Prl signal peptide, we extracted rough microsomes first with puromycin and 600 mM salt to remove the ribosomes and peripherally associated proteins and then with alkali to wash out lumenal proteins (Hamman et al., 1998; Nicchitta and Blobel, 1990). The resulting PKXRMs were still active and processed p-Prl/30 to the ~20-residue fragment (Fig. 2.1.5 B, lane 4). We then solubilized the residual ER membrane proteins with the detergent CHAPS. When p-Prl/30 was incubated with the detergent-solubilized proteins, it was processed to the ~20-residue fragment, as with intact, untreated microsomes (Fig. 2.1.5 B, lane 5). (Z-LL)$_2$ ketone inhibited the processing reaction, although a ~27-residue fragment was obtained instead (Fig. 2.1.5 B, lane 7). The latter cut was most likely performed by signal peptidase that, when solubilized, may have access to a second potential consensus site in the C-terminal extension of the p-Prl signal peptide (von Heijne, 1985). These results indicate that "intramembrane proteolysis" can be re-constituted using detergent-solubilized membranes.
Figure 2.1.5 The p-Prl signal peptide is processed in the center of the transmembrane region. A, parallel electrophoretic analysis of the signal peptide fragment obtained after puromycin release of p-Prl/86 chains (lane 3) compared with in vitro translated reference peptides corresponding to the N-terminal 30-, 18-, 20-, and 25-amino acid residues of the p-Prl signal peptide (lanes 1, 2, 4, and 5). B, signal peptide processing with detergent-solubilized ER membrane proteins. In vitro translated signal peptide of p-Prl (p-Prl/30, lane 1) was incubated with rough microsomes (lane 3), puromycin/high salt and alkali-treated microsomes (PKXRMs, lane 4), and CHAPS-solubilized PKXRMs (lanes 5 and 7). To one sample, 1 μM (Z-LL)₂ ketone was added (lane 7). The electrophoretic mobility of the resulting cleavage products is compared with the signal peptide fragment obtained after puromycin release of p-Prl/86 chains into rough microsomes (lane 2), and 20- and 27-residue-long reference peptides (lanes 6 and 8).
2.1.4. Discussion

The role of signal sequences in protein targeting and membrane insertion is well established (Stroud and Walter, 1999). The fate of signal peptides beyond cleavage from the pre-protein, however, remains unclear. Degradation may be the immediate destination for most signal peptides that are cleared from the ER membrane by as of yet unidentified proteases. However, in some cases signal peptides have been shown to perform functions downstream of precursor protein processing. Dissecting the pathway followed by signal peptides, as presented here, reveals a possible mechanism for how the ER membrane is cleared from peptides and indicates an approach toward the identification of the distinct components involved in the release process.

*Signal Peptide Processing Includes a Novel Type of Intramembrane Proteolysis*—Cleavage of proteins in transmembrane regions and concomitant release of protein domains or peptides from the membrane, as reported here for the signal peptide of p-Prl, has become increasingly evident and is involved in cellular differentiation, lipid metabolism, and presumably, the unfolded protein response (Brown et al., 2000; Martoglio, 1999). The process was named intramembrane proteolysis, assuming that cleavage occurs in the plain of the lipid bilayer, although the proof of proteolysis within the membrane is not provided so far (Brown et al., 2000).

In animal cells, at least three proteins undergo intramembrane proteolysis according to the definition given above. These proteins are SREBP(s) (sterol regulatory element-binding proteins) and Notch, transmembrane proteins of the ER and Golgi/plasma membrane, respectively, whose cytosolic transcription factor domains are liberated upon activation, and APP (β-amyloid precursor protein), which can be processed to the amyloid peptide Aβ suspected to cause Alzheimer's disease (Brown and Goldstein, 1997; Selkoe, 1999).
SREBPs and Notch are cleaved close to the cytosolic end of their respective transmembrane regions of type II topology (N in, C out) and type I topology (N out, C in), respectively. In contrast, APP is cleaved in the center of its transmembrane region, which has type I topology. The latter type of cleavage is analogous to the processing of the preprolactin signal peptide, which is also cleaved in the center of the transmembrane region. However, the membrane orientation of the signal peptide is opposite to that of APP. This finding adds a missing link to the group of proteases that perform intramembrane proteolysis: a protease that cleaves in the center of a type II-oriented transmembrane region (Martoglio, 1999).

With the exception of S2P, proteases that promote cleavage in transmembrane regions have not been identified so far. This putative metalloprotease catalyzes cleavage within one of the two membrane anchors of SREBPs and was identified by complementation cloning (Rawson et al., 1997). Data base searches revealed a family of S2P-like metalloproteases with the common, unusual feature of a HExxH motive within a transmembrane region, suggesting that intramembrane proteolysis is a process conserved in evolution from bacteria to man (Rudner et al., 1999).

Signal peptide processing is inhibited by cysteine protease inhibitors. Inhibition is particularly efficient with the novel (Z-LL)_2 ketone, designed according to the expected cleavage site within the signal peptide. Metalloprotease inhibitors such as EDTA (25 mM) and o-phenanthroline (5 mM) have no effect (not shown). These findings suggest that the unidentified signal peptide peptidase belongs to another class of proteases that cleave presumably in the plane of the lipid bilayer (Martoglio, 1999). The molecular identification of signal peptide peptidase remains a challenging task for future research.

Intramembrane proteolysis and concomitant release of a functional peptide also occurs in bacteria. The eubacterium Enterococcus faecalis secretes an
octapeptide pheromone cAF1 that is derived from a signal sequence (An et al., 1999). The pheromone is generated from a precursor protein by cleavages at two sites. First the signal sequence, which contains the octapeptide, is cleaved off by signal peptidase at the extracellular side of the plasma membrane. Then the signal peptide is processed within the transmembrane region by a designated protease Eep that resembles S2P (An et al., 1999; Brown et al., 2000).

The processes of SREBP activation and cAF1 release are strikingly similar to the generation of the p-Prl signal peptide fragment described herein. Signal peptidase cleaves the translocating p-Prl chain in the ER lumen and liberates the signal peptide that becomes anchored in the ER membrane. A subsequent cut within the transmembrane region favors the release of the N-terminal peptide portion toward the cytosol. This signal peptide fragment binds to Ca²⁺/calmodulin in vitro upon release from the membrane, which led to the speculation that the released peptide may influence calmodulin-dependent signal transduction pathways in a cell (Martoglio et al., 1997).

Signal peptides of other proteins may likewise have functions beyond protein targeting and membrane insertion (Martoglio and Dobberstein, 1998). Signal peptide processing could promote the release of these peptides from the ER membrane, as shown here for the preprolactin signal peptide. Liberated into the cytosol or the exoplasmic space, signal peptide fragments can report on the synthesis of major histocompatibility complex class I molecules (Borrego et al., 1998; Braud et al., 1998a) or inducing a mating response (An et al., 1999). Specific protease inhibitors such as (Z-LL)₂ ketone are powerful tools to further elucidate the role of signal peptide processing in vitro and possibly in living cells. Furthermore, such inhibitors may facilitate the biochemical identification of the elusive signal peptide peptidase, particularly in combination with the assay that allows monitoring of signal peptide processing using detergent-solubilized ER membrane proteins. The approach to address intramembrane proteolysis presented here may be applied
similarly to identify related proteases such as γ-secretase- and S2P-like proteases.
2.1.5. Materials and Methods

Synthesis of (Z-LL)$_2$ Ketone-- The ketone inhibitor (Z-LL)$_2$ ketone was synthesized in a single step by coupling an excess of commercially available Z-LL-OH with diamino acetone using the coupling reagent HATU and collidine (Fig. 2.1.1 B). The resulting product was then isolated by column chromatography and identified by NMR and mass spectrometry. The biotin derivative, (Bio-LL)$_2$ ketone, was synthesized as the corresponding di-$t$-butoxycarbonyl-capped derivative (BOC-LL)$_2$ ketone as described for (Z-LL)$_2$ ketone, except that BOC-LL-OH was used in place of Z-LL-OH. The resulting BOC-capped derivative was de-protected by brief trifluoroacetic acid treatment followed by precipitation in ether. The activated, nitrophenyl ester of biotin was used to alkylate both free N termini, resulting in (Bio-LL)$_2$ ketone. The identity of the compound was confirmed by NMR and mass spectrometry.

Competition Experiments-- Extracts were prepared from the dendritic cell line DC2.4 and the fibroblast cell line NIH-3T3 by mechanical disruption using glass beads in buffer A (50 mM Tris, pH 5.5, 1 mM dithiothreitol, 5 mM MgCl$_2$, 250 mM sucrose) at pH 5.5 (DC2.4 cells) or pH 7.4 (NIH-3T3 cells). Protein concentrations were determined using BCA reagents. Samples of DC2.4 lysates (100 µg of total protein) were incubated with increasing concentrations (as indicated) of the two ketone inhibitors (Bio-LL)$_2$ ketone and (Z-LL)$_2$ ketone and the peptide aldehyde leupeptin for 30 min at room temperature. Cysteine proteases were then labeled by the addition of $^{125}$I-JPM-565 (~106 cpm/sample) followed by separation by SDS-PAGE and analysis by autoradiography. The same protocol was used for analysis of proteasome activity in NIH-3T3 cells except the peptide aldehyde MG-132 was used instead of leupeptin, and samples were labeled with $^{125}$I-NLVS (Bogyo et al., 1997) (data not shown).
Plasmids and Transcription-- The HindIII/EcoRI fragment of pGEM4/p-Prl (High et al., 1993) coding for p-Prl was transferred into pGEM3Z (Promega) under the control of the SP6 promoter to give pGEM3Z/p-Prl. To prepare mRNA coding for full-length p-Prl, plasmid pGEM3Z/p-Prl was linearized with EcoRI and transcribed in vitro with SP6 RNA polymerase at 42 °C in the presence of 500 μM m7G(5’ppp(5’))G CAP analogue (New England Biolabs) (Gilmore et al., 1991). To prepare mRNA coding for p-Prl/86, the respective coding region was amplified with polymerase chain reaction using Pfu DNA polymerase (Stratagene), SP6 primer, and a reverse primer starting at the 5’-end with the triplet that corresponds to the 86th amino acid residue. When translation was supposed to terminate by proper termination of translation at a given residue (e.g. for synthesis of reference peptides), a TAG stop codon was introduced at the relevant position, and a reverse primer starting with 5’-NNNNNNNNNCTA- was used for polymerase chain reaction. Polymerase chain reaction-amplified DNA fragments were transcribed in vitro with SP6 RNA polymerase as described above (Nilsson and von Heijne, 1993).

In Vitro Translation and Translocation-- Translations of mRNA coding for p-Prl were performed in 25 μl of reticulocyte lysate (Promega) containing [35S]methionine (Amersham Pharmacia Biotech) and, where indicated, 1.5 eq of nuclease-treated rough microsomes prepared from dog pancreas (Walter and Blobel, 1983), (Z-LL)2 ketone (0.25 μl of a 500 μM stock solution in Me2SO), or Me2SO (0.25 μl). Samples were incubated for 30 min at 30 °C. Samples containing microsomes were next diluted with 25 μl of RM buffer (50 mM HEPES-KOH, pH 7.6, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol, 250 mM sucrose), and the salt concentration was raised to 500 mM potassium acetate. After incubation for 5 min on ice, membranes were separated by a 3-min centrifugation through a 100-μl sucrose cushion (RM buffer with 500 mM potassium acetate and 500 mM sucrose) at 48,000 rpm and 4 °C in a Beckman TLA100 rotor (Lyko et al., 1995). Samples without microsomes were supplemented with 50 μl of saturated (NH4)2SO4 to precipitate the bulk of proteins. The precipitate was
recovered by centrifugation and washed once with 500 µl of 5% trichloroacetic acid and twice with 150 µl of acetone. Membrane and protein pellets were prepared for SDS-PAGE as described below.

Truncated mRNA coding for p-Prl/86 was translated for 10 min at 30 °C in 50 µl of reticulocyte lysate containing [³⁵S]methionine and nuclease-treated rough microsomes (3 eq). After translation, samples were diluted with 50 µl of RM buffer, and microsomes were treated with 500 mM potassium acetate and recovered by centrifugation through a sucrose cushion as described above. Microsomes were resuspended in 120 µl of RM buffer, and samples were split in 20-µl aliquots. To one aliquot, 0.5 µl of Me₂SO was added, and the sample was placed on ice; to other aliquots, 0.5 µl of 40 µM (Z-LL)₂ ketone, 400 µM (Bio-LL)₂ ketone, 20 mM calpain inhibitor I, or 20 mM dichloroisocoumarin (all in Me₂SO) was added, and the samples were pre-incubated for 3 min at 22 °C. Nascent chains were released by adding 1 µl of 100 mM puromycin and further incubation at 22 °C for 15 min. Proteins were next either precipitated by adding trichloroacetic acid to 10% and prepared for SDS-PAGE (see below) or membranes were separated by a 10-min centrifugation at 100,000 rpm and 4 °C in a Beckman TLA100 rotor, and the membrane pellet and supernatant were prepared for SDS-PAGE as described below. Translations of mRNAs coding for reference peptides (p-Prl/18, p-Prl/20, p-Prl/25, p-Prl/27, and p-Prl/30) were performed in 25 µl of wheat germ extract containing [³⁵S]methionine (Martoglio et al., 1998).

Signal Peptide Processing with Solubilized ER Membrane Proteins-- To obtain solubilized ER membrane proteins, rough microsomes were first prepared from dog pancreas (Martoglio et al., 1998). Associated ribosomes and peripheral membrane proteins were removed by treatment with puromycin/high salt (Hauser et al., 1995), and lumenal proteins were depleted by exposure of microsomes to alkaline pH (Nicchitta and Blobel, 1990). Membranes were next resuspended (1-2 eq/µl) by using a Dounce homogenizer in solubilization buffer containing 50 mM Tris-HCl, pH
7.8, 50 mM potassium acetate, 2 mM magnesium acetate, 125 mM sucrose, 1 mM dithiothreitol, and 2% CHAPS. The sample was incubated for 30 min on ice, and non-solubilized proteins were subsequently removed by centrifugation in a TLA100.4 rotor (Beckman Instruments) for 30 min at 75,000 rpm and 4 °C.

For the processing assay, 2 µl of p-Prl/30 translation mixture (see above) were added to 35 µl of assay buffer (25 mM HEPES-KOH, pH 7.6, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol). Where indicated, 1 µl of 40 µM (Z-LL)2 ketone was added; 1 µl Me2SO was added to all the other samples. The reaction was started by the addition of 2 µl (2 eq) of rough microsomes, puromycin-treated, and content-depleted microsomes or detergent-solubilized microsomes. After incubation at 30 °C for 1 h, proteins were precipitated by adding trichloroacetic acid to 10%, and samples were prepared for SDS-PAGE (see below).

**Electrophoresis**—Proteins and peptides were analyzed by SDS-PAGE using Tris-Bicine gels (Wiltfang et al., 1997). Membrane pellets were solubilized in 12 µl of sample buffer containing 360 mM BisTris, 160 mM bicine, 1% SDS, 50 mM dithiothreitol, 15% sucrose, 0.01% bromphenol blue, and 0.004% Serva blue. Supernatants (20 µl) obtained after treatment with puromycin were supplemented with 1 µl of wheat germ extract, and proteins were precipitated by adding trichloroacetic acid to 10%. The precipitate was recovered by centrifugation, washed twice with 150 µl of acetone, and solubilized in 12 µl of sample buffer. All samples were incubated for 20 min at 65 °C. Proteins were finally separated on 14.25% acrylamide, 0.75% bis-acrylamide, 8 M urea gels (70 x 80 x 1 mm). Labeled proteins were visualized by a STORM Phosphorlmager (Molecular Dynamics).
2.1.6. Acknowledgments

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2.1.7. Thesis author’s work

This part contains many results performed before I started my thesis. I contributed with the improvement of the cell-free *in vitro* SPP assay and the preparation of solubilised ER membrane proteins. The contributions were the basis for the subsequent identification of SPP.
2.2. Result Part II:
Identification of Signal Peptide Peptidase, a Presenilin-Type Aspartic Protease

Andreas Weihofen¹, Kathleen Binns², Marius K. Lemberg¹, Keith Ashman², Bruno Martoglio¹

¹ Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
² Samuel Lunenfeld Institute, Proteomics, 600 University Avenue, Toronto Ontario, Canada

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2.2.1. Abstract

Signal peptide peptidase (SPP) catalyzes intramembrane proteolysis of some signal peptides after they have been cleaved from a preprotein. In humans, SPP activity is required to generate signal sequence-derived human lymphocyte antigen-E epitopes that are recognized by the immune system, and to process hepatitis C virus core protein. We have identified human SPP as a polytopic membrane protein with sequence motifs characteristic of the presenilin-type aspartic proteases. SPP and potential eukaryotic homologs may represent another family of aspartic proteases that promote intramembrane proteolysis to release biologically important peptides.
2.2.2. Introduction

The discovery of intramembrane proteolysis has revealed alternative pathways in cell signaling, cell regulation, and protein processing (Brown et al., 2000). Dormant, membrane-bound transcription factors, like sterol regulatory element-binding protein (Brown and Goldstein, 1999), activating transcription factor-6 (Haze et al., 1999), and NOTCH (Chan and Jan, 1999), or the growth factor Spitz in Drosophila (Lee et al., 2001), are activated and liberated in regulated processes that culminate in proteolytic cleavage within their membrane anchor. Similarly, β-amyloid (Aβ) peptides, which are believed to be the main toxic component in Alzheimer's disease, are generated from membrane-anchored β-amyloid precursor protein (β-APP) (Selkoe, 1999). The critical cleavage in the membrane anchor of β-APP is thought to be catalyzed by the aspartic protease presenilin (Steiner and Haass, 2000).

Processing of signal peptides by an SPP is related to protein cleavage by presenilin. Both proteases cleave their substrates within the center of a transmembrane region (Steiner and Haass, 2000; Weihofen et al., 2000). The discovery of posttargeting functions of signal peptides, which are required primarily for the biosynthesis of secretory and membrane proteins, has pointed to a central role for SPP activity (Martoglio and Dobberstein, 1998). Generation of cell surface histocompatibility antigen (HLA)-E epitopes in humans requires processing of signal peptides by SPP (Lemberg et al., 2001). HLA-E epitopes originate from the signal sequence of polymorphic major histocompatibility complex (MHC) class I molecules and report biosynthesis of these molecules to the immune system. SPP activity is also required for processing hepatitis C virus polyprotein and hence is exploited by the pathogen to produce viral components (McLauchlan et al., 2002). It is thought that SPP promotes the liberation of functional signal peptide fragments from the endoplasmic reticulum (ER) membrane.
2.2.3. Results

To identify human SPP, we synthesized a ligand affinity probe based on the SPP inhibitor \((Z\text{-}LL)\text{_2-ketone}\), which is thought to reversibly attack an active-site residue of the protease with its central ketone moiety (Weihofen et al., 2000). We synthesized a diazirine-containing derivative of \((Z\text{-}LL)\text{_2-ketone}\), TBL\(_4\)K (Fig. 2.2.1 A), because the photo-reactive compound might also be directed to the active site of the protease but irreversibly bind on activation with ultraviolet (UV) light (Brunner, 1989). A biotin moiety was also incorporated into the probe to facilitate identification of the adduct.

The effect of TBL\(_4\)K on SPP activity was examined \textit{in vitro} with detergent-solubilized SPP activity isolated from canine pancreas ER membranes; the \textit{in vitro}-synthesized substrates HLA-A/24 corresponding to the signal peptide of HLA-A\(^\text{*0301}\); and HLA-A\(^\text{ext/30}\), a mutant signal peptide containing an extended \(\text{NH}_2\)-region that allowed fixation of the cleavage product (HLA-A\(^\text{ext/20}\)) on the gel (Lemberg et al., 2001) (Fig. 2.2.1 B). Addition of TBL\(_4\)K to reactions inhibited cleavage of the peptides in a dose-dependent manner. The apparent median inhibitory concentration (IC\(_{50}\)) value (\(\sim 50 \text{ nM}\)) and the concentration that abolished SPP activity (1 \(\mu\)M) were within the range for inhibition of SPP by \((Z\text{-}LL)\text{_2-ketone}\) (Weihofen et al., 2000), indicating that modifications of TBL\(_4\)K did not affect targeting to SPP.

To identify protein(s) to which TBL\(_4\)K binds, we mixed detergent-solubilized ER membrane proteins with the inhibitor and then exposed the mixture to UV light. Western blot analysis with a biotin-specific antibody revealed a major and a minor product of about 42 and 40 kD, respectively (Fig. 2.2.1 C). Specificity of TBL\(_4\)K for these species was confirmed by competition with \((Z\text{-}LL)\text{_2-ketone}\). Endoglycosidase H treatment shifted both TBL\(_4\)K-labeled proteins into a single band of \(\sim 38 \text{ kD}\), suggesting that they are differentially glycosylated forms of the same protein (Fig. 2.2.1 D).
Figure 2.2.1 Inhibition of SPP activity and photolabeling with TBL4K. (A) Diagram of TBL4K. (B) TBL4K inhibits signal peptide processing. Amino acid sequences of substrates HLA-A24 and HLA-Aext30. Arrows indicate approximate SPP cleavage sites in the transmembrane region (underlined). HLA-Aext20, cleavage product of HLA-Aext30. Substrates were incubated in the presence of detergent (CHAPS)-solubilized SPP activity (lanes 2 to 6) or buffer only (lane 1). TBL4K was added at indicated concentrations. (C) TBL4K labels a 42-kD protein. CHAPS-solubilized ER membrane proteins were incubated with 50 nM TBL4K (lanes 2 to 8) and (Z-LL)2-ketone at indicated concentrations (lanes 4 to 8). Samples were irradiated with UV light (lanes 1 and 3 to 8) to activate TBL4K. (D) TBL4K-labeled protein is N-glycosylated. After labeling with TBL4K, samples were treated with endoglycosidase H (lane 2) or buffer only (lane 1). (E) Monitoring of purification by SDS-polyacrylamide gel electrophoresis-silver staining and Western blot analysis with biotin-specific antibody. Lane 1, CHAPS-solubilized ER membrane proteins; lanes 2 and 3, Concanavalin A-Sepharose flow-through and eluate pool; lanes 4 and 5, hydroxyapatite flow-through and eluate pool; lanes 6 and 7, reversed-phase flow-through and peak fraction; lane 8, reversed-phase peak fraction, but five times the equivalents loaded in lane 7. The arrow indicates the position of the TBL4K-labeled protein.
To identify the 42-kD species, we performed photolabeling with TBL₄K on a preparative scale and purified the labeled protein by chromatography (Fig. 2.2.1 E). At each purification step, the presence of TBL₄K-labeled protein was confirmed by Western blot analysis with a biotin-specific antibody. Analysis of the final pooled fractions revealed five proteins (Fig. 2.2.1 E, lane 8). The TBL₄K-labeled protein was identified by Western blot analysis, excised from a preparative gel, and analyzed by mass spectrometry. Sequences were obtained for six peptides that were compared with predicted translated products in the National Center for Biotechnology Information non-redundant and Expressed Sequence Tags databases. With the exception of two amino acid residues, all of the peptide sequences matched a hypothetical human protein of unknown function.

In the absence of homology to other proteins, we screened sequence databases for homologous genes in other species. Potential orthologs of the identified protein were found in higher eukaryotes, but no functions have yet been described for any of these hypothetical proteins. The most conserved regions contain the motifs YD and LGLGD within two putative transmembrane segments (Fig.S1/Fig. 2.2.2, regions 1 and 2). Such motifs are characteristic of the presenilin type of aspartic proteases (Steiner et al., 2000; Wolfe et al., 1999c). In presenilin, the two aspartic acid residues within these motifs are required for proteolytic activity and are thought to reside in the protease active site. Apart from these motifs, there is no particular homology between presenilins and the putative SPP. Also, no other potentially conserved catalytic residues such as serine, histidine, or cysteine were found.
Figure S1 Multiple sequence alignment (ClustalW 1.4) of predicted amino acid sequences of human SPP (CAD13132.1) and potential orthologues in *Mus musculus* (BAB25172), *Drosophila melanogaster* (AAL48184), *Caenorhabditis elegans* (P49049), and *Arabidopsis thaliana* (AAL38345). Conserved residues are highlighted; the "YD" and "LGLGD" motifs are underlined in red. Accession numbers refer to the EMBL/GenBank/DDBJ database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).
**Figure 2.2.2** Amino acid comparisons of conserved motifs in members of the SPP clan of putative aspartic proteases. Multiple alignment of predicted amino acid sequences of human SPP and its homologs across species. Most conserved regions are shown. Shading indicates residues highly conserved in all homologs (dark gray) and those conserved in the subfamilies (light gray). Subfamily SPP, potential SPP orthologs; subfamilies SPL1-4, SPP-like proteases; h, hydrophobic amino acid; Hs., Homo sapiens; Mm., Mus musculus; Dm., Drosophila melanogaster; Ce., Caenorhabditis elegans; At., Arabidopsis thaliana; Sp., S. pombe; Sc., S. cerevisiae. Accession numbers refer to the EMBL/GenBank/DNA Data Bank of Japan database (http://www.ncbi.nlm.nih.gov/Genbank/index.html)
Predicted amino acid sequences of more than 15 proteins of unknown function were homologous to human SPP and could be subdivided into at least five subfamilies on the basis of phylogenetic tree analysis (ClustalW 1.4) (Fig. 2.2.2). The first subfamily comprises proteins with a COOH-terminal ER-retrieval signal, KKXX, and includes SPP. Members of this subfamily are potential orthologs and share homology (up to 94% identity) with SPP throughout the whole amino acid sequence as illustrated in Figure S1. They are found only in higher eukaryotes. The other four subfamilies comprise SPP homologues without obvious intracellular localization signals. Also, they are homologous to SPP only in the COOH-terminal half of the protein and show substantial variation in the NH₂-terminal regions. This result suggests that the COOH-terminal portions containing the YD and LGLGD motifs and a highly conserved sequence [QPALLYhhP ; Fig. 2.2.2, region 3] form the proteolytic (sub)domain, whereas the NH₂-terminal parts define the specific function of the respective proteins.

Potential orthologs of SPP were not found in the genomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, suggesting that SPP function was acquired late in evolution, possibly to gain signal peptide-derived peptides for signaling or regulatory events, because it seems typical for processes that include intramembrane proteolysis (Huppert and Kopan, 2001). To confirm that the identified protein targeted by TBL₄K is an SPP, we expressed the protein in yeast *S. cerevisiae*. We cloned and sequenced the human cDNA from a HeLa cell cDNA library with partial expressed sequence tag sequences from public databases, and expressed the protein encoded by the recovered cDNA [submitted to the European Molecular Biology Laboratory (EMBL) database; accession number AJ420895]. Yeast microsomal membrane proteins were isolated and solubilized with detergent, and associated proteins were either labeled with TBL₄K or tested for protease activity. The TBL₄K-labeled protein appeared as a glycosylated and a nonglycosylated protein, which were not present in control yeast membranes (Fig. 2.2.3 A). Furthermore, solubilized membrane proteins containing the human protein showed SPP activity that was sensitive to TBL₄K (Fig. 2.2.3 B).
Mutation of the conserved aspartic acid residue at position 265 of the LGLGD motif to alanine did not affect its labeling with TBL₄K, indicating that substrate binding was not disrupted, but catalytic activity was abolished in the mutant (Szelke, 1985) (Fig. 2.2.3, A and B). These results suggest that the human TBL₄K-binding protein is SPP and likely an aspartic protease.

Analysis of the amino acid sequence of the predicted protein revealed seven putative transmembrane regions, according to the TMHMM 2.0 prediction program (Moller et al., 2001): four potential N-glycosylation sites, YD and LGLGD aspartic protease motifs, and the ER retrieval signal KKXX. To assess the topology of SPP, we determined its sites of glycosylation. At Asn⁶², which lies in the loop between putative transmembrane regions one and two, glycosylation is unlikely. This residue is part of a peptide identified by mass spectrometry and hence not modified and presumably facing the cytosol. To test the two N-glycosylation sites Asn¹⁰ and Asn²⁰, we mutated these residues to glutamines (N10Q/N20Q). When wild-type (WT) SPP was expressed in yeast, the TBL₄K-labeled protein appeared as two bands corresponding to glycosylated and nonglycosylated forms of the protein. In contrast, expression of the N10Q/N20Q mutant resulted in a single protein with the expected molecular size of nonglycosylated WT SPP, indicating that the NH₂-terminus of SPP had been translocated into the ER lumen (Fig. 2.2.3 C). Glycosylation of Asn¹⁰ and Asn²⁰ was also confirmed in vitro (not shown): The N-terminal 150 residues of wt and N10Q/N20Q SPP were synthesised in a cell-free in vitro translation system. When ER-derived rough microsomes were added to reactions, two additional bands with higher molecular weight appeared when the wt protein was synthesized, compared to translations in the absence of microsomes. These bands did not appear when the glycosylation inhibitor N-benzoyl-Asn-Leu-Thr-methylamide (Martoglio et al., 1998) was added, indicating that the wt SPP fragment is glycosylated up to two times. In contrast, when the N10Q/N20Q mutant SPP fragment was synthesised, the translation product had always the same apparent molecular weight, irrespective of whether microsomes were present or not.
Figure 2.2.3 Expression of human SPP in yeast and predicted topology. (A) TBL₄K labeling of human SPP expressed in S. cerevisiae. Yeast microsomes were isolated, solubilized with CHAPS, and labeled with TBL₄K. Wild-type (WT) SPP (lane 2); mutant D265A SPP (lane 3); control strain transformed with vector only (lane 1). (B) Signal peptide processing with recombinant SPP. HLA-A/24 and HLA-Aext/30 were incubated in the presence of CHAPS-solubilized yeast microsomes isolated from the control strain (lane 2), and the strains expressing WT SPP (lanes 3 and 4) and mutant D265A SPP (lane 5), or buffer only (lane 1). To one sample, 1 μM TBL₄K was added (lane 4). (C) Glycosylation of human SPP expressed in yeast. Yeast microsomes were isolated, solubilized with CHAPS, and labeled with TBL₄K. WT SPP (lane 2); mutant N10Q/N20Q SPP (lane 3); control strain transformed with vector only (lane 1). Dot indicates glycosylated protein. (D) Predicted topology of human SPP compared with presenilin-1. Arrows indicate the orientation of transmembrane regions containing the catalytic site motifs YD and LGLGD. Arrows to the right indicate the orientation of the cognate substrate. KKXX, ER retrieval signal.
On the basis of the analysis of glycosylation sites and the prediction of transmembrane regions, we propose a seven-transmembrane topology for SPP with the NH$_2$-terminus in the ER lumen, the COOH-terminus containing the ER retrieval signal in the cytosol, and the active-site motifs YD and LGLGD in the center of adjacent transmembrane regions (Fig. 2.2.3 D). Such motifs are also present in adjacent transmembrane regions of presenilins (Chan and Jan, 1999; Selkoe, 1999; Steiner and Haass, 2000). However, the predicted orientation of the transmembrane regions containing the YD and LGLGD motifs is opposite in presenilins compared with that of SPP, in accordance with the opposite orientation of the substrates. The substrates of presenilins, NOTCH-1 and β-APP, are type I membrane proteins, whereas SPP substrates have a type II orientation (Fig. 2.2.3 D).
2.2.4. Discussion

Genetic evidence supports the explanation that presenilins are γ-secretases, which catalyze cleavage of β-APP in its transmembrane region and liberate Aβ peptides (Steiner and Haass, 2000). Recent observations, however, question presenilins as the proteolytic components that cleave β-APP and NOTCH and suggest alternative functions, such as that of a molecular chaperone for membrane proteins (Sisodia et al., 2001). The identification of SPP as a presenilin-type aspartic protease favors the former view that presenilins are proteases. The identification of potential SPP homologs may in fact expand the number of potential proteases, which may account for γ-secretase activity in systems that exclude the action of presenilins (Sisodia et al., 2001). Identification of functional human SPP may allow elucidation of the mechanism of intramembrane proteolysis and address the still-unsolved question of how the cleavage of peptide bonds can be achieved in an environment that is thought to preclude hydrolysis.
2.2.5. Materials and Methods

Synthesis of TBL₄K

TBL₄K was synthesised by initially coupling an excess of BOC-LeuLeu-OH (Bachem) with diamino acetone using the coupling reagent HATU and collidine (all from Fluka). The resulting product was isolated by silica gel column chromatography and identified by nuclear magnetic resonance spectrometry (NMR). The BOC-capped derivative was de-protected by brief treatment with trifluoroacetic acid followed by precipitation in hexane. The two free amino termini of (H₂N-LeuLeu)₂ ketone were next modified with 1 equivalent of 4-(3'-trifluoromethyl-3H-diazirin-3-yl)benzoic acid N-hydroxysuccinimidyl ester (TDBAOSu; gift from Josef Brunner, ETH, Zurich) and 1 equivalent of biotinamidocapronic acid N-hydroxysuccinimidyl ester (Biotin-X-Osu; Molecular Probes). (TDBA-LeuLeu)(Biotin-X-LeuLeu) ketone (= TBL₄K) was isolated by preparative thin layer chromatography.

Inhibition and photo-labeling of SPP with TBL₄K

The inhibitory effect of TBL₄K on SPP was tested in a previously established assay (Weihofen et al., 2000) with HLA/24 and HLA-Aext/30 as substrates (Lemberg et al., 2001). 2 μl of the translation mixture, containing radio-labeled p-Pri₃⁷/30 peptide, were diluted with 35 μl SPP buffer (25 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2 mM Mg(OAc)₂, 1 mM DTT) and 0.8 μl of TBL₄K, dissolved as a 50x stock in DMSO, was added. Reactions were initiated by addition of 2 μl CHAPS-solubilised rER membrane proteins and samples were incubated for 1 hour at 30 °C. Proteins were precipitated by adding 4 μl of 100 % trichloroacetic acid, and analysed by SDS-PAGE using Tris-bicine-urea acrylamide gels (15 % T or 10 % T, 5 % C; 8 M urea) (Wiltfang et al., 1997).

For analytical labeling, 2 μl of CHAPS-solubilised rER membrane proteins were diluted with 16 μl of SPP buffer and supplemented with 50 nM TBL₄K (dissolved in DMSO). Samples were incubated at 30 °C for 1 hour and subsequently irradiated with UV light (30 seconds; 350 W high pressure
mercury lamp with a Pyrex filter, 10 cm distance to lamp) (Durrer et al., 1995). Proteins were precipitated with 10 % trichloroacetic acid and resolved on Tris-glycine acrylamide gels (10 % T, 2.7 % C (Laemmli, 1970). Biotinylated proteins were visualised by Western blotting with a polyclonal anti-biotin antibody (Bethyl), the appropriate secondary antibody conjugated with horseradish peroxidase, and enhanced chemiluminescence (Amersham-Pharmacia).

For preparative labeling, membrane proteins were reacted with TBL₄K on a large scale. 15 ml of CHAPS-solubilised rER membrane proteins (prepared from 15-20'000 eq RMs; (Weihofen et al., 2000) were diluted with 120 ml of SPP buffer and supplemented with 20 µl of 500 µM TBL₄K in DMSO. The sample was incubated at 30 °C for 1 hour. For irradiation with UV light, the sample was distributed between four 50 ml polypropylene tubes and irradiated for 30 seconds. The aliquots were then pooled and proteins separated by column chromatography.

**Isolation of SPP**

- **ConA Sepharose**  CHAPS-solubilised ER membrane proteins (135 ml) containing TBL₄K-labeled species were supplemented with 15 ml of 5 M NaCl and 2.6 ml of 20 % reduced Triton X-100 (Sigma). The sample was applied to a 1 ml Concanavalin A-sepharose column (Amersham-Pharmacia), that had been equilibrated with EQ buffer I (50 mM HEPES-KOH, pH 7.6, 500 mM NaCl, 20 mM sucrose, 1 mM DTT, 0.35 % reduced Triton X-100). The sample was circulated 5 times over the column at 0.2 ml/min and 4 °C. The column was next washed with 5 ml of EQ buffer I, and bound proteins containing TBL₄K-labeled species were eluted at room temperature with 15 ml elution buffer (1 M methyl-α-D-glucopyranoside, 50 mM HEPES-KOH, pH 7.6, 500 mM NaCl, 1 mM DTT, 0.35 % reduced Triton X-100).

- **Hydroxyapatite**  Eluate (15 ml) from the ConA column was diluted 10 times with 50 mM HEPES-KOH, pH 7.6, 1 mM DTT, 0.35 % reduced Triton X-100 to reduce salt concentration. The sample was applied to a 2.5 ml hydroxyapatite
column (BioRad), which had been equilibrated with EQ buffer II (50 mM HEPES-KOH, pH 7.6, 50 mM KOAc, 1 mM DTT, 0.35 % reduced Triton X-100) at 0.2 ml/min and 4°C. The column was next washed with 12.5 ml of EQ buffer II, and bound proteins containing TBL4K-labeled species were eluted at room temperature with 3 ml elution buffer (50 mM HEPES-KOH, pH 7.6, 500 mM KOAc, 200 mM KP, 1 mM DTT, 0.35 % reduced Triton X-100).

-Reversed Phase RP4 Eluate (3 ml) from the hydroxyapatite column was supplemented with 300 µl of 100 % trichloroacetic acid to precipitate proteins. After centrifugation (Eppendorf centrifuge, 14’000 rpm, 4 °C, 5 min), the protein pellet was washed with acetone and resuspended in 50 % formic acid in H2O. The sample was applied to a RP4 reversed phase HPLC column (CC 125/4 Nucleosil 300-5 C4; Machery Nagel), which had been equilibrated with 50 % formic acid. The flow rate was 1 ml/min. Proteins were first eluted with a linear gradient of 50 % formic acid in H2O to 50 % formic acid in acetonitrile. The column was next re-equilibrated with 50 % formic acid in H2O and residual proteins including TBL4K-labeled species were eluted with a linear gradient of 50 % formic acid in H2O to 50 % formic acid in propan-2-ol.

-SDS-PAGE Fractions containing the TBL4K-labeled species were pooled and proteins were resolved by SDS-PAGE using a Tris-glycine acrylamide gel (10 % T, 2.7 % C) (Laemmli, 1970). Proteins were visualized by coomassie blue staining. Western blotting using anti-biotin antibody identified the TBL4K-labeled protein. The corresponding coomassie stained band was excised from the gel and subjected to sequencing by mass spectrometry.

Sequencing by Mass Spectrometry
Proteins were reduced, alkylated and in-gel trypsin digested as described previously (Shevchenko et al., 1996). Peptides were extracted from the gel and desalted using ZipTip desalting columns (Millipore) equilibrated in 5 % formic acid, washed with equilibration buffer and eluted with 5 % formic acid, 60 % methanol (v/v). Tandem mass spectrometry (MS/MS) analysis was performed using a nanoelectrospray source (Protana A/S) coupled to the high
performance hybrid quadrupole time of flight API QSTAR™ Pulsar mass spectrometer (MDS-Sciex). Doubly or triply charged tryptic parent ion candidates were selected and product ion spectra generated by collision induced dissociation (CID). Sequence and mass information derived from the parent and fragment ions were used to screen the NRDB and dbEST databases. Databases searches were performed using the Mascot MS/MS search engine (Matrix Science). Comparison of the retrieved peptide sequence and masses with the tandem mass spectrum identified the sequences NASDMPETITSR, QYQLLTQGSGENK, LVFPQDLLEK, and GEVTEMFSYEEENPK from gi:14772424, and sequences FFPANFPNR and EEIINYEFATK with homologous peptides in gi:14772424 (FFPAEFPNR, EEIINYEFDTK).

Plasmid constructs and expression of human SPP in yeast
Human SPP cDNA is based on human EST fragments from the NCBI database coding for human protein gi:14772424. The cDNA was amplified from a HeLa cell-derived cDNA library (gift from Ulrike Kutay, ETH, Zurich) with Pfu DNA polymerase (Stratagene) and the PCR primes 5’-ACGACTAGTTCCATGGACTCGGCCCTCAGC-S’ and 5’-TGGAAGCTTCCTGAGAGCTCGGCACCAGC-3’. The resulting 1181bp fragment was cloned into the SpeI/HindIII sites of the yeast expression vector p426gal1 (Mumberg et al., 1995) yielding pDAW300. The sequence was confirmed by sequencing and deposited in EMBL databank (AJ420895). SPP mutants D265A and N10Q/N20Q were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) resulting in pDAW302, and pDAW306. Plasmids were next transformed into S. cerevisiae strain BY4742 (MATα; his3Δ; leu2Δ0; lys2Δ0; ura3Δ0) by lithium acetate transformation to give yeast strains DAW300 (MATα; his3Δ; leu2Δ0; lys2Δ0; ura3Δ0; p(spp, gal1,2u,ura3)), DAW302 and DAW306. Strains were grown at 23 °C in SC-medium with 2 % raffinose, 4 % galactose and appropriate supplements for selective growth until an OD600 of ~0.9 was reached.
To prepare microsomes\textsuperscript{6}, cells were washed with 50 mM HEPES-KOH pH 7.5, 10 % glycerol, 25 mM KOAc, 5 mM Mg(OAc)\textsubscript{2}, 1 mM EDTA, 1 mM DTT, 10 \(\mu\)g/ml PMSF, and complete protease inhibitor cocktail (Roche), and broken up by vigorous vortexing with glass beads for 3 min at 4 °C (Panzner et al., 1995). After sedimentation of beads and unbroken cells (centrifugation at 1'000 g, 5 min, 4 °C), microsomes were recovered by centrifugation at 100'000 g for 45 min at 4 °C in a Beckman TLA 120.2 rotor. Microsomes were next solubilised with CHAPS, tested for activity, and labeled with TBL\textsubscript{4}K as described above and previously (Weihofen et al., 2000).

\textit{In vitro translation and translocation}

\textit{In vitro} translations were performed in reticulocyte lysate (Promega) as previously described (Lemberg et al., 2001). To prime the system with mRNA, the cDNAs of wt and N10Q/N20Q mutant SPP were first subcloned into the pSV-SPORT vector (Life Technologies) under the control of the SP6 promoter, and the regions coding for the N-terminal 150 residues of the respective protein, were amplified by PCR. mRNAs were then produced by run-off transcription using SP6 RNA polymerase. For protein translocation, ER-derived rough microsomes prepared from canine pancreas, were added to translation reactions and incubated with or without the glycosylation inhibitor N-benzoyl-Asn-Leu-Thr-methylamide as described (Lemberg et al., 2001; Martoglio et al., 1998).

\textsuperscript{6} Improved preparation see Chapter 5
2.2.6. Acknowledgments

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2.2.7. Remarks

In this version the supplementary figure S1 is included (http://www.sciencemag.org/cgi/content/full/296/5576/2215/DC1)

2.2.8. Thesis author work

This part is the thesis core and is mainly performed by myself. Contributions from other people comprise the synthesis of TBL\textsubscript{4}K, protein sequencing by mass spectrometry and \textit{in vitro} confirmation of glycosylation sites (supplementary information; not shown).
2.3. Result Part III
Targeting Presenilin-Type Aspartic Protease
Signal Peptide Peptidase with γ-secretase Inhibitors

Andreas Weihofen*, Marius K. Lemberg*, Elena A. Friedmann1, Heinrich Rueeger2, Albert Schmitz2, Paolo Paganetti, Giorgio Rovelli2 and Bruno Martoglio1

1 Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
2 Novartis Pharma AG, Nervous System Research, 4002 Basel, Switzerland

* These authors contributed equally to the work

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2.3.1. Abstract

Presenilin is implicated in the pathogenesis of Alzheimer’s disease. It is thought to constitute the catalytic subunit of the γ-secretase complex that catalyzes intramembrane cleavage of β-amyloid precursor protein, the last step in the generation of amyloidogenic Aβ peptides. The latter are major constituents of amyloid plaques in the brain of Alzheimer’s disease patients. Inhibitors of γ-secretase are considered potential therapeutics for the treatment of this disease because they prevent production of Aβ peptides. Recently, we discovered a family of presenilin-type aspartic proteases. The founding member, signal peptide peptidase, catalyzes intramembrane cleavage of distinct signal peptides in the endoplasmic reticulum membrane of animals. In humans, the protease plays a crucial role in the immune system. Moreover, it is exploited by the hepatitis C virus for the processing of the structural components of the virion, and hence is an attractive target for antiinfective intervention. Signal peptide peptidase and presenilin share identical active site motifs and both catalyze intramembrane proteolysis. These common features let us speculate that γ-secretase inhibitors directed against presenilin, may also inhibit signal peptide peptidase. Here we demonstrate that some of the most potent known γ-secretase inhibitors efficiently inhibit signal peptide peptidase. However, we found compounds that showed higher specificity for one or the other protease. Our findings highlight the possibility to develop selective inhibitors aimed at reducing Aβ generation without affecting other intramembrane-cleaving aspartic proteases.
2.3.2. Introduction

Alzheimer's disease (AD) is characterized by the formation of senile plaques in the brain. Major constituents of these plaques are the amyloidogenic 40 and 42 residues long Aβ peptides Aβ_{40} and Aβ_{42}, respectively (Glenner and Wong, 1984). The amyloidogenic hypothesis casually links the generation of amyloid plaques with the neuropathological changes accompanying the symptoms typical of this disease (Selkoe, 2001). Aβ peptides are generated from the type I transmembrane protein β-APP (for β-amyloid precursor protein) by sequential proteolysis (Kang et al., 1987). The protein is first cleaved in the exoplasmic domain by the β-site APP cleaving enzyme (BACE) to release the ectodomain (Sinha et al., 1999; Vassar et al., 1999). The residual membrane anchored stub of 99 residues (C99) is subsequently cleaved in the center of the transmembrane region by γ-secretase (Haass and Steiner, 2002). The resulting cleavage products, an Aβ peptide and the amyloid intracellular domain (AICD), are liberated from the lipid bilayer toward the exoplasm and cytosol, respectively (Cao and Sudhof, 2001; Kimberly et al., 2001; Leissring et al., 2002).

To date, the majority of characterized familial AD (FAD) mutations are clustered along the presenilin-1 (PS1) gene (Levy-Lahad et al., 1995; Sherrington et al., 1995). They are thought to accelerate disease onset by increasing the Aβ_{42}/Aβ_{40} ratio (Selkoe, 1999). It is not well understood how these mutations, which are essentially scattered along the entire PS1 gene, can lead to a specific increase in the production of the 42 residue long peptide that corresponds to the most amyloidogenic form of Aβ (Jarrett et al., 1993). It has been shown that PS1 plays a key role in transport and maturation of β-APP (Kaether et al., 2002). It is also an essential component of the γ-secretase complex (Haass and Steiner, 2002), and several lines of evidences suggest that PS1 could constitute the catalytic subunit of this multi-subunit protease (Wolfe, 2001). For example, several aspartic protease transition state analogues were found to inhibit γ-secretase activity and target PS1
(Esler et al., 2000; Li et al., 2000; Shearman et al., 2000; Wolfe et al., 1998; Wolfe et al., 1999), and conservative mutations of putative active site aspartates in PS1 resulted in the loss of γ-secretase activity (Steiner et al., 1999; Wolfe et al., 1999). Thus, in the recent years, the development of small molecular weight compounds aimed at reducing γ-secretase/PS1 activity as a possible therapeutic strategy for AD has attracted major attention. Several potent inhibitors that affect γ-secretase/PS1 in cellular assays have been reported, and at least one compound was shown to reduce plaque load in a transgenic animal model for AD-type amyloidosis (Josien, 2002). The major concern related to this approach is that γ-secretase/PS1 is not only catalyzing the processing of C99, but it is also required for the processing of other transmembrane proteins, such as CD44 (Lammich et al., 2002), the tyrosine kinase receptor Erb4 (Lee et al., 2002; Ni et al., 2001), and the Notch receptor family (De Strooper et al., 1999; Mumm and Kopan, 2000).

Recently, we identified the intramembrane-cleaving protease SPP (for signal peptide peptidase) that contains motifs YD and LGLGD characteristic for GxGD aspartic proteases (Weihofen et al., 2002). These motifs are identically present in predicted transmembrane regions of PS1 supporting its function as an intramembrane-cleaving aspartic protease and hence catalytic subunit of the γ-secretase/PS1 complex (Haass and Steiner, 2002; Weihofen and Martoglio, 2003). SPP promotes intramembrane proteolysis of distinct signal peptides after they have been cleaved off from newly synthesized secretory or membrane proteins in the endoplasmic reticulum (ER) membrane of higher eukaryotes (Lemberg and Martoglio, 2002; Weihofen et al., 2002). In humans, SPP is essential for the generation of signal sequence-derived human lymphocyte antigen (HLA)-E epitopes and thus plays a crucial role in our immune system (Lemberg et al., 2001). Furthermore, SPP promotes cleavage at an internal signal sequence in the hepatitis C virus (HCV) polyprotein and is essential for proper maturation of the viral core protein (McLauchlan et al., 2002). Inhibitors of SPP may thus be considered as potential therapeutics for the treatment of HCV infection. The common features of SPP and PS1 raise the question whether γ-secretase/PS1 inhibitors directed against the putative
active site of PS1 – for example aspartic protease transition state analogues – are also acting against SPP and hence affect intramembrane cleavage of signal peptides. In the present study, we investigated the effects of representative, potent γ-secretase/PS1 inhibitors on SPP activity. We first tested the compounds for their potency in blocking Aβ generation in intact cells as well as inhibiting solubilized γ-secretase activity in a cell free *in vitro* assay. In the same type of assays, we then investigated the effect of these compounds on SPP activity and assessed their propensity to compete with active site labeling.
2.3.3. Results

Introducing γ-secretase/PS1 inhibitors and potency of SPP inhibitor against γ-secretase/PS1 The potency of γ-secretase/PS1 inhibitors L-685,458 (Shearman et al., 2000), L-852,646 (Li et al., 2000) and DAPT (WO 9822494), second generation compounds LY411575 (WO 9828268), a more potent analog of DAPT, and a novel compound NVP-AHW700-NX, a derivative of L-685,458, as well as the SPP inhibitors (Z-LL)₂-ketone (Weihofen et al., 2000) and TBL₄K (Weihofen et al., 2002) were investigated in this study (Fig. 2.3.1A). In a first series of experiments, we tested whether NVP-AHW700-NX and (Z-LL)₂-ketone function as γ-secretase/PS1 inhibitors and affect generation of Aβ peptides, and compared the potency of the two compounds with known γ-secretase/PS1 inhibitors DAPT, L-685,458 and LY411575 (Fig. 2.3.2B). Stably transfected human embryonic kidney (HEK) cells expressing β-APP were treated with various concentrations of inhibitor. Following incubation for 24 hours, media was removed and analyzed for Aβ peptides in a sandwich ELISA. Compounds LY411575 and NVP-AHW700-NX efficiently inhibited Aβ generation with IC₅₀ values of 0.4 nM and 0.62 pM, respectively, as well as the previously described inhibitors L-685,458 (0.46 µM) and DAPT (0.17 µM). In contrast, (Z-LL)₂-ketone did not inhibit generation of soluble Aβ₄₀ up to a concentration of 100 µM.

The latter finding was confirmed in a cell free in vitro assay using detergent-solubilized HEK cell membranes containing γ-secretase/PS1 activity (Fig. 2.3.1C). As a substrate, we used Met-C99, which was synthesized by cell-free in vitro translation. This peptide corresponded to the natural substrate of γ-secretase/PS1, C99, with an additional N-terminal methionine required to initiate peptide synthesis. After incubation, samples were subjected to immunoprecipitation with an Aβ₄₀ specific antiserum, and analyzed by SDS-PAGE and phosphorimaging. As expected, the γ-secretase/PS1 inhibitor DAPT (5 µM) blocked generation of Aβ₄₀. The SPP inhibitor (Z-LL)₂-ketone, in contrast, did not affect production of Aβ₄₀ up to a concentration of 100 µM.
Fig. 2.3.1 Inhibitory potency of γ-secretase/PS1 inhibitors and (Z-LL)₃-ketone. A, Chemical structure of inhibitors and photoaffinity labels used in this study. B, Inhibition of γ-secretase/PS1 in live cells. Amyloid precursor protein carrying the Swedish mutation was expressed in human embryonic kidney (HEK) cells in the presence of indicated inhibitors. IC₅₀ values were determined by measuring levels of Aβ40 secreted into the medium. C, Effect of (Z-LL)₃-ketone on detergent-solubilized γ-secretase/PS1 activity. Radio-labeled γ-secretase/PS1 substrate Met-C99 was added to CHAPSO-solubilized HEK cell membranes and incubated in the presence of 5 μM DAPT or 100 μM (Z-LL)₃-ketone. Samples were immunoprecipitated with Aβ-40 specific antibody. ref, reference peptide Met-Aβ40.
Inhibition of detergent-solubilized SPP We next investigated the effect of γ-secretase/PS1 inhibitors on SPP activity, first in a previously described cell free in vitro assay (Weihofen et al., 2000). A radiolabeled SPP substrate, peptide p-PrlPP29/30 (Lemberg and Martoglio, 2002), was prepared by cell-free in vitro translation in wheat germ extract, and incubated with detergent-solubilized ER membrane proteins containing SPP. Cleavage of the 30 residue long p-PrlPP29/30 by SPP resulted in the generation of ~20 residue long product that was readily detected and quantified by SDS-PAGE and phosphorimaging (Fig. 2.3.2). Addition of the SPP inhibitors (Z-LL)2-ketone and TBL4K, and the γ-secretase/PS1 inhibitors L-685,458, L-852,646, LY411575, and NVP-AHW700-NX efficiently inhibited cleavage of p-PrlPP29 with apparent IC50 values ranging from 8 to ~100 nM. Interestingly, the γ-secretase/PS1 inhibitor DAPT, which is a less potent derivative of LY411575, had no effect on SPP activity at concentrations up to 100 μM (Fig. 2.3.2). Also Pepstatin A and JKL2, which both were reported to inhibit γ-secretase/PS1 activity (Li et al., 2000a; Petit et al., 2001) did not affect SPP at concentrations up to 100 μM.

Active-site labeling of SPP and competition with γ-secretase/PS1 inhibitors To test whether the effective γ-secretase/PS1 inhibitors affect SPP by binding to the active site of SPP, we labeled the protease with the previously described photo-affinity label TBL4K (Weihofen et al., 2002) in the presence of increasing amounts of inhibitors (Fig. 2.3.3A). The central ketone moiety of TBL4K – a derivative of (Z-LL)2-ketone – is thought to be converted in situ to a transition state mimicking gem-diol upon binding to the SPP active site. As expected, increasing concentration of the transition state analogues L-685,458 and NVP-AHW700-NX progressively displaced TBL4K from SPP (Fig. 2.3.3A). Likewise, the most potent γ-secretase/PS1 inhibitor tested, LY411575, reduced labeling of SPP in a dose dependent manner. In agreement with what we observed in the cell free in vitro SPP assay, DAPT (Fig. 2.3.3A), Pepstatin A and JKL2 (not shown) did not influence labeling of SPP.
To further demonstrate that some of the γ-secretase/PS1 inhibitors target SPP, we made use of the photo-reactive compound L-852,646, a derivative of L-685,458, that was previously applied to label PS1 in detergent-solubilized HeLa total cell membranes (Li et al., 2000b). When incubated with detergent-solubilized ER membrane proteins and activated with UV light, L-852,646 selectively labeled a ~40 kDa protein, like TBL\textsubscript{4}K (Fig. 2.3.3B). Addition of increasing amounts of the SPP inhibitor (Z-LL)\textsubscript{2}-ketone progressively reduced labeling. Consistently, compounds that inhibited SPP in the cell free \textit{in vitro} assay, competed with TBL\textsubscript{4}K and L-852,646 for binding to the SPP active site. This finding is further evidence that PS1 and SPP are of the same type of aspartic protease (Steiner et al., 2000; Weihofen and Martoglio, 2003; Wolfe and Selkoe, 2002).
Fig 2.3.2. Inhibition of detergent-solubilized SPP with γ-secretase/PS1 inhibitors. Radiolabeled SPP substrate p-PPriPP39/30 (SP30) was incubated with detergent-solubilized SPP activity in the presence of inhibitor at indicated concentrations. Samples were analyzed by SDS-PAGE and phosphorimaging (example shown for L-685,458). For quantification of signal peptide processing, the amount of cleavage product, SP20, obtained in the presence of inhibitor is expressed as % of that obtained without inhibitor. L-685,458 (filled squares), L-852,646 (filled circles), DAPT (open triangles), LY411575 (filled triangles), NVP-AHW700-NX (filled diamonds), (Z-LL)2-ketone (asterisks), TBL4K (crosses), Pepstatin A (open diamonds), and JKL2 (open circles).
Fig 2.3.3 Photoaffinity-labeling of SPP and competition with γ-secretase/PS1 inhibitors. A, Labeling with TBL₄K and competition. SPP was labeled with TBL₄K in the presence of γ-secretase/PS1 inhibitors L-685,458, NVP-AHW700-NX, LY411575, and DAPT at indicated concentrations. B, Labeling with L-852,646 and competition with the SPP inhibitor (Z-LL)₂-ketone. No labeling of SPP was observed in the controls without activation of the reagent (−UV) and in the absence of label. The high molecular weight band observed in all lanes corresponds to a biotinylated protein present in the solubilized material.
Potency of γ-secretase/PS1 inhibitors on SPP in live cells We next tested the inhibitory potency of γ-secretase/PS1 inhibitors on SPP in a cellular assay system. Besides cleaving signal peptides, SPP also catalyzes processing of HCV core protein, and promotes its release from the ER membrane and trafficking to lipid droplets in the cytosol (McLauchlan et al., 2002). When SPP is inhibited, the core protein is not processed and remains anchored in the ER membrane by the C-terminal hydrophobic transmembrane region. We therefore can investigate SPP activity in tissue culture cells expressing HCV proteins, and monitor processing of core protein, either by detecting core protein by Western blot analysis (Fig. 2.3.4A), or by visualizing its intracellular localization using indirect immunofluorescence (Fig. 2.3.4B and C).

As depicted in figure 2.3.4A, (Z-LL)$_2$-ketone, L-685,458, NVP-AHW700-NX, and LY411575 inhibited processing of HCV core protein. Apparent IC$_{50}$ values varied from ~10 nM (for LY411575) to ~5 μM (for L-685,458). IC$_{50}$ values observed with the less membrane permeable compounds (Z-LL)$_2$-ketone and L-685,458, were much higher than in the in vitro assays. These compounds most likely penetrate the plasma membrane to a lower extent compared to the less peptidic and therefore more permeable compounds LY411575 and NVP-AHW700-NX, which showed comparable IC$_{50}$ values in both assays. DAPT and Pepstatin A did not inhibit processing of HCV core protein, and hence did not affect SPP, as already observed in the cell free in vitro assay. Also JKL2 did not affect processing of HCV core protein at concentrations up to ~10 μM, at which the compound started to become cytotoxic (not shown).
Fig 2.3.4 Effect of γ-secretase/PS1 inhibitors on SPP activity in live cells. A, Western blot analysis of core protein processing. Hepatitis C virus (HCV) core-E1-E2 polyprotein was expressed in baby hamster kidney (BHK) cells in the presence of protease inhibitors at the indicated concentrations. C/191 indicates core protein processed by signal peptidase; C/179 mature core protein processed by signal peptidase and SPP. B, Analysis of cells by immunofluorescence. HCV core-E1-E2 was expressed in BHK cells, and probed with a core-specific antibody and staining of lipid droplets. C, Immunofluorescence of core protein and staining of lipid droplets with cells expressing HCV core-E1-E2 in the presence of inhibitors.
The consequences of SPP inhibition on the processing of HCV core protein were next visualized by indirect immunofluorescence. When processed and released from the ER membrane, core protein was found associated at the surface of lipid droplets in the cytosol and appeared in characteristic ring-like structures (Fig. 2.3.4B). When expressed in the presence of (Z-LL)_2-ketone, L-685,458, NVP-AHW700-NX, and LY411575, which all inhibit SPP, HCV core protein did not localize to lipid droplets and appeared in a reticular staining pattern, indicating retention in the ER membrane. DAPT and Pepstatin A, which do not affect processing of HCV core protein, had also no effect on its intracellular distribution. Taken together, (Z-LL)_2-ketone and the γ-secretase/PS1 inhibitors L-685,458, LY411575, and NVP-AHW700-NX efficiently inhibit SPP in the detergent-solubilized state as well as in living cells. These compounds prevented intramembrane proteolysis of SPP substrates that, in turn, cannot be released from the ER membrane and fulfill associated functions in the cell (Hegde, 2002).
2.3.4. Discussion

In the present study we demonstrated that aspartic protease inhibitors directed against γ-secretase/PS1 are not necessarily specific and can affect the related intramembrane-cleaving aspartic protease SPP. This finding has implications for the therapeutic strategy for treatment of AD. To date, the therapeutic potential of small compound inhibitors of γ-secretase/PS1 was mainly scored against the possible side effects that could be expected by the concomitant inhibition on the Notch-1 signaling pathway (Mumm and Kopan, 2000). This was evaluated by measuring inhibition of fetal T cell maturation in presence of γ-secretase/PS1 inhibitors (Doerfler et al., 2001; Hadland et al., 2001; Radtke et al., 2002). However, the results presented in this study suggest that some of the most potent γ-secretase/PS1 inhibitors can also block SPP. At first glance, our data are discouraging in respect of developing γ-secretase/PS1 inhibitors as therapeutics, because SPP plays a key role in the processing of distinct signal peptides (Weihofen and Martoglio, 2003), which can have post-targeting functions such as that of reporting proper biosynthesis of antigen-presenting major histocompatibility (MHC) class I molecules to the immune system (Braud et al., 1998; Lemberg et al., 2001).

Our study, however, also identified compounds that are more selective against either γ-secretase/PS1 or SPP indicating that specific inhibitors may be designed, but need to be tested against the individual intramembrane-cleaving aspartic proteases.

The nature of the catalytic site of the γ-secretase complex has been intensively probed, but still remains somewhat controversial. Biotinylated photo-affinity labels based on aspartic protease transition-state analogues that mimic the γ-secretase cleavage site in β-APP/C99 can be covalently cross-linked to PS1 (Esler et al., 2000; Li et al., 2000). Furthermore, γ-secretase activity is abolished by mutations of two critical aspartate residues (D-257 and D-385) located in predicted transmembrane domains of PS1 (Steiner et al., 2000; Wolfe et al., 1999). Although such findings supported the hypothesis that PS1 is the catalytic component of the complex, this notion
was hampered by the fact that PS1 did not share any sequence homology with other known aspartic proteases. The discovery of SPP, an intramembrane-cleaving aspartic protease with active site motifs identical to the putative ones in PS1 (Weihofen et al., 2002), overruled this objection and provided further evidence that PS1 is a protease.

Additional indirect evidence that PS1 is a protease, is provided by the present study reporting on overlapping inhibitor activities. Compounds, including transition state analogues, were found to efficiently inhibit both, γ-secretase/PS1 and SPP. Furthermore, the active site directed affinity probe L-852,646 previously applied to label PS1 in solubilized total cell membranes (Li et al., 2000), selectively labeled SPP when applied on detergent solubilized ER membrane proteins. The latter also contained PS (not shown) but only in the unprocessed form, which cannot be labeled by L-852,646 (Li et al., 2000). In fact, all the effective inhibitors competed with labeling of SPP by the transition state analogue L-852,646 and the photo-affinity label TBL4K, which mimics the gem-diol intermediate upon hydration in the active site. These results suggest that the compounds investigated in this study target the active site of SPP, and it is likely that they similarly interact with PS1.

While three compounds, Pepstatin A, DAPT and (Z-LL)₂-ketone, could discriminate between γ-secretase/PS1 and SPP, the other tested inhibitors affected both proteases to a variable degree. Thus despite overlapping inhibitor activities, the two proteases clearly differ in the way they interact with the inhibitors. The small number of compounds investigated, however, does not allow us make predictions about the specificity of a particular compound. Modifications on a lead compound may not only significantly increase its inhibitory potency, but also can influence compound selectivity, as shown for DAPT and its second-generation derivative LY411575. The new derivative is indeed ~400x more potent against γ-secretase/PS1, but it also became an efficient inhibitor of SPP. The potency of LY411575 against SPP, however, was lower than against γ-secretase/PS1. Similarly, the transition state analogue L-685,458 was less potent against SPP, whereas the related
compound NVP-AHW700-NX was equally effective against SPP and γ-secretase/PS1. Thus, SPP and γ-secretase/PS1 interact differently with various compounds, but to determine what makes an inhibitor selective against one or the other protease will be a major challenge for future drug design.

SPP and γ-secretase/PS1 are both of pharmaceutical interest. SPP is essential for the processing of the HCV core protein (McLauchlan et al., 2002); γ-secretase/PS1 is implicated in the cause of AD (Wolfe, 2002). Drugs against either protease may be useful for the treatment of HCV infection or AD, but they should discriminate between the two proteases in order to minimize side effects. More complicating though, the human genome encodes four additional homologues of SPP (Grigorenko et al., 2002; Ponting et al., 2002; Weihofen et al., 2002). It is likely that these candidate aspartic proteases catalyze intramembrane proteolysis of so far unidentified substrate proteins. In analogy to known intramembrane-cleaving proteases, they may promote the release of bioactive peptides and proteins such as signaling molecules and transcription factors (Weihofen and Martoglio, 2003). Because all these proteins contain motifs identical to the active site motifs of SPP and γ-secretase/PS1, compounds like the ones tested in the present study, may well target the SPP-like candidate proteases too. Therefore, compound specificity will be even more important. In the future, the development of effective therapeutic agents targeting γ-secretase/PS1 or SPP will challenge the chemists and may require systematic probing of all human intramembrane-cleaving aspartic proteases.
2.3.5. Materials & Methods

Synthesis of inhibitors
L-658,485 (Shearman et al., 2000), L-852,646 (Li et al., 2000c), DAPT (WO 9822494), LY411575 (WO 9828268), (Z-LL)₂-ketone (Weihofen et al., 2000), and TBL₄K (Weihofen et al., 2002) were synthesized as previously described. NVP-AHW700-NX was synthesized according to methods reported for a related compound (Beher et al., 2001). The purity of each compound was checked by ¹H nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy, high-pressure liquid chromatography (HPLC) and thin-layer chromatography, and results were consistent with the expected structures. JLK2 (Petit et al., 2001) was kindly provided by F. Checler; Pepstatin A purchased from Sigma.

γ-Secretase assays
Inhibition of γ-secretase activity in live cells was assayed by quantifying the generation of secreted Ab. In brief, human embryonic kidney cells (HEK)-293 cells stably transfected with β-APP carrying the Swedish mutation (Mullan et al., 1992; Schrader-Fischer and Paganetti, 1996) were plated in microtiter plates. After one day, the inhibitors were added in fresh medium and the cells were incubated for another 24 hours. 10 μl of conditioned medium were removed for determination of Aβ-levels by sandwich ELISA using the Aβ40-specific monoclonal antibody 25H10 raised against the free C-terminal peptide, MVGGVV, of Aβ40. The monoclonal b₁ antibody (Schrader-Fischer and Paganetti, 1996) was biotinylated and used as detection antibody with alkaline phosphatase coupled to streptavidin. For chemiluminescence, substrate CSPD and the enhancer EmeraldI (Tropix) were applied. Standard curves with synthetic Aβ₄₀ peptide (Bachem) were run in parallel.

For testing γ-secretase in vitro, detergent solubilized γ-secretase activity was prepared from HEK-293 cells (Li et al., 2000) and incubated with substrate Met-C99, which was synthesized by in vitro translation (see below), and either
DMSO (2 %) or inhibitor at the indicated concentration. After incubation, samples were subjected to immunoprecipitation with antibody 25H10 and analyzed by SDS-PAGE and phosphorimaging using 15 % polyacrylamide Tris-Bicine-urea acrylamide gels (Wiltfang et al., 1997) and a STORM Phospholmager (Molecular Dynamics). Reference peptide Met-Ab40 was synthesized by in vitro translation.

**SPP assay and affinity labeling**

γ-Secretase inhibitors were tested on SPP in a previously established in vitro assay (Weihofen et al., 2000). In brief, 2 μl cell-free translation mixture containing [35S]-methionine labeled peptide p-Prl^{PP29/30} (Lemberg and Martoglio, 2002), were diluted with 35 μl SPP buffer (25 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2 mM Mg(OAc)_2, 1 mM DTT), and supplemented with 1 μl 100x concentrated inhibitor in DMSO. Reactions were initiated by addition of 2 μl CHAPS-solubilized ER membrane proteins and samples were incubated for 1 hour at 30 °C. Samples were next analyzed by SDS-PAGE and phosphorimaging using 15 % polyacrylamide Tris-Bicine-urea acrylamide gels (Wiltfang et al., 1997) and a STORM Phospholmager (Molecular Dynamics). Quantification was performed with IQMac V1.2 software (Molecular Dynamics). For affinity labeling, CHAPS-solubilized ER membrane proteins were incubated in SPP buffer in the presence of 50 nM TBL4K or 25 nM L-852,646 and indicated concentrations of competitor (Weihofen et al., 2002). Samples were incubated at 30°C for 30 minutes and subsequently irradiated with UV light (30 seconds for TBL4K, 5 minutes for L-852,646; 350 high pressure mercury lamp, 10 cm distance to lamp) (Weihofen et al., 2002). Samples were analyzed by SDS-PAGE on 12% polyacrylamide Tris-glycine gels (Laemmli, 1970) and biotinylated proteins were visualized by enhance chemiluninescence (Amersham-Pharmacia) after Western blotting with a polyclonal anti-biotin antibody (Bethyl) (Weihofen et al., 2002).
Inhibition of SPP in tissue culture cells and indirect immunofluorescence.

Hepatitis C virus structural proteins C, E1 and E2 were transiently expressed in baby hamster kidney C13 cells as previously described (McLauchlan et al., 2002). Following electroporation with in vitro transcribed mRNA encoding the CE1E2 polyprotein, cells were diluted in growth medium at a concentration of \( \sim 10^6 \) cells/ml. 0.25 ml cell suspension was diluted with 0.25 ml growth medium containing either 2 % DMSO, or 2 % 100x concentrated inhibitor dissolved in DMSO, and seeded in 24-well tissue culture plates. After incubation at 37 °C for 10 hours, cells were either solubilized in SDS-PAGE sample buffer, or fixed for indirect immunofluorescence analysis with monoclonal core-specific antibody JM122 (Hope and McLauchlan, 2000) (gift from J. McLauchlan) and staining of lipid droplets (Hope and McLauchlan, 2000). For Western blot analysis, proteins were first separated by SDS-PAGE using 13 % polyacrylamide Tris-glycine gels, transferred to PVDF membranes, and probed with polyclonal core-specific antibody R308 (Hope and McLauchlan, 2000) (gift from J. McLauchlan). Bound antibody was detected by enhanced chemiluminescence.

2.3.6. Acknowledgements

We thank J. McLauchlan for antibodies JM122 and R308, F. Checler for compound JKL2, and R. Ortmann and U. Neumann for development of the Ab ELISA methodology.

2.3.7. Thesis author’s work

I performed cell-free in vitro SPP inhibitor assays and competition experiments.
3. Discussion

Intramembrane cleaving proteases: controlled liberation of proteins and bioactive peptides

Andreas Weihofen and Bruno Martoglio

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This review summarizes recent findings and common principles for intramembrane-cleaving proteases that catalyse critical steps in cell regulation and signalling and which are involved in diseases such as Alzheimer's disease and hepatitis C virus infection.

Intramembrane-cleaving proteases (I-CLiPs) catalyse peptide bond hydrolysis in the plane of cellular membranes. They thereby promote controlled release of membrane-anchored proteins such as transcription factors or epidermal growth factor (EGF) ligands. Recently, a new I-CLiP family, with members in higher eukaryotes, was discovered. They catalyse the liberation of small peptides from cellular membranes. These peptides can function as reporters or effectors of signalling pathways. It appears, therefore, that I-CLiPs are versatile key elements of cell regulation, signalling and protein processing. This review summarizes the families and features of I-CLiPs and highlights common principles of the reactions they catalyse.

During the past decade, a wealth of data acquired by many groups revealed that proteases not only act in aqueous environments such as cellular compartments and the extracellular space, but also that they can reach the interior of lipid bilayers to effect peptide bond hydrolysis. Many investigators have identified cleavage products containing remnants of former transmembrane regions, processes comprising a protease-mediated release of membrane-anchored precursor proteins and multispanning candidate proteases with catalytic site motifs embedded in predicted transmembrane regions. All this indirect evidence led to the description of 'intramembrane-cleaving proteases', the so-called I-CLiPs (Box 1) (Wolfe et al., 1999a).

I-CLiPs that have so far been identified play pivotal roles in cell regulation and signalling (Table 3.1). The reactions they catalyse are in most cases part of highly controlled processes. Therefore, I-CLiPs are considered to promote so-called 'regulated intramembrane proteolysis' (RIP) (Brown et al., 2000). Several of the more recently discovered intramembrane cleavage reactions do not seem as obviously regulated as the archetypal process, which is the
activation of the mammalian sterol regulatory element-binding protein (SREBP) (Brown and Goldstein, 1997). Nevertheless, all known and suspected processes that include the action of an I-CLiP comprise control mechanisms to avoid disordered attack on membrane proteins and to guarantee controlled liberation of cleavage products either into the cytosol or out of the cell.

3.1. I-CLiPs: founding members and families

3.1.1. S2P-type metalloproteases

The first protein to be convincingly identified as an I-CLiP was the mammalian metalloprotease site-2 protease (S2P) (Rawson et al., 1997). S2P was discovered by complementation cloning as a protein essential for the proteolytic activation of the transcription factor SREBP. SREBP regulates expression of genes required for cholesterol and fatty acid biosynthesis in animals (Brown and Goldstein, 1997; Rawson et al., 1997). More recent data indicate that S2P is also required for the activation of other membrane-bound transcription factors such as ATF6, an inducer of chaperone expression in the mammalian unfolded protein response (UPR) (Ye et al., 2000b).

S2P is a multispansing membrane protein containing the classical consensus HEExxH motif of a metalloprotease, as well as an additional conserved sequence, LDG, which provides an aspartate as the third coordinating residue for the zinc atom at the active site (Table 3.1). Mutational analysis in a cellular assay system revealed the requirement for predicted active-site residues and provided genetic evidence for the proteolytic activity of S2P (Rawson et al., 1997). Additional studies on the topology of S2P indicated that its catalytic-site motifs are embedded in the membrane close to the cytosolic surface (Zelenski et al., 1999)(Table 3.1), consistent with the observed cleavage of substrates in transmembrane regions close to the interface with the cytosol (Duncan et al., 1998).
S2P and its homologues can be divided into six subfamilies (Lewis and Thomas, 1999). Whereas S2P is found in animals only, the homologues are also found in bacteria, archaea and plants, but not in protozoa and fungi (Lewis and Thomas, 1999; Rudner et al., 1999)(Table 3.1). They all contain the conserved active-site motifs HexxH and LDG, but these are only located unequivocally in predicted transmembrane regions in members of the S2P subfamily. Members of the other five subfamilies are all putative multisingning membrane proteins with catalytic-site motifs close to, but not necessarily positioned within, a membrane. Some of these candidate proteases may not fulfil the criteria of bona fide l-CLiPs (Box 3.1). They may be ancestors of the S2P-type proteases and represent intermediate stages on evolution's way to exploit the interior of lipid bilayers for proteolysis.

3.1.2. Presenilins and SPP-type aspartic proteases

The presenilins PS-1 and PS-2 are currently the most prominent candidate l-CLiPs because they are intimately involved in the pathogenesis of Alzheimer's disease (reviewed in (Selkoe, 2001)). They are considered to function as the catalytic subunit of the γ-secretase complex (reviewed in (Haass and Steiner, 2002)), which promotes intramembrane cleavage of β-amyloid precursor protein (β-APP) and participates in the generation of amyloidogenic Aβ peptides. In addition, presenilins are involved in the activation of a variety of membrane-tethered signalling factors (reviewed in Fortini, 2002) (Table 3.1). Not surprisingly, investigators discovered the role of presenilins as candidate proteases engaged in RIP from studies of a nuclear signalling factor, NOTCH-1/LIN-12 (De Strooper et al., 1999; Levitan and Greenwald, 1998). Signalling via the transmembrane receptor NOTCH-1 involves ligand-induced activation, which includes presenilin-dependent intramembrane cleavage and results in the release of its intracellular domain NICD (Table 3.1). NICD then translocates into the nucleus and modifies the transcription of target genes (reviewed in Mumm and Kopan, 2000). Likewise, the cytosolic domains of other presenilin substrates, such as AICD, resulting from cleavage of β-APP
(Cao and Sudhof, 2001), function, or are suspected to function, as molecular signals (Table 3.1).

Initiated by a study demonstrating reduced Aβ production in PS-1 null cells (De Strooper et al., 1998), research by many groups provided evidence that presenilins act as proteases (reviewed in Steiner and Haass, 2000). Presenilins are the only components of γ-secretase complexes that contain motifs similar to those found in other proteases. Although these motifs, YD and LGLGD, do not match those of classical aspartic proteases, D(T/S)G(T/S), they are similar to the active site of the bacterial type-4 prepilin peptidase (Steiner et al., 2000), which is a multispansning membrane protease with active-site motifs close to but not within predicted transmembrane regions, and of the more recently identified signal peptide peptidase (SPP) (see below). Consistent with their proposed I-CLiP activity, the YD and LGLGD motifs of presenilins are located in predicted transmembrane regions (Table 3.1). Furthermore, aspartic protease transition-state analogues inhibited γ-secretase activity and targeted presenilin (Esler et al., 2000; Li et al., 2000c) and mutations of active-site aspartates abolished activity (Wolfe et al., 1999c). Despite such convincing but indirect evidence, the function of presenilins as proteases is a matter of debate (Sisodia and St George-Hyslop, 2002).

Recently, the presenilin-related aspartic protease signal peptide peptidase (SPP) was discovered (Weihofen et al., 2002). SPP promotes intramembrane cleavage of certain signal peptides after they have been cleaved from newly synthesized secretory or membrane proteins (Lemberg and Martoglio, 2002). It is essential for the generation of signal sequence-derived human lymphocyte antigen E (HLA-E) epitopes and thus plays a crucial role in the human immune system (Braud et al., 1998a; Lemberg et al., 2001). Furthermore, it promotes cleavage at an internal signal sequence in the hepatitis C virus (HCV) polyprotein and is essential for proper maturation of the viral core protein (McLauchlan et al., 2002).
SPP was identified on the basis of a biochemical approach and its proteolytic activity was demonstrated by expressing human SPP in the yeast *Saccharomyces cerevisiae*, which does not contain an SPP orthologue (Weihofen et al., 2002). Like presenilins, SPP contains the aspartic protease motifs YD and LGLGD, which are located in predicted transmembrane regions (Table 3.1). Moreover, aspartic protease transition-state analogues and active-site mutations abolish SPP activity. The striking difference between SPP and presenilins is the opposite orientation of the transmembrane regions containing the active-site motif (Table 3.1), but this seems intuitive as it correlates with the opposite topology of the transmembrane region of the respective substrates (Table 3.1). It appears, therefore, that a particular I-CLiP only attacks substrates of one particular orientation – e.g. either type I (N-terminus in the exoplasm, C-terminus in the cytosol) or type II (N-terminus in the cytosol, C-terminus in the exoplasm) oriented substrates – but not both types.

Presenilins and SPP do not share a particular sequence homology, with the exception of the conserved active-site motifs and the conserved motifs QPALLYLxxP in SPP and PALP in presenilins (Ponting et al., 2002; Weihofen et al., 2002). The latter motifs may have functional similarity. Presenilins are found only in animals and plants, whereas orthologues of SPP are found in animals and plants, and additional homologues in all kingdoms except bacteria (Table 3.1). In humans, four SPP-like I-CLiPs are expressed in addition to SPP and the two presenilins, implying that many more intramembrane proteolysis events are catalysed by aspartic I-CLiPs.

### 3.1.3. Rhomboid-type serine proteases

Rhomboid is a multispanning membrane protein that is required for the establishment of the dorsoventral axis and development of the peripheral nervous system in the fruit fly, *Drosophila melanogaster* (Bier et al., 1990). After more than 10 years of extensive research aimed at identifying its cellular function, it became evident that Rhomboid-1 is an I-CLiP with the motifs of a
serine protease (Table 3.1) (Urban et al., 2001). Located in the Golgi apparatus, Rhomboid-1 promotes the release of Spitz, which is a membrane-bound ligand for the EGF receptor. The activated ligand is secreted and activates EGF receptors on neighbouring cells (Lee et al., 2001).

Evidence that Rhomboid-1 is a serine protease was provided by mutational analysis in a cell-based assay system (Urban et al., 2001). Drosophila Rhomboid-1 and homologous proteins from other species contain the conserved sequence GxSG, which is characteristic for serine proteases of the chymotrypsin/trypsin/elastase family. In addition, Rhomboid-1 contains conserved asparagine and histidine residues, which together with the serine in GxSG makes up the catalytic triad Asp/Asn–His–Ser typical for serine proteases. All these residues are embedded within predicted transmembrane regions (Table 3.1). Also, serine protease inhibitors affected Spitz activation, and the cleavage product contained part of the former transmembrane region, consistent with the intramembrane-cleaving activity of Rhomboid-1.

Rhomboid-like proteins are conserved throughout evolution from bacteria to humans (Table 3.1). They exist in multiple copies in all examined genomes (Gallio and Kylsten, 2000; Wasserman et al., 2000). In Drosophila, for example, seven Rhomboid-like proteins were predicted, four of which were found to be associated with EGF signalling (Urban et al., 2002a). In addition, recent studies revealed a role for a Rhomboid-like protein in extracellular signalling in the Gram-negative bacterium Providencia stuartii (Gallio et al., 2002) and in leader sequence processing in yeast mitochondria (Esser et al., 2002)(Table 3.1).
<table>
<thead>
<tr>
<th>I-CLiP</th>
<th>Catalytic motifs (and topology of substrates)</th>
<th>Substrates</th>
<th>Cleavage products</th>
<th>Function, pathway</th>
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<td>NOTCH 1-4</td>
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Table 3.1 Illustrations show the positioning of active site motifs within predicted transmembrane regions (gray barrels). Arrows highlight the orientation of the respective transmembrane region. Arrows in red barrels highlight the orientation of the scissile transmembrane region of the cognate I-CLiP substrates. SPP cleaves within the transmembrane region of the signal sequence of these proteins. SPP cleaves within the internal signal sequence between core protein and the following glycoproteins. S2P cleaves SREBP within the first of the two transmembrane regions. Eep cleaves within the transmembrane region of the signal sequence of cAD1 precursor. PCP1 cleaves within the transmembrane region of the leader sequence of Ccp1. Abbreviations: APP, amyloid precursor protein; Cad, cadherin; CaM, calmodulin; EGF, epidermal growth factor; HCV, hepatitis C virus; HLA, human lymphocyte antigen; ICD, intracellular domain; I-CLiP, intramembrane cleaving protease; LRP, LDL receptor-related protein; MHC, major histocompatibility complex; SPP, signal peptide peptidase; SREBP, sterol regulatory element-binding protein; TPA, tris phorbol acetate.
### Chapter 3

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<th>I-CLiP</th>
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3.2. Expanding diversity of regulated intramembrane proteolysis (RIP)

3.2.1. Controlled release of proteins for activation and degradation

The classical form of RIP is the activation of a dormant, membrane-bound transcription or signalling factor in a regulated process that culminates in its liberation upon intramembrane cleavage (reviewed in Refs (Brown et al., 2000; Mumm and Kopan, 2000)). This basic principle was first discovered for the activation of SREBP and NOTCH, but many other signalling and regulatory factors appear to be activated by RIP, from bacteria to humans (Table 3.1). Intra- or extracellular signals can induce activation of dormant factors, which are released into either the cell or the exoplasm and might promote intracellular events or intercellular communication (Fig. 3.1) (Urban and Freeman, 2002). As examples, we will describe recently revealed events during activation of mammalian and bacterial stress genes.

In eukaryotic cells, the endoplasmic reticulum (ER) provides a folding compartment for proteins destined for secretion and for organelles of the exocytic pathway (Ellgaard and Helenius, 2001). Folding factors, such as molecular chaperones and oxidoreductases, assist newly synthesized proteins in acquiring the correct three-dimensional fold, and a complex quality-control system guarantees that only correctly folded proteins exit the ER. If proteins fail to fold properly, they are cleared from the ER by ER associated protein degradation.

The folding environment of the ER is carefully monitored and adjusted to need. When misfolded proteins accumulate, UPR is induced (Patil and Walter, 2001). In mammalian cells, one such UPR signal comprises activation of the membrane-bound transcription factor ATF6 (Fig. 3.1a). The regulatory step in this process requires the molecular chaperone BiP, which plays a crucial role in measuring the content of unfolded proteins within the ER. BiP also binds to
the lumenal domain of ATF6 and thereby retains the dormant transcription factor within the ER. When misfolded proteins accumulate and sequester BiP, the chaperone dissociates from ATF6, which, in turn, is liberated for trafficking to the Golgi apparatus (Chen et al., 2002; Shen et al., 2002). There, it meets the proteases S1P and S2P, which subsequently cleave the newly arrived ATF6, first in the exoplasmic domain and then within the transmembrane region (Ye et al., 2000b). The activated cytosolic transcription factor domain is released from the Golgi membrane and, upon transport into the nucleus, induces expression of ER stress gene products.

Stress response pathways that sense protein misfolding and make use of RIP to activate transcription factors also occur in bacteria. In the Gram-negative bacterium *Escherichia coli*, this response is initiated by activating the $\sigma^E$ transcription factor RpoE, which directs the expression of various genes encoding, for example, envelope-localized chaperones (Fig. 3.1b). As discovered recently, the principal sequence of events—the signal induced release of a membrane-anchored protein by an l-CLiP—is analogous to the activation of transcription factors described above, but in this case the l-CLiP substrate is not cleaved to become an active component; rather, it is cleaved and released for degradation (Alba et al., 2002; Kanehara et al., 2002).

In the resting state, RpoE is retained at the plasma membrane by the anti-$\sigma^E$ factor RseA, which in turn associates with the chaperone RseB (Clausen et al., 2002). The extracytoplasmic stress response is induced by excessive amounts of unfolded proteins in the envelope of the cell (Mecsas et al., 1993). These proteins recruit RseB and dissociate the chaperone from the RseA–RpoE complex. In a subsequent two-step proteolytic process, proteases DegS and YaeL cleave RseA in the periplasm and the transmembrane region, respectively. The residual RseA–RpoE complex is released into the cytosol where it dissociates; RpoE is then free to activate stress genes, whereas RseA is degraded (Fig. 3.1b). The characterization of this bacterial stress response pathway may point to a variant of RIP that is the I-CLiP-induced degradation of a membrane protein. Proteins such as RseA
may fulfil a function when bound to a membrane. If they are no longer required, degradation is initiated and an I-CLiP could then liberate the protein from the membrane for complete breakdown in the cytosol.
Variations of RIP-induced liberation of proteins

(a) Activation of transcription factor

![Diagram of transcription factor activation]

**Fig. 3.1 a** Activation of dormant transcription factors illustrated for the mammalian chaperone inductor ATF6. Resting ATF6 is retained in the endoplasmic reticulum (ER) membrane by the lumenal chaperone BiP. When unfolded proteins accumulate in the ER lumen and recruit chaperones, BiP dissociates from ATF6, which in turn is liberated for trafficking to the Golgi apparatus. There, the proteases S1P and S2P cut newly arriving ATF6 and liberate the cytosolic transcription factor domain that is transported into the nucleus and activates ER stress genes.

(b) Release for degradation

![Diagram of protease degradation]

**Fig. 3.1 b** Intramembrane-cleaving protease (IClip)-initiated degradation illustrated for the anti-σ^E^ factor RseA of the Gram-negative bacterium *Escherichia coli*. In the resting state, RseA is located at the plasma membrane in a complex with the chaperone RseB and the σ^E^ factor RpoE bound to the cytosolic domain. Accumulation of unfolded proteins in the envelope recruits RseB, which dissociates from RseA–RpoE, and initiates cleavage of RseA, first by DegS and then by YaeL. The cleavage product with associated RpoE is liberated into the cytosol where it dissociates. RpoE is then free to activate stress genes, whereas the RseA fragment is degraded.
Fig. 3.1 c Release and secretion of ligands for intercellular signalling. The example illustrates secretion of the epidermal growth factor (EGF) ligand Spitz in *Drosophila*. Its membrane-bound precursor is transported to the Golgi apparatus in a process mediated by Star. Rhomboid-1, located in the Golgi, cleaves Spitz, and the luminal cleavage product is secreted along the secretory pathway and can activate EGF receptors on neighbouring cells.

### 3.2.2. Liberation of bioactive peptides as effectors and reporters

I-CLiP-catalysed reactions are not limited to the release of membrane-anchored proteins. Identification of SPP, which promotes the release of bioactive signal peptide fragments from the ER membrane in humans, revealed that intramembrane proteolysis is more versatile and might be a critical element of many diverse intra- and intercellular signalling events mediated by small peptides (Fig. 3.2). For example, calmodulin (CaM)-binding effector peptides can be released from the ER membrane by SPP and might influence CaM-dependent enzymes in higher eukaryotes (Martoglio et al., 1997).

A recent study might be the first conclusive example of an I-CLiP-induced release of an effector peptide in mammals. The MHC class II chaperone invariant chain (li) controls, among other functions, the differentiation of B cells from the immature stage to the mature stage (Matza et al., 2001). Presumably, in endosomal/lysosomal compartments of immature B cells,
endosomal proteases remove the luminal domain of IL, and, upon intramembrane cleavage by a currently unidentified I-CLiP, the cytosolic ~40-residue tail is liberated and activates NF-κB, which in turn mediates the transcription of genes required for B-cell differentiation (Fig. 3.2 a) (Matza et al., 2002). The released peptide is rapidly degraded to turn off the signal, indicating that the release of the peptide also determined its degradation.

I-CLiP cleavage products can also function as reporter signals. In humans, antigen-presenting MHC class I molecules are crucial components of immune defence. Their biosynthesis is carefully monitored, and cells with impaired MHC class I molecule synthesis are sorted out by natural killer (NK) cells. For the purpose of control, all the polymorphic MHC class I molecules are synthesized with a signal sequence that contains a highly conserved segment of nine residues (Braud et al., 1997). These signal peptide fragments become presented as so-called HLA-E epitopes at the surface of almost every nucleated cell (Braud et al., 1998a). They are generated from the signal sequences in a process that requires intramembrane cleavage by SPP (Lemberg et al., 2001) (Fig. 3.2 b). At the cell surface, the epitopes are recognized by receptors on NK cells and thereby report to the immune system that the cell has properly synthesized MHC class I molecules.

A similar type of signal peptide processing is catalysed by the S2P-like protease Eep in the Gram-positive bacterium Enterococcus faecalis. The signal peptide-derived bacterial octapeptide pheromone cAD1 is released from cells by a two-step cleavage of the cAD1 precursor protein, first by signal peptidase and then by the I-CLiP Eep (Fig. 3.2 c) (An et al., 1999). The resulting pheromone, which comprises the C-terminal half of the former signal peptide, is liberated from the plasma membrane toward the extracellular space and is free to enter neighbouring bacteria and induce a mating response.
Variations of RIP-induced liberation of bioactive peptides

(a) Intracellular release of effector peptide

Fig. 3.2 a Liberation of effector peptides. In immature human B cells, the protein invariant chain (li) is processed presumably in the endosomal/lysosomal MHC class II compartment. An unidentified intramembrane-cleaving protease (I-CLiP) promotes the liberation of the cytosolic tail, which activates differentiation of B cells via the NF-κB signal transduction pathway.

(b) Generation of reporter peptide

Fig. 3.2 b Generation of reporter peptides. Human lymphocyte antigen E (HLA-E) epitopes indirectly report biosynthesis of antigen-presenting MHC class I molecules to the human immune system. They are produced from the signal sequence of polymorphic MHC class I molecules in a process that includes cleavage, first by signal peptidase (SPase) and then by signal peptide peptidase (SPP). The resulting product is further processed in the cytosol and transported via the transporter associated with antigen processing (TAP) back into the lumen of the endoplasmic reticulum (ER) where it binds to a HLA-E molecule. The resulting complex is transported along the secretory pathway to the cell surface for presentation to CD94/NKG2 inhibitory receptors on natural killer (NK) cells.
Chapter 3

(c) Release of pheromone

Fig. 3.2 c Secretion of pheromones. The pheromone cAD1 of the Gram-positive bacterium Enterococcus faecalis is generated from the signal sequence of the cAD1 precursor by two cleavages: first by SPase and then by Eep. The exoplasmic cleavage product, pheromone cAD1, is liberated from the cell and taken up by nearby bacteria, in which it binds to the negative regulator protein TraA and thereby induces conjugation.

3.3. Emerging common principles

Knowledge about the mechanism of intramembrane proteolysis is limited, and our understanding of such events is rudimentary. Many candidate I-CLiPs were discovered only recently, and there are only a few assay systems to study intramembrane cleavage at the molecular level. From the available data, however, we can envision common principles of how I-CLiPs recognize a substrate protein and how I-CLiPs are integrated in a process of RIP.

3.3.1. Preparing the substrate for I-CLiP attack

Proteolysis in the plane of a lipid bilayer raises the question of how I-CLiPs recognize a scissile transmembrane region but avoid random attack on other transmembrane proteins. The most simple answer would be the recognition of a distinct sequence motif on the substrate, as is known for many other proteases. Most I-CLiPs, however, do not recognize specific sequences.
Mutational analysis with substrates for S2P, SPP and presenilin revealed that a variety of amino acid residues at the respective cleavage site are tolerated without affecting intramembrane cleavage (Duncan et al., 1998; Lemberg and Martoglio, 2002; Sastre et al., 2001; Struhl and Adachi, 2000; Weidemann et al., 2002). Mutations, however, might influence the efficiency of the cleavage reaction. In the case of NOTCH processing, a conserved valine at the cleavage site close to the cytosolic end of the transmembrane region (Fig. 3.3) is essential for efficient cleavage (Schroeter et al., 1998). However, similar mutations at the respective cleavage site in ß-APP did not affect cleavage. Rather than specific consensus sites, less well-defined parameters such as topology, length, flexibility, mobility and structure seem critical for cleavage by I-CLiPs, possibly to disrupt the rigid helical structure of typical transmembrane regions and disclose peptide bonds for proteolytic attack (Lemberg and Martoglio, 2002; Struhl and Adachi, 2000; Ye et al., 2000a).

One method of control relies on the preparation of a substrate for I-CLiP attack. Substrates of S2P, SPP and presenilin-type I-CLiPs are not immediately cleaved within the transmembrane region. These I-CLiPs act in processes that comprise a two-step proteolytic event (Sakai et al., 1996) (this is not yet known for rhomboids). The cut in the transmembrane region is preceded by another cleavage outside the membrane, but close to the exoplasmic end of the transmembrane region. Proteases such as S1P (Sakai et al., 1998), signal peptidase (Dalbey et al., 1997), ADAM proteases (Mumm et al., 2000) and ß-secretase (Vassar et al., 1999) catalyse the first cuts and recognize rather distinct motifs in the respective substrate. These first cleavages generate the immediate I-CLiP substrates, which could gain higher lateral mobility within the lipid bilayer, or be liberated out of a complex, and hence become more accessible for the respective I-CLiP.
3.3.2. More than one cut in the transmembrane region?

I-CLiP cleavage sites are typically deduced from analysis of cleavage products obtained in cell-based or cell-free assay systems. In many cases, only one of the two possible cleavage products has been analysed, using techniques such as polyacrylamide gel electrophoresis, sequencing or mass spectrometry. From such experiments we can envisage three principal types of I-CLiPs: those that cleave substrates in the centre of a transmembrane region (e.g. SPP); those that cleave substrates close to the cytosolic end of the membrane anchor (e.g. S2P); and those that may promote dual cleavage and cut substrates both in the central portion and close to the cytosolic end of the transmembrane region (e.g. presenilins) (Fig. 3.3).
Fig. 3.3 Intramembrane-cleaving protease (I-CLiP) cleavage sites. Transmembrane regions of presenilin, signal peptide peptidase (SPP) and site-2 protease (S2P) substrates are illustrated. Cleavage products that have been identified are highlighted in bold. Black arrows indicate major presenilin, approximate SPP, and S2P cleavage sites. The red arrow in one SPP substrate indicates a subsequent cleavage by a cytosolic protease.

References: APP (Sastre et al., 2001; Weidemann et al., 2002), NOTCH-1 (Okochi et al., 2002; Schroeter et al., 1998), CD44 (Lammich et al., 2002; Okamoto et al., 2001), HLA-A (Braud et al., 1997; Lemberg et al., 2001), p-Prl (Weihofen et al., 2000), HCV core-E1 (McLauchlan et al., 2002), SREBP-2 (Duncan et al., 1998).
Interestingly, I-CLiP cleavage products found in the cytosol contain only a remnant of three hydrophobic residues of the former transmembrane region, irrespective of whether it is an N-terminal or a C-terminal product (Fig. 3.3). We can speculate that former transmembrane regions must be shortened to that size so that liberated products become sufficiently soluble for functioning in the cytosol. I-CLiPs such as S2P may thus directly generate an appropriate cleavage product, whereas cytosolic products resulting from cleavage in the centre of a transmembrane region could undergo concomitant or further processing. Presenilins appear to catalyse cleavage in two regions – i.e. in the centre and close to the cytosolic surface of the membrane (reviewed in Fortini, 2002) (Fig. 3.3). For some substrates of SPP, however, an additional protease located in the cytosol shortens the hydrophobic stub to the appropriate length (F. Bland et al., unpublished).

3.4. Concluding remarks

Intramembrane-cleaving proteases, the I-CLiPs, represent a group of proteolytic enzymes that catalyse the hydrolysis of proteins within the plane of lipid bilayers. As a defining feature, their catalytic site motifs are located within predicted transmembrane regions. This is consistent with cleavage of substrate proteins in a membrane-spanning region and the resulting release of cleavage products from cellular membranes. Protein cleavage in transmembrane regions is not just a random event. It appears that I-CLiPs are engaged in highly controlled processes. I-CLiPs can liberate membrane-tethered proteins such that they are activated to promote functions in the cytosol or outside the cell. I-CLiPs can also inactivate membrane proteins by initiating their degradation. In addition to the liberation of proteins, I-CLiPs promote the controlled release of small bioactive peptides, which can affect intra- or intercellular signalling pathways, or function as reporters. Furthermore, pathogens such as HCV can exploit I-CLiPs of a host cell for the processing of their components (McLauchlan et al., 2002). Since the function of most I-CLiPs is not yet known, we can foresee the discovery of many more processes that depend on these unique proteases. Increasing knowledge
about the t-CliPs might also allow us to elucidate the unexplained mechanism of peptide bond hydrolysis within the hydrophobic environment of a lipid bilayer.

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Chapter 4

4. Concluding Remarks and Perspectives
The work presented in this thesis reports the molecular identification of the novel aspartic I-CliP SPP that cleaves some, but not all signal peptides within the centre of their transmembrane segment and thereby releases bioactive peptides from the ER membrane. In addition, the identification of SPP revealed a novel family of aspartic proteases with presenilin type motifs in their potential active site. The similarity of SPP to PS provides strong evidence that PS is the catalytic components of the \( \gamma \)-secretase complex. Furthermore, this work has revealed that \( \gamma \)-secretase inhibitors that are potential drug candidates for AD also target SPP.

The results have been already discussed in the publications (Chapter 2) and more general in the accompanying review (Chapter 3). This chapter provides an additional discussion, remarks and perspectives for future work.

4.1. Signal peptide processing

The main function of signal sequences is to target nascent polypeptide chains to translocation sites at the ER. Signal peptides were also implicated in post-targeting functions (Kim et al., 2002; Martoglio and Dobberstein, 1998). Recent data (Lemberg and Martoglio, 2002) as well as this thesis provide mechanistic insights on how signal peptides act beyond protein targeting.

Two-step proteolytic production of signal peptide-derived bioactive peptides shows analogy to RIP. This thesis has shown that the second cleavage, which triggers the release of the peptides from the membrane, is catalysed by an I-CliP, namely SPP, in agreement with other RIP processes. Consistent with the requirement of ectodomain shedding for other I-CliP substrates, cleavage by SPC of pre-proteins is a prerequisite for cleavage by SPP (Lemberg et Martoglio, 2002). But signal peptide processing and RIP are also distinct in important aspects. In contrast to other known processes comprising RIP, signal peptide processing appears not regulated. The first cleavage by SPC generating the I-CliP substrate occurs as soon as the substrate becomes
available and does not appear to be signal-induced as in other RIP processes. Thus, signal-peptide derived bioactive peptide generation is directly linked to the synthesis of its precursor. As exemplified by SPP catalysed HLA-E epitope generation (see chapter 3), this can be used to monitor the translation of the precursor. Furthermore signal peptide processing generates peptides, whereas most other RIP processes liberate large protein domains. Peptides do not comprise such complex biological information as for instance a transcription factor. But they may influence biological processes as effectors or reporter molecules (see Chapter 3).

Whether signal peptide processing by SPP primarily generates bioactive peptides or serves as a signal peptide-disposal process in eukaryotes, needs further examination. There are arguments against the latter. An SPP orthologue has not been identified in all eukaryotes and not all signal peptides are processed by SPP (Lemberg and Martoglio, 2002). Both findings indicate that an alternative pathway for removal of "normal" signal peptides may exist. On the other hand, it is possible that intramembrane proteolysis evolved initially as a means for degrading membrane-spanning stubs. Once this mechanism was established, it would be a small step to engraft a functional role to the cleavage products.

Likely, there are many more signal peptides with functions beyond protein targeting awaiting discovery. To date only two endogenous SPP substrates have unequivocally been identified, namely the signal peptides of p-Prl (Lyko et al., 1995) and of MHC class I molecules (Lemberg et al., 2001). These substrates are only found in higher animals, but SPP is likely present in all animals, indicating more SPP substrates. Recent exploration of requirements for SPP-dependent signal peptide processing may help to identify the additional SPP substrates (Lemberg and Martoglio, 2002).
Presently, all identified l-CliPs are only potential proteases, because the formal proof for their proteolytic activity is still missing. The proof would be the biochemical reconstitution of proteolytic activity with purified enzymes. This thesis does not provide the definitive proof for proteolytic activity of the identified SPP, but provides strong biochemical and genetical evidence that the identified protein is an aspartic l-CliP (see 2.2 and 2.3). Betting against it means in fact believing that the exogenous expression of the identified protein in *S. cerevisiae* is not directly responsible for the detected SPP activity, but activates another protein to become SPP. It means further that by a remarkable coincidence this hypothetical activator protein is inhibited by several aspartic protease transition states and requires two conserved aspartic residues for its proper function. Because this is a rather implausible scenario, it is safe to say that the identified protein is SPP.

The first result section gives the impression that SPP activity may be catalysed by a cysteine protease. This was concluded from the reaction of the peptidomimetic SPP inhibitor (ZLL)$_2$-ketone. Symmetrical ketones are potent cysteine protease inhibitors. They bind via nucleophilic attack of their carbonyl group to the catalytic thiol residue of cysteine proteases (Marquis et al., 1999; Marquis et al., 1998). As shown in the section 2.1 (ZLL)$_2$-ketone inhibits indeed the lysosomal cysteine protease Cathepsin B. But a carbonyl group of such a ketone can be hydrated in the active site of an aspartic protease and is thereby converted into a high-energy gem-diol intermediate that represents an aspartic protease transition state analogue (see below, Szelke, 1985). Therefore (ZLL)$_2$-ketone is not only a cysteine but as well an aspartic protease inhibitor, consistent with the actual findings (Section 2.2).

The catalytic centre of aspartic proteases is composed of two aspartic carboxyl groups. They polarise a water molecule stimulating its attack on the carbonyl carbon of the scissile peptide bond. Nucleophilic attack of the water molecule results in a tetrahedral gem-diol intermediate of the substrate that is
stabilised by hydrogen bonds from the gem-diol unit to the aspartic residues. (Veerapandian et al., 1992). Proton abstraction from a hydroxyl group of the gem-diol subunit by a negatively charged catalytic aspartic residue completes peptide bond hydrolysis (reviewed in Dunn, 2002). In this thesis, it has been shown that mutation of the C-terminal catalytic aspartic residue to an alanine within the LGLGD\textsubscript{269} motif of human SPP completely abolishes SPP’s activity, but not its capacity to bind TBL4K. The second proposed catalytic aspartic residue within the YD\textsubscript{219} motif has not been addressed in this thesis. As anticipated, mutation of this aspartate to an alanine results in complete abrogation of SPP activity (unpublished result). On the other hand, compared to SPP D269A, this mutation also results in loss of its capacity to bind TBL4K (unpublished result). The simplest explanation for the binding capacity loss is that the mutation D219A disturbs the structure of the active site. Alternatively it can be speculated that the conversion of TBL4K into an aspartic transition-state could depend on D219. Thus, L-852,646 that directs SPP and already represents a transition state analogue might still label SPP D219A (work in progress). This experiment might functionally distinguish the two aspartic catalytic residues and could be a start to explore the catalytic mechanism of SPP.

What are the further steps in characterising SPP? The possibility to express recombinant human SPP in yeast and monitor its activity is a powerful tool to analyse essential amino acids in the proposed active site motifs. So far, only the two aspartic residues have been analysed. Further, the complete conservation of the QPALLY motif is striking. Site-directed mutagenesis of this motif will show whether or not it is required for activity. Interestingly, the similar PALP motif in PS has been connected with γ-secretase complex formation and maturation (Takasugi et al., 2002; Tomita et al., 2001). Does this indicate that SPP is as well part of a protein complex? Recombinant expression of active human SPP in yeast suggests that SPP is proteolytically active independently of any further components, but nevertheless regulatory components may associate with SPP.
Structural information about any l-CLiP will help to understand the mechanism of intramembrane proteolysis. In order to obtain structural information by protein crystallography or NMR, a high amount of to homogeneity purified native l-CLiP is required. Presently, no l-CLiP has been purified in a native state, mainly because of the inherent difficulties of working with these polytopic membrane proteins and of the lack of suitable assays to monitor their activity in a detergent solubilised state. Indeed, a purified active l-CLiP would also be the ultimate proof for its proteolytic activity, an aim that is especially desirable for the controversial PS. At the moment, SPP is for several reasons the most promising candidate to be structurally characterised. Firstly, SPP apparently acts alone whereas for instance γ-secretase activity requires at least three essential co-factors in addition to PS that are all membrane proteins. Secondly, SPP activity can easily be monitored with detergent solubilised proteins, a criterion that is required for the purification under native conditions and is so far not fulfilled for S2P and rhomboid like l-CLiPs. Nevertheless, because SPP is a highly hydrophobic protein that easily sticks to chromatography column resins such as Superdex, MonoS, MonoQ (unpublished observation), the purification by itself will be an ambitious project.

SPP orthologues are present in M. musculus, D. melanogaster, C. elegans and A. thaliana and likely in all higher eukaryotes. This suggestion derives from the phylogenetic tree analysis based on a sequence alignment (ClustalW) shown in section 2.2. They show all the characteristics motifs of the SPP/SPPL family (see below). In contrast to SPP-like (SPPL) proteins, they contain all a C-terminal ER retrieval signal. Initially, the yeast Schizosaccharomyces pombe SPP homologue has been aligned to the SPPL1 subfamily. But based on a phylogenetic tree building algorithme (UPGMA, see Appendix III), this protein is a SPP orthologue. This finding suggests that SPP activity emerged earlier during evolution as initially proposed (Section 2.2). Presently, the C.elegans SPP (SPP Ce)7 is the only orthologue that has been characterised.

7 T05E11.5 (www.wormbase.org)
to some extent. The data is derived from genome-wide analysis using RNAi and DNA microarray experiments (Hill et al., 2000; Kamath et al., 2003; Kim et al., 2001c). The systematic functional analysis of the C. elegans genome using RNAi revealed morphological and movement defects in SPP-deficient larval and adult animals (Kamath et al., 2003). Furthermore, SPP Ce cDNA is expressed in all growth stages with a strikingly increased expression in the embryonic stage (Hill et al., 2000), and seems to be co-regulated with biosynthesis, protein expression and heat shock genes (Kim et al., 2001c). These data provide a starting point for characterising SPP in vivo and the examination of other SPP orthologues will hopefully be helpful to reveal more physiological and functional roles of SPP.

4.3. Chances and limitations of SPP as drug target

SPP is considered as potential drug target for HCV therapy (McLauchlan et al., 2002). Chronic HCV infection is the major cause of liver disease that may lead to cirrhosis and hepatocellular carcinoma (Kuo, 1989). HCV is a small enveloped virus that belongs to the group of positive stranded RNA viruses. Its genome encodes a single polyprotein that is cleaved co- and post-translationally by host and viral proteases yielding the mature proteins such as the structural glycoproteins and the core protein (reviewed in Dubuisson et al., 2002). As shown recently, SPP is one of the host proteases (McLauchlan et al., 2002).

The HCV core protein, which is located at the N-terminus of the viral polyprotein, precedes an internal ER signal sequence. After co-translationally translocation of the polyprotein into the ER, the core protein and the internal signal sequence are removed from the polyprotein by SPC. Facing the cytosol, the core remains anchored at the ER membrane via the signal sequence. Intramembrane proteolysis of the signal sequence catalysed by SPP then promotes budding of the core protein from the membrane into lipid droplets (McLauchlan et al., 2002). Lipid droplets are storage compartments
consisting of neutral lipids surrounded by a monolayer of phospholipids (Murphy and Vance, 1999). As shown in Result section 2.3 (results from M.K.Lemberg), inhibition of SPP by SPP/γ-secretase inhibitors and the SPP specific inhibitor (ZLL)₂-ketone prevent budding of core protein into lipid droplets. Assuming that SPP mediated lipid droplet association of the core protein is an essential process in the live cycle of HCV, specific SPP inhibitors could be potent HCV therapeutic compounds.

There are factors questioning SPP to be a HCV therapeutic drug target to date. Presently there is no suitable system to explore HCV in vivo. This makes it difficult to figure out whether lipid droplet association of the HCV core proteins is essential for HCV replication. Further, SPP inhibitors may have adverse affects. Firstly, SPP is likely involved in many biological pathways that could be essential in the living organism. For instance, SPP inhibition could disarrange HLA-E signalling resulting in a strong autoimmune response (Lemberg et al., 2001). Secondly, as provided by this thesis, SPP inhibitors could also interfere with essential pathways involving PS and likely SPPL proteins. On the other hand, considering the severity of HCV infection, adverse affects of therapeutical compounds are in fact tolerable.

4.4. SPP/SPPL-family

SPP belongs to a conserved family of polytopic membrane proteins with members in archaea, fungi, arthropods, plants and vertebrates (this thesis, Grigorenko et al., 2002; Ponting et al., 2002). SPP homologues that were not suggested SPP orthologues were termed SPP-like (SPPL) proteins. SPPL proteins were further divided into subfamilies based on phylogenetic tree analysis (see Appendix III). Typical for SPP/SPPL family members are 3 highly conserved motifs: YDxxVF, LGxGDxxPG and QPALLYxxP (see Fig. 2.2.2). The former two motifs contain putative catalytic aspartic residues and are embedded in predicted transmembrane segments. The latter motif, QPALLYxxP is not unequivocally embedded in transmembrane segments. By
way of an exception, the putative complex formation motif (see above) is not present in archaea SPPL proteins (Ponting et al., 2002) and SPPL2c contains a FDxxxVF motif instead of an YdxxxVF motif. In contrast to SPP, the SPPL proteins have no obvious localisation signal. It is speculated that this proteins are located in a distinct organelle than the ER. The human genome encodes 5 members of the SPP/SPPL family. Interestingly, SPPL2a, SPPL2b and SPPL2c, which are likely only present in mammals (Appendix III), contain a so-called Protease associated (PA) domain\(^8\). This domain has been found as an insert domain in diverse proteases, but also in a plant vacuolar sorting receptor and a ring finger protein of unknown function. The location of the PA domain varies and may be outside of the catalytic domain (Mahon and Bateman, 2000). In addition to the conserved motifs, comprising the putative catalytic residues, the presence of the PA domain in some SPPL proteins provides further evidence that these proteins are proteases and it is suggested that all SPPL proteins are aspartic l-CliPs.

What are the substrates of these putative aspartic l-CliPs? This question is the opposite of the question that has been addressed in this thesis: We now know putative proteases but not the substrates. It is tempting to speculate that substrates for SPPL proteins are not signal peptides but membrane proteins such as for example dormant transcription factors or nuclear signalling molecules. Although the striking homology of SPP-like proteins to SPP suggests the same topology, an opposite orientation of the catalytic motifs containing membrane domains in SPP-like proteins cannot be excluded (see Appendix II). An opposite orientation would result in considering type I membrane proteins as potential SPPL substrates. For instance divergent membrane protein topology has been reported for the *E. coli* membrane proteins RnfA and RnfE even though they display more than 35% sequence identity over a stretch of five transmembrane regions (Saaf et al., 1999). Therefore, the characterisation of the topology of SPPL proteins is a

\(^8\) pfam 02225.6 (Conserved domain database, NCBI)
challenge for the future that will not only tell us more about the proteases but also about their substrates.

What are the approaches to reveal SPPL substrates? Whether SPPL substrates must fulfill similar requirements as SPP-substrates is presently only part of speculations. Helix-breaking residues are likely a common feature of most l-CliP substrates: Transmembrane segments are supposed to form helical structures that make peptide bonds hardly accessible for proteolysis since they are stabilized by hydrogen bonds (Hubbard, 1998; Paetzel et al., 1998). Thus helix-breaking residues might turn small segments of α-helices into random coil segments that are accessible to proteolysis. Thus, an initial approach to identify SPP-like substrates might be looking for helix breaking residues within transmembrane segments in protein databases.

An alternative approach to identify substrates for SPPL and also for SPP is for instance the proteomic analysis of SPP/SPPL-activity deficient cells; in analogy to the approach used to identify the epidermal growth factor ErbB4 as a γ-secretase substrate (Lee et al., 2002). Lee and co-workers compared subcellular fractions of γ-secretase inhibitor-treated and -untreated cells by 2D PAGE. Thereby, they have found ErbB4 only in the membrane fraction of cells treated with γ-secretase inhibitors, indicating that γ-secretase is required for the release of ErbB4 from the membrane. A similar approach could be applied to identify SPP/SPPL substrates using (ZLL)₂-ketone or RNAi to inactivate SPP/SPPL activity. Obviously, this approach demands that the putative substrates are not alternatively degraded and present in sufficient amount in order to detect them by 2D PAGE analysis. Furthermore, the substrates cleavage must not be dependent on specific induction conditions. Many substrates however will not fulfill all these requirements and escape detection.
4.5. Presenilin and SPP

Recent observation questioned PS to be the catalytic subunit of the γ-secretase complex (see 1.2.2.3, reviewed in Sisodia et al., 2001; Sisodia and St George-Hyslop, 2002). One main argument was that PS does not look like a typical protease. However, this thesis shows that among the members of the γ-secretase complex, only PS resembles a protease, namely SPP. Furthermore compounds mimicking aspartic transition-state analogous are directed to both PS and SPP, providing further evidence that PS is a protease. Nevertheless further work is necessary to prove the proteolytic activity of PS and also SPP.

Even though presenilin and SPP share similarities, they are distinct from each other in important aspects. Firstly, PS cleaves type I membrane proteins, whereas SPP cleaves type II orientated peptides. This observation is consistent with the orientation of the transmembrane segments containing the putative active site residues: In PS the orientation of TMD6 containing the YD motif is lumen to cytoplasm, and the orientation of TMD7 containing the LGLGD motif is cytoplasm to lumen. On the contrary, the orientation of SPP TMD4 containing the YD motif is cytoplasm to ER lumen, and TMD5 containing the LGLGD motif is lumen to cytoplasm. This consistence is meaningful as long as no type I orientated SPP substrate or type II orientated PS substrate is identified. Secondly, SPP but not PS is N-glycosylated. Initial observations suggest that N-glycosylation at the N-terminus is a conserved feature of SPP/SPPL proteins and that it is at least not essential for proteolytic activity of SPP (unpublished observation). Furthermore, PS is active as stable heterodimer, consisting of an N-terminal and C-terminal fragment, which are generated by endoproteolysis of PS in its cytosolic loop between TMD6 and TMD7. On the other hand, SPP acts as full-length protein. This suggests that PS has to be proteolytically activated but not SPP. In addition, SPP seems to act without additional proteins, whereas PS requires association with other proteins for activity. More, it has been suggested that PS cleaves its substrate
simultaneously at two distinct sites, namely at the \( \gamma \)-processing site in the middle of the substrate TMD and at the \( \varepsilon \)-processing site close to the cytosolic interphase of the substrate TMD. Cleavage of substrates at two topologically distinct sites might be explained by multiple active site confirmations of PS (Haass and Steiner, 2002). On the contrary, SPP apparently cleaves only in the middle of its substrate TMD. Last, this thesis has shown that some inhibitors can discriminate between PS and SPP. Even PS and SPP distinguish each other in important aspects, they likely have a similar catalytic mechanism. Further characterisation of SPP will therefore also provide important data for PS research and vice versa.

### 4.6. GxGD-type aspartic proteases

SPP/SPPL proteins and PS do not contain the classical (D(T/S)G(T/S)) signature motif of aspartic proteases. In contrast, they contain a LGLGD motif, comprising a putative catalytic aspartic residue. A similar motif, GYGDF, comprising a catalytic aspartic residue, is also present in the family of the bacterial aspartic type IV prepilin peptidases (TFPPs). TFPP's are polytopic membrane proteins that catalyse two reactions: One reaction catalyses the cleavage of prepilin peptides, and the other methylates the free \( \alpha \)-amino group of the newly generated substrate. The former reaction is essential for type IV pilus biogenesis and type II protein secretion. Like for PS and SPP, evidence for proteolytic activity of TFFPs derives from site-directed mutational analysis of putative catalytic residues and inhibitor studies (LaPointe and Taylor, 2000). In agreement with the type IV prepilin leader peptide processing site, the catalytic motifs of TFPPs occur close, but not within a transmembrane segment. Thus TFPPs are no I-CliPs. In further contrast to the aspartic I-CliPs, the two catalytic aspartic residues of TFPPs are separated by two TMD. Because so far all identified polytopic aspartic proteases, namely SPP/SPPL proteins, PS and TFPPs, share the catalytic consensus GxGD motif, they have been termed GxGD-type aspartic proteases (Haass and Steiner, 2002). In the protease database MEROPS the
GxGD-type aspartic proteases have been assigned to the AD clan of aspartic proteases. The clan is subdivided into two families. The family A24 comprises the TFPP's and the family A22 the aspartic I-CliPs. The latter is further subdivided into A22A, comprising PS, and into A22B, comprising SPP and SPPL proteins. It can not be excluded that there are more GxGD-type aspartic proteases, which are distinct from PS, SPP/SPPL and TFFPs, awaiting discovery.

The question arises whether or not GxGD-type aspartic proteases are homologues, meaning that they are derived from a single ancestral precursor, which share similarities only in some domains due to convergent evolution. Phylogenetic tree analysis shows that PS, SPP/SPPL and TFFP are three well-separated protein families (see Appendix III). A global hidden markov model that is derived from a multiple sequence alignment of the SPP/SPPL family shows significant homology with a plant presenilin homologue (Ponting et al., 2002). But more detailed evolutionary analysis is required to confirm this proposed homology between PS and SPP/SPPL (Grigorenko et al., 2002). A homology between the aspartic I-CliPs and TFPP's has not been detected. Thus, nature independently evolved at least two times, maybe three times, a GxGD-type catalytic mechanism.

4.7. γ-secretase – a true AD drug target?

γ-secretase inhibitors are considered as potential therapeutic compounds to treat AD because they prevent Aβ formation. This thesis provides results that could advance the development of specific γ-secretase inhibitors. Molecules that direct the active site of an enzyme, thereby preventing the binding of the natural substrate, are potent enzyme inhibitors. In the case of the γ-secretase complex the nature of the protein comprising the active site is controversial and thus the design of active-site directed γ-secretase inhibitors is difficult. Providing strong evidence that PS is the catalytic subunit of the γ-secretase complex, this thesis points to the drug target, namely PS. Furthermore, it has
been shown that some γ-secretase inhibitors also target SPP. This shows that in future all potential therapeutic γ-secretase inhibitors must be probed for their SPP and SPPL inhibition potency. In addition, the thesis strongly suggests that DAPT and its second generation derivative LY411575, which are both potent γ-secretase inhibitors whose reaction mechanism is unknown, are directed to the active site of PS. This is deduced from the affinity-labelling competition experiments showing that all examined SPP/γ-secretase inhibitors, including known transition-state analogous, compete for the same binding site of SPP.

Recently, it emerged that γ-secretase is involved in many important signalling pathways (see Chapter 3 and Fortini, 2002), questioning γ-secretase inhibitors to be useful compounds to treat AD. Preferably, therapeutical compounds should lower Aβ42 production without disrupting the function of other γ-secretase targets. Indeed, FAD mutations in PS shifting the cleavage of APP from Aβ40 to Aβ42 suggest that small changes in the conformation of the γ-secretase complex can differentially modulate cleavage activity. Inhibitors that selectively inhibit Aβ production without affecting Notch cleavage by γ-secretase have been also reported (Petite and al, 2001). Thus, there is hope for compounds only affecting Aβ42 production. Nevertheless, β-secretase, which also plays a direct role in Aβ formation, is likely the more promising drug target than γ-secretase since it has so far not been associated to other signalling pathways and mice live well without β-secretase (reviewed in Vassar, 2002).
4.8. Conclusions

How I-Clips catalyse intramembrane proteolysis is a fascinating biochemical question, but we are far away from understanding this process. How the active site attracts both a water molecule, which is required for catalysis, and a hydrophobic substrate is not yet clear. It has been speculated that membrane-embedded substrates, which can only move in the two-dimensional environment of the lipid bilayer, access an initial docking site of an I-Clip and then a conformational change would allow the substrate to enter the active site (Wolfe and Selkoe, 2002). Clearly, structural information about an I-Clip will be necessary to discern the details of the mechanism of intramembrane proteolysis.

The I-Clips and RIP processes so far discovered probably represent just the tip of the proverbial iceberg. Thus likely, a new chapter in biochemistry and cell biology has just been started, absorbing many biologists in the future. Simply the characterisation of the SPP/SPPL family and their substrates requires huge manpower, but it will hopefully be profitable for scientists and society.
Chapter 5

5. Materials and Methods
Methods are described here that have been established or improved by myself but are not described in detail in chapter 2 or in literature.

5.1. Materials

5.1.1. Solutions

1M DTT: To make ten 1 ml aliquots, fill up 1.54 g DTT to 10 ml water and store 1ml aliquots at -20°C.

500 mM EDTA pH 8.0: To make 100 ml of this solution, dissolve 18.6 g Na₂EDTA₂H₂O in water, adjust pH to 8.0 with NaOH solution, and complete to 100 ml with water and autoclave.

Energy mix: 50 mM HEPES-KOH pH 7.6, 12.5 nm ATP, 0.25 mM GTP, 110 mM creatine phosphate, 10 mg/ml creatine kinase, and 0.25 mM of each amino acid, except methionine. To make 1 ml of this solution, dissolve 41 mg creatine phosphate (disodium salt: 4H₂O) and 10 mg creatine kinase in 590 µl water and add 50 µl 1M HEPES-KOH pH 7.6, 125 µl 100 mM ATP solution, 2.5 µl 100 mM GTP solution and 250 µl 19 amino acid mix without methionine. The energy mix is stored at -20°C in aliquots. Do not refreeze!

1M HEPES-KOH, pH 7.6: To make 200 ml of the buffer, dissolve 47.7 g HEPES in water, adjust pH to 7.6 with KOH solution, and complete to 200 ml and autoclave.

4M KOAc: To make 250 of the solution, dissolve 98.25 g KOAc in water, neutralize to pH 7 with diluted acetic acid, and complete to 250 ml with water and autoclave.
1M Mg(OAc)\(_2\): To make 50 ml of this solution, dissolve 10.75 g Mg(OAc)\(_2\) \(\cdot\) 4H\(_2\)O in water, neutralize to pH 7 with diluted acetic acid, and complete to 50 ml with water and autoclave.

10mg/ml PMSF: To make 5 ml of the solution, dissolve 50 mg PMSF in 5 ml isopropanol. Do not filter and prepare just before use.

100 mM Puromycin: To make 360 μl of the solution, dissolve 20 mg puromycin salt in 360 μl water. Do not filter!

0.8M sorbitol: To make 250 ml of the solution, dissolve 36.44 g D-Sorbitol in water and complete to 250 ml. Filter sterile.

2M sucrose: To make 200 of the solution, dissolve 136.9 g sucrose in water and complete to 200 ml. Filter sterile.

100% (w/v) TCA: To make 100 ml of the solution, dissolve 100 g trichloroacetic acid in water and complete to 100 ml.

20% (w/v) TCA: To make 100 ml of the solution, add 20 ml 100% TCA to 80 ml water.
5.1.2. Buffers

Depletion buffer: 50 mM CAPS-KOH, 50 mM HEPES-KOH, 10μg/ml PMSF, 1mM DTT, pH 9.6. To make 500 ml of the solution dissolve 5.53g CAPS and 5.96 g HEPES in water, add 500 μl 1M DTT and 500 μl 10 mg/ml PMSF, adjust pH to 9.6 with KOH solution and complete to 500 ml with water. Filter sterile.

High Salt Buffer: 250 mM sucrose, 50 mM HEPES-KOH, pH7.6, 500 mM KOAc, 2 mM Mg(OAc)₂, 1mM DTT, 10 μg/ml PMSF. To make 50 ml of the buffer, add 6.25 ml 2M sucrose, 2.5 ml 1M HEPES-KOH, pH 7.6, 6.25 ml 4M KOAc 100 μl 1M Mg(OAc)₂, 50 μl 1M DTT, 50 μl 10 mg/ml PMSF and complete to 50 ml with water.

Homogenisation buffer: 50 mM HEPES-KOH, pH 7.6, 0.2M Sorbitol, 25 mM KOAc, 5 mM Mg(OAc)₂, 2mM DTT, 20 μl/ml PMSF, 1 U Complete Mini Cocktail. To make 10 ml of this buffer add 500 μl 1 M HEPES-KOH, pH 7.6, 62.5 μl 4 M KOAc, 50 μl 1M Mg(OAc)₂, 20 μl 1M DTT, 20 μl 10 mg/ml PMSF, 1 pill Complete Mini Cocktail to 2.5 ml 0.8 M sorbitol, and complete to 10 ml with water.

Lysis buffer: 0.7 M D-sorbitol, 1% Yeast extract, 2% BactoPeptone, 1% Dextrose (or Galactose), 10 mM TRIS-HCl, pH 7.4, 5 mM DTT. To make 50 ml of the buffer dissolve 0.5 g Yeast extract, 1 g BactoPeptone, 0.5 g Dextrose (or Galactose) in 43.75 ml 0.8M Sorbitol, add 500 μl 1M TRIS-HCl, pH 7.4, 250 μl 1 M DTT, and complete to 50 ml with water.

Lysis buffer with Lyticase: 4000-5000 U/ml Lyticase, 0.7 M D-sorbitol, 1% Yeast extract, 2% BactoPeptone, 1% Dextrose (or Galactose), 10 mM TRIS-HCl, pH 7.4, 5 mM DTT. To make 5 ml of this buffer dissolve 8'000-10'000 U Lyticase in 5 ml Lysis Buffer.
**RM Buffer:** 250 mM sucrose, 50 mM HEPES-KOH, pH 7.6, 50 mM KOAc, 2 mM Mg(OAc)$_2$, 1 mM DTT, 10 µg/ml PMSF. To make 250 ml of the buffer, add 31.25 ml 2M sucrose, 12.5 ml 1M HEPES-KOH, pH 7.6, 3.125 ml 4M KOAc, 500 µl 1M Mg(OAc)$_2$, 250 µl 1M DTT, 250 µl 10 mg/ml PMSF, and complete to 250 ml with water. Filter sterile.

**Salt mix:** 500 mM HEPES-KOH, pH 7.6, 1 M KOAc, 50 mM Mg(OAc)$_2$. To make 1 ml of this solution, add 500 µl 1M HEPES-KOH, pH 7.6, 250 µl KOAc, 70 µl Mg(OAc)$_2$ to 180 µl water.

**Solubilisation buffer:** 125 mM sucrose, 50 mM HEPES-KOH, pH 7.6, 50 mM KOAc, 2 mM MgAc, 1 mM DTT, 10 µg/ml PMSF, 2%(w/v) CHAPS. To make 50 ml of the buffer, add 3.125 ml 2M sucrose, 2.5 ml 1M HEPES-KOH, pH 7.6, 625 µl 4M KOAc, 100 µl 1M Mg(OAc)$_2$, 50 µl 1 M DTT, 50 µl 10 mg/ml PMSF, 1g CHAPS and complete to 50 ml with water.

**SPP buffer:** 25 mM HEPES-KOH pH 7.6, 100 mM KOAc, 2 mM Mg(OAc)$_2$, 1 mM DTT. To make 10 ml of this solution, add 250 µl 1 M HEPES-KOH, pH 7.6, 250 µl KOAc, 20 µl Mg(OAc)$_2$, 10 µl 1 M DTT, and complete with water to 10 ml.

**SRP buffer:** 20 mM HEPES-KOH, pH 7.6, 650 mM KOAc, 5 mM Mg(OAc)$_2$. To make 1 ml of this solution, add 20 µl 1 M HEPES-KOH, pH 7.6, 162.5 µl KOAc, 5 µl Mg(OAc)$_2$ to 812.5 µl water.

**0.5M Sucrose Cushion:** 500 mM sucrose, 50 mM HEPES-KOH, pH 7.6, 50 mM KOAc, 2 mM Mg(OAc)$_2$, 1 mM DTT, 10 µg/ml PMSF. To make 50 ml of the cushion, add 12.5 ml 2M sucrose, 2.5 ml 1M HEPES-KOH, pH 7.6, 625 µl 4M KOAc, 100 µl 1M Mg(OAc)$_2$, 50 µl 1 M DTT, 50 µl 10 mg/ml PMSF and complete to 50 ml with water. Filter sterile.
1.5M Sucrose Cushion: 1.5 mM sucrose, 50 mM HEPES-KOH, pH 7.6, 500 mM KOAc, 2 mM Mg(OAc)₂, 1 mM DTT, 10 µg/ml PMSF. To make 50 ml of the cushion, add 37.5 ml 2M sucrose, 2.5 ml 1M HEPES-KOH, pH 7.6, 6.25 ml 4M KOAc, 100 µl 1M Mg(OAc)₂, 50 µl 1M DTT, 50 µl 10 mg/ml PMSF and complete to 50 ml with water. Filter sterile.

**TD buffer:** 100 mM TRIS-HCl, pH 9.4, 10 DTT mM. To make 10 ml of the buffer, add 1 ml 1M TRIS-HCl, pH 9.4, 100 µl 1M DTT to 8.9 ml water.

### 5.1.3. Medium

**Minimal liquid medium:** 1x Yeast amino acids, 1x YNB, 1-2% sugar (such as Glucose, Galactose or Sucrose). To make 1000 ml of this medium, add 10 ml 100x Yeast amino acids, 50 ml 20x YNB, 10-20 g sugar, and complete to 1000 ml with water. Filter sterile!

100x Yeast amino acids: ADE (4 g/l), URA (2 g/l), TRP (2 g/l), HIS (2 g/l), LYS (3 g/l), LEU (3 g/l), MET (2 g/l). To make 50 ml of this solution add 200 mg ADE, 100 mg URA, 100 mg TRP, 100 mg HIS, 150 mg LYS, 150 mg LEU, 100 mg MET to water and complete to 50 ml. Note: For selection, just add the reagents your yeast strain requires minus the reagent you selecting for. For example for the strain BY4742 transformed with pDAW300, add 100 mg HIS, 150 mg LYS, and 150 mg LEU to water and complete to 50 ml. Filter sterile!

20x YNB: Add 67 g to water and complete to 500 ml. Filter sterile!
5.2. Methods

5.2.1. Preparation of detergent solubilised rough ER membrane proteins (sol.PKXRM)

Required component: ER derived rough microsomes prepared as described in detail in (Martoglio et al., 1998).

Steps:

Samples and buffers should be kept on ice unless otherwise stated.

1. To wash microsomes, resuspend 18'000 eq RM's with RM Buffer to a final volume of 52.6 ml using a dounce homogenizer.

2. Distribute the RM suspension equally to two 26.3 ml polycarbonate bottle assembly tubes for a Type 70Ti rotor.

3. Centrifuge at 36'000 rpm for 1 hr at 4°C in a Beckman Type 70Ti rotor.

4. Discard the supernatant and resuspend the pellet with high salt buffer to a final volume of 15ml using a Dounce Homogenizer.

5. Add 360 ml 100 mM Puromycin (to 2 mM) and 50 ml 100 mM GTP (to 0.33 mM) and incubate at RT for 20 min on a turning wheel.

6. Add 13.8 g fine crystals sucrose and further incubate at RT until sucrose is dissolved. The final volume is 24 ml with 1.8 M sucrose.

7. Distribute the sample equally to four 13.2 ml Ultra Clear tubes for a SW41 Ti rotor.
8. Overlay each sample with 3 ml 1.5 sucrose cushion and fill up with 3.5 ml RM buffer. Avoid mixing!

9. Centrifuge at 36'000 rpm for 16-18 hrs at 4°C in a Beckman SW41 Ti rotor. Low accel and deccel.

10. Collect the Puromycin/High Salt treated rough microsomes (PKRM) layer on the top of the 1.5M cushion. Avoid mixing with the ribosomal layer!


12. Optional: Wash PKRM's (see step 1-3) and resuspend in 26.2 ml RM buffer.

13. Fill up sample with depletion buffer to a final volume of 288 ml and incubate for 30 min. on ice.

14. In the meantime, prepare six 70 ml polycarbonate bottle assembly tubes for the Type 70Ti rotor and add 22 ml 500 mM sucrose cushion per tube.

15. After incubation apply the sample equally, without mixing, onto the 22-ml sucrose cushion.

16. Centrifuge at 40'000 rpm (186'000g) for 1 hr at 4°C in a Beckman Type 45Ti rotor.

17. Discard the supernatant and wash the pellet (PKXRM's) as described in step 1-3. Note: The supernatent contains the luminal proteins.
18. Resuspend the pellet in 18 ml solubilisation buffer using a dounce homogenizer and incubate at 4°C for 1-2 hrs on a turning wheel.

19. Distribute the sample equally to six 3.2 ml polycarbonate tubes for the TLA-100.4 rotor.

20. Centrifuge at 75'000 rpm (305'550g) for 1 hr at 4°C in a Beckman TLA100.4 rotor.

21. Collect the supernatant \(\rightarrow\) sol. PKXRM. Freeze 1ml aliquots in liquid nitrogen and store at \(-80°C\) until use.
5.2.2. Preparation of detergent solubilised human SPP expressed in *S. cerevisiae*

Required components: *S. cerevisiae* strain BY4742 (MATα; his3Δ; leu2Δ0; lys2Δ0; ura3Δ0) from Euroscarf, Yeast expression vector encoding human SPP pDAW300 (for cloning procedure see 2.2)

Steps:

Samples and buffers should be kept on ice unless otherwise stated.

1. Transformation of pDAW300 into BY4742 by standard lithium acetate transformation and selection on agar plates (-URA, 2% Galactose).

2. Inoculate a fresh transformed colony in 5 ml liquid minimal medium (-URA, 2% Galactose) and grow at RT to log phase (OD₆₀₀ 0.6-0.8).

3. Inoculate the preculture in 150 ml liquid minimal medium (-URA, 2% Galactose) and grow at RT to log phase (OD₆₀₀ 0.6-0.8).

4. Harvest the cells by centrifugation at 2000 rpm for 5 min at RT in a Falcon centrifuge.

5. Discard supernatant. To wash cells, resuspend cells in 50 ml water and centrifuge at 2000 rpm for 5 min at RT in a Falcon centrifuge.

6. Discard supernatant. Resuspend cells in 25 ml 0.8M Sorbitol and centrifuge at 3000 rpm for 5 min at RT in a Falcon centrifuge.

7. Discard supernatant. Resuspend cells in 2 ml TD buffer (50-60 OD/ml) and incubate for 10 min at RT.
8. Centrifuge at 2000 rpm for 5 min at RT in an eppendorf centrifuge.

9. Discard supernatant. To prepare sheroplasts, resuspend cells in 1ml Lysis Buffer (100-120 OD/ml) with Lyticase (40 U/OD), transfer to 15 ml falcon tube, and incubate for 1 hr at 25°C under shaking (350 rpm).

10. Centrifuge at 4000 rpm for 5 min at 4°C

11. Resuspend cells in 4 ml Homogenisation buffer (25-30 OD/ml) and break cells using a dounce homogenizer (20 strokes).

12. Centrifuge at 5000 rpm for 5 min at 4°C


14. Centrifuge at 40'000 rpm (100'000g) for 30 min at 4°C in a Beckman TLA100.4 rotor.

15. Perform one of the following steps to yield crude yeast microsomes (YM/SPP), solubilised crude yeast microsomes (sol. YM/SPP) or solubilised depleted crude yeast microsomes (sol. YXM/SPP):

- Resuspend pellet in 500 μl RM buffer
  \[\rightarrow\text{YM/SPP}\]

- Resuspend pellet in 500 μl Solubilisation buffer, incubate for 2 hrs at 4°C on a turning wheel, centrifuge at 75'000 for 30 min at 4°C in a Beckman TLA100.1 rotor, Collect supernatant
  \[\rightarrow\text{sol. YM/SPP}\]

- Resuspend pellet in 5 ml Depletion buffer, incubate on ice for 30 min, centrifuge at 60'000 for 30 min at 4°C in a Beckman TLA100.4 rotor, resuspend pellet in 500 μl Solubilisation buffer,
incubate for 2 hrs at 4°C on a turning wheel, centrifuge at 75'000 for 30 min at 4°C in a Beckman TLA100.1 rotor, Collect supernatant

→ sol. YXM/SPP

16. Freeze samples in liquid nitrogen and store at −80°C until use.

Note: Best results in SPP assay and TBL4K labelling were obtained with sol. YM (see Result Part II).
5.2.3. Signal peptide processing assay with detergent solubilised SPP activity

Required components: Solubilised SPP activity (see above) or microsomes (see (Martoglio et al., 1998)), capped mRNA encoding a signal peptide with proper termination codon or in a truncated form, wheat germ extract (Martoglio et al., 1998)

Steps:

Samples and buffers should be kept on ice unless otherwise stated.

*In vitro translation:*

1. Mix on ice: 6 µl water
   1 µl salt mix
   2 µl energy mix
   10 µl wheat germ extract
   2 µl SRP buffer
   2 µl RM buffer
   1 µl [\(^{35}\)S]methionine (>1000Ci/mmol)
   1 µl capped mRNA
   25 µl total

2. Incubate for 15 min at 25°C

3. Optional: Freeze sample in liquid nitrogen and store until use at –80°C
**SPP assay:**

1. Mix on ice: 4µl 0.5M EDTA, pH 8
   31µl SPP buffer
   1µl DMSO or Inhibitor dissolved in DMSO
   2µl sol. SPP activity (for exp. sol. PKXRM) or microsomes (for exp. RM's (1 eq/µl)
   2µl Translation mix
   40µl Total

2. Incubate for 60 min at 30°C

3. Add 40 µl 20% TCA to sample, vortex, and incubate on ice for 30 min

4. Centrifuge at 14'000 rpm for 5 min at 4°C in an eppendorf centrifuge.

5. Discard supernatant. Wash pellet with 150 µl Aceton.

6. Centrifuge at 14'000 rpm for 2 min at 4°C in an eppendorf centrifuge.


8. Centrifuge at 14'000 rpm for 1 min at 4°C in an eppendorf centrifuge.

9. Incubate sample for 20 sec at 65 °C

10. Add 16 µl WF sample buffer, vortex, and incubate for 15 min at 65°C

11. Analyse sample by SDS-PAGE using Tris-Bicine gels (Wiltfang et al., 1997). The radiolabeled proteins can be visualised by autoradiography using a Phosphorimager.
5.2.4. Labelling of detergent solubilised SPP by TBL₄K or L-858,646

Required components: sol. SPP activity (see above), TBL₄K or L-858,646

Steps:

Samples and buffers should be kept on ice unless otherwise stated.

Labelling reaction:

1. Mix on ice together: 12μl sol. SPP activity (for exp. sol. PKXRM)
   158μl SPP buffer
   20μl 0.5 M EDTA, pH 8.0
   8μl DMSO or Inhibitor (Competition experiment)
   2μl 5μM TBL₄K or 5μM L-858,646 (in DMSO)
   200μl Total

2. Incubate for 30 min at 30°C

3. Irradiate sample with UV light (30 seconds for TBL₄K, 5 min for L-858,646;
   350W high pressure mercury lamp with pyrex filter, 10 cm distance to
   lamp).

4. Add 20 μl 100% TCA to sample, vortex, and incubate on ice for 30 min.

5. Centrifuge at 14’000 rpm for 5 min at 4°C in an eppendorf centrifuge.


7. Centrifuge at 14’000 rpm for 2 min at 4°C in an eppendorf centrifuge.

9. Centrifuge at 14'000 rpm for 1 min at 4°C in an eppendorf centrifuge.

10. Incubate sample for 20 sec at 65 °C

11. Add 20 µl WF sample buffer, vortex, and incubate for 15 min at 65°C

12. Separate sample by 10% SDS-PAGE. Visualize labelled proteins by Western Blotting with a polyclonal rabbit anti-biotin antibody (1:1000 in 5% BSA TBS-T for 1 hr) (Bethyl).
6. References


References


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References


Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P.,


7. Appendix
### 7.1. Appendix I: Swiss Prot Entry SPP

**SPP/HM13_Human** PS13°/PSH3° (Q8TCT9)

- **ID**: HM13_HUMAN STANDARD; PRT: 377 AA.
- **AC**: Q8TCT9; Q9SH73; Q9H110; Q9H111.
- **DT**: 15-JUN-2002 (Release 41, Created).
- **DT**: 15-JUN-2002 (Release 41, Last sequence update).
- **DT**: 15-JUN-2002 (Release 41, Last annotation update).
- **DE**: Minor histocompatibility antigen H13 (EC 3.4.99.-) (Signal peptide peptidase) (Presenilin-like protein 3).
- **GN**: HM13 OR H13 OR SPP OR PSL3.
- **OS**: Homo sapiens (Human).
- **OC**: Mammalia; Eutheria; Primates; Catarrhini; Homo.
- **OX**: NCBI TaxID=9606.
- **RN [1]**
  - **RP**: SEQUENCE FROM N.A. (ISOFORM 1).
  - **RA**: Irmler M., Tomauk S., Korner M.R., Hofmann K., Conradt M.;
  - **RT**: "Characterization of a new protein family with homology to presenilins."
  - **RL**: Submitted (SEP-2001) to the EMBL/GenBank/DDBJ databases.
- **RN [2]**
  - **RP**: SEQUENCE FROM N.A. (ISOFORM 1), PARTIAL SEQUENCE, FUNCTION,
    - **RT**: H-GLYCOSYLATION, AND MUTAGENESIS OF ASN-10; ASN-20 AND ASP-265.
  - **BC**: TISSUE=Cervical carcinoma;
  - **BX**: PubMed=12077416; [NCBI, EBI]
  - **RA**: Weihofen A., Binns K., Lemberg M.K., Ashman K., Martoglio B.;
  - **RT**: "Identification of signal peptide peptidase, a presenilin-type aspartic protease."
- **RN [3]**
  - **RP**: SEQUENCE FROM N.A.
  - **RA**: Brown A.C., Roopeman P.C.;
  - **RT**: "Genomic analysis of the H13 minor histocompatibility antigen gene."
  - **RL**: Submitted (MAR-2002) to the EMBL/GenBank/DDBJ databases.
- **RN [4]**
  - **RP**: SEQUENCE FROM N.A. (ISOFORMS 1 AND 3).
  - **BC**: TISSUE=Mammary gland, and Retinoblastoma;
  - **RA**: Isogai T., Ota T., Nishikawa T., Hayashi K., Otsuki T., Sugiyama T.,
    - **RT**: Sasaki Y., Hattori A., Okumura K., Iwayanagi T., Ninomiya K.;
  - **RT**: "NEDO human cDNA sequencing project."
  - **RL**: Submitted (MAR-2002) to the EMBL/GenBank/DDBJ databases.
- **RN [5]**
  - **RP**: SEQUENCE FROM N.A. (ISOFORMS 1 AND 2).
  - **RX**: MEDLINE=21638749; PubMed=11780052; [NCBI, ExPASy, EBI, Israel, Japan]
  - **RA**: Deloukas P., Matthews L.H., Ashurst J., Burton J., Gilbert J.G.R.,
    - **RT**: Jones M., Stavrinides G., Almeida J.P., Babbage A.K., Baguley C.L.,
    - **RT**: Bailey J., Barlow K.P., Bates K.N., Beard L.M., Beare D.M.,
    - **RT**: Beasley O.P., Bird C.P., Blakey S.E., Bridgeman A.M., Brown A.J.,
    - **RT**: Buck D., Burrell W.D., Butler A.P., Carter C., Carter N.P.,
    - **RT**: Chapman J.C., Clapp M., Clark G., Clark L.N., Clark S.X., Clise C.M.,
    - **RT**: Cleug S., Colby V.E., Collier R.E., Connor R.E., Corby N.R.,
    - **RT**: Coulson A., Coville G.J., Deadman R., Dham P.D., Dunn M.,
    - **RT**: Elliot A.G., Frankland J.A., Fraser A., French L., Garner P.,
    - **RT**: Graham D.V., Griffiths C., Griffiths M.N.D., Gwilliam R., Hall R.E.,
    - **RT**: Hammond S., Harley J.L., Heath P.D., Ho S., Holden J.L., Howden P.J.,

A small peptide of mouse SPP serves as H13 minor histocompatibility antigen. Because of automatic computational annotation SPP is therefore termed in databases also as HM13, even it makes no sense.

Presenilin-like (PSL); unpublished term for SPP/SP-like family.


SEQUENCE FROM N.A. (ISOFORM 1).

TISSUE=Muscle;

Submitted (MAY-2001) to the EMBL/GenBank/DDBJ databases.

FUNCTION.

PubMed=11714810; [NCBI, EBI]


FUNCTION.

PubMed=12145199; [NCBI, EBI]


- FUNCTION: Catalyzes intramembrane proteolysis of some signal peptides after they have been cleaved from a preprotein, resulting in the release of the fragment from the ER membrane into the cytoplasm. Required to generate lymphocyte cell surface (HLA-E) epitopes derived from MHC class I signal peptides. May play a role in graft rejection (By similarity). May be necessary for the removal of the signal peptide that remains attached to the hepatitis C virus core protein after the initial proteolytic processing of the polyprotein.

- SUBCELLULAR LOCATION: Integral membrane protein, reticulum (Probable).

- ALTERNATIVE PRODUCTS: 3 isoforms; 1 (shown here), produced by alternative splicing.

- PTM: N-glycosylated.

- SIMILARITY: BELONGS TO THE PRESENILIN FAMILY.

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DR BLOCKS: Q8TCT9.
DR Protomapper: Q8TCT9.
DR Freebase: Q8TCT9.
DR SWISS-2DPAGE: GET REGION ON 2D PAGE.
KW Hydrolase; Protease; Transmembrane; Glycoprotein; Polymorphism; Alternative splicing.

FT DOMAIN 1 31 LUMENAL (POTENTIAL).
FT TRANSMEM 32 52 POTENTIAL.
FT DOMAIN 53 77 CYTOPLASMIC (POTENTIAL).
FT TRANSMEM 78 98 POTENTIAL.
FT DOMAIN 99 100 LUMENAL (POTENTIAL).
FT TRANSMEM 101 121 POTENTIAL.
FT DOMAIN 122 209 CYTOPLASMIC (POTENTIAL).
FT TRANSMEM 210 256 POTENTIAL.
FT TRANSMEM 257 277 POTENTIAL.
FT DOMAIN 278 291 CYTOPLASMIC (POTENTIAL).
FT TRANSMEM 292 312 POTENTIAL.
FT TRANSMEM 313 315 LUMENAL (POTENTIAL).
FT TRANSMEM 316 336 POTENTIAL.
FT DOMAIN 337 377 CYTOPLASMIC (POTENTIAL).
FT CARBOHYD 10 16 N-LINKED (GLCNAC...)(POTENTIAL).
FT CARBOHYD 20 20 N-LINKED (GLCNAC...)(POTENTIAL).
FT VARSPLIC 347 347 E -> ESSARILHPFLHPPPPSGPASLDSMQQKLAG
FT VARSPLIC 122 143 PRRRPPQPASATE (IN ISOFORM 2).
FT VARSPLIC 259 259 A -> P (IN DBSNP:1044419).
FT VARSPLIC 144 377 MISSING (IN ISOFORM 3).
FT VARIANT 259 259 A -> P (IN DBSNP:1044419).
FT MUTAGEN 10 10 N->Q: ADOBILISES N-GLYCOSYLATION; WHEN
FT MUTAGEN 10 10 N->Q: ADOBILISES N-GLYCOSYLATION; WHEN
FT MUTAGEN 20 20 N->Q: ADOBILISES N-GLYCOSYLATION; WHEN
FT MUTAGEN 265 265 D->A: NO EFFECT ON INHIBITOR BINDING;
FT MUTAGEN 265 265 D->A: NO EFFECT ON INHIBITOR BINDING;
FT CONFLICT 150 150 K -> R (IN REF. 4).
FT CONFLICT 235 235 S -> P (IN REF. 6).
SQ SEQUENCE 377 AA; 41488 MW; 322D31B52B33118 CRC64;
MDASLDDHH GSAEAGPTNT STRTPPEFE GIALAYGSSL LMAALLPIFF ALRSVRCARG
MNASDMMFTI TRSEARPFPI IASCTLLGIP LPPHFSPQKE INLLISMYFF VGLIALTSET
ISYPHNKFPP AYPHPQPYQL RFTQGSSKN KFIVZONEPOT CRIYVCGSIS TVQVYLLARK
HWINANLFLG AFSNLKGVGL HKNYVSGC/T LLGGGFYDVP FWPFGTNMV TVAKSFEAPI
KLVPQGGLEK KLLENNFAM LGLEDVVTPG IFIALLIRFD ISLKKVHTY FYTSSFAAYIF
GLOUTIFIMF IPFHRQPFALL YLVFACGFFP VVVALARGEV TEFSTSESH PNCPFAAFTES
XEGTEASASK GLEKREK

//
7.2. Appendix II: SPP/SPP-like proteins

7.2.1. SPP (Homo sapiens)

- Also known as PSL3, HM_13 and PSH3 (Ponting et al., 2002)
- Accession: Protein CAD13132, DNA AJ420895 (EMBL Database), SwissProt Q8TCT9
- Chromosome: 20, 20q11.21
- Calculated molecular weight: 41485.28 Da; Estimated pl: 5.99
- Predicted Transmembrane segments (THMM2.0) (underlined):
- N-terminus is in the ER lumen and the C-terminus in the cytosol
- The catalytic motifs are shown in bold
- 2 N-Glycosylation sites: N10, N20
- C-terminal ER retrieval signal

```
  10  20  30  40  50
MDASALDSDPHNGSAEAGGPNSTTRPSTPEGIALAYGSLALLIMALLPPFPG
  60  70  80  90 100
ALREVRCAEGSNASIMPETITISDAARFPNITSCTLLGLLFFKIFSQQEV
 110 120 130 140 150
1NNLSMYPPVGLALALHFTISPPFMTFPPNNRQCVQLTFTQQSGSRENK
 160 170 180 190 200
EEIINEFPTMDDLVSGLSSVVGVLALLKHANLNLGLAPLNLNEGVEV
 210 220 230 240 250
HLNIVSTGCILLGILPFTYDVYFVFTYFHLFTVAKSFSAIFKLVVQDILLE
 260 270 280 290 300
KGLEANNFMGLODVIISPQIALLLRVDLSKKNTYFTHSFYAPF
 310 320 330 340 350
GLGLTFTMIFPPKAQALLLVLVFACIGPPVIVALAKGEVTIMFSEESN
 360 370
FKDPAAVYESKREIASAKGLENKK
```
7.2.2. SPPL2a (Homo sapiens)

- Also known as PSL2 and PSH5 (Ponting et al., 2002)
- Accession: Protein CAD13133, DNA AJ420696 (EMBL Database)
- Chromosome: 15, 15q15.2
- Calculated molecular weight: 46343.22 Da; Estimated pl: 9.07
- Predicted Transmembrane segments (THMM2.0) (underlined):
  60-82, 103-125, 135-157, 178-195, 200-217, 229-251, 289-311, 324-346, 351-370
- According to prediction the N-terminal sequence is outside in ER lumen
  → The catalytic motifs (bold) have the same orientation as in SPP.
- According to prediction there are 4 potential N-Glycosylation sites: N5,N15,N38,N44
- A protease-associated domain (PA) has been detected! (73-144)

```
MLVNNNSVLFPPSQRSEETFVKILIAF13133, DNA AJ420696 (EMBL Database)
KDYDFRBMSQFLGDNITVEKY
SPSNHFDYMTYIFVIIAVTVALGQYWSQKLYKLENL8AVT5DREMKK
KEEYLFSPFTLTVIFVIVCVCNV5KLYFFYKWLYVM1ASAMSLY
NCLAALIIKYIQCTIACRG0NSVRL1FLSGLCIAAVAVA5FRENNEI
WAM1LQDPILG1AFCLNLH4ILKLH2SCV1LG1LLYDVFFVFITPFI
TKNGESINVLAAGPSH8KLRT8VIV8F1YFSVNM1CNPVSILFPG
DIVPGLIIIAYCRRFDVQ8SSYIY5TS5VAYA9H5LSFVVL1MKG
QPALLYLVCPH1SITASVVA9R8KMKKFWKGSYQMDHLCATNEENPV
ISGEQ1VQQ
```
7.2.3. SPPL2b (Homo sapiens)

- Also known as PSL1 and PSH4 (Ponting et al., 2002)
- Accession: Protein CAD13134, DNA AJ420697 (EMBL Database)
- Chromosome: 19, 19p13.3
- Calculated molecular weight: 61894.22 Da; Estimated pl: 9.31
- According to prediction the N-terminal sequence is outside
  - The catalytic motifs (bold) have opposite orientation as in SPP!
- According to prediction there are 3 potential N-Glycosylation sites outside N41, N69, N101 and 2 inside N255, N375
- A protease-associated domain (PA) has been detected! (48-124)

```
10  20  30  40  50
MVHVSQAGGPEGKDYCILINFOQWAHLPHDSLSKASPLQELNWTASLLCSA

60  70  80  90  100
ADLPAEGSNNPQIPVARGNCTFYMRRKQLQSSGARGLLIVSVRELVPFGG

110 120 130 140 150
NKTQYIDGIPVALLSYKIMLGDFTRGFTVAALYAPKREPDIDLDYNMVII

160 170 180 190 200
FINAVQTVAILGSXSGSCRKDKROMHESDQEREAEAVDADPFTVCMV

210 220 230 240 250
FVVNCQGSMVLHVVFYVLVVVGIYICQASCSDATQKGRKLDPFK

260 270 280 290 300
CRIYNSNLFPYTKHKRQPAMRLLALFCTVAVSVVVGFCRKEDQVAWVLQDAL

310 320 330 340 350
GIAFCMYMTLKFLIPFPACTLILLVFLYDYFFVFITFFLTKSSSSHIV

360 370 380 390 400
EVATGPSGSSATREKLPWLVKVRFLNSSLACDPRPSLQGDGDILLQGVLL

410 420 430 440 450
VAYCHRPVQVQSSPVVTVPACTIAAYGVVILTVFVALALMQRGQPALLVV

460 470 480 490 500
FPCLVTSCAVALMRRKELGVWRTGSGFARLVPFPANAPAYFAGDQPPQPSA

510 520 530 540 550
TLPSPQQFSPSEEPATSPPMSPQSKRTSSEEMIAQAPMRPEPSGSPAESGRD

560
QAQPSFVTQPGASA
```
7.2.4. SPPL3 (Homo sapiens)

- Also known as PSL4 and PSH1 (Ponting et al., 2002)
- Accession: Protein CAD13135, DNA AJ420898 (EMBL Database)
- Chromosome: 12 (12q24.31)
- Calculated molecular weight: 42560.28 Da; Estimated pI: 9.24
- Predicted Transmembrane segments (THMM2.0) (underlined):
- According to THMM prediction, the N-terminal sequence is outside, but in contrast SignalP predicts N-terminal signal sequence.
- According to the predicted potential N-Glycosylation sites N50, N305 are inside.

```
10 20 30 40 50
MAEQTYSWAYSLVDSSQUSTFLISILLIVGYGSFRLNMDFENQDRKDN

60 70 80 90 100
SSGSFNGEQRPIIGFQPMOSTRASPFLMGACVSLLVFMHFFSVQYVPT

110 120 130 140 150
ICTAVLATICAFALLFLLLPMCYLTFCSPQMISFGCGRFTAAELLSFSL

160 170 180 190 200
SVMLYLTVLGTGLALANDALCVAMIAFYRFLPSLKVSCLLILLSLL

210 220 230 240 250
DVFWVFFSAYIFRSNVMKVDATQPAFMPLDVLSLRLHLGHPVREDFVPR

260 270 280 290 300
LPGLNVFDSSGTSGFGMLGIQDIYMPGGLLCPVLRDNYFQASGSGCSGA

310 320 330 340 350
PGPANIGPQFTYFHTLLCYGPVGLLTATVASEHRSAQPALLFLVYF

360 370 380
TLLPLILHAYLKGDLRRMWSKFPHSKSSS5RFLEV
```
7.2.5. PSH2/SPPL2c (Homo sapiens)

- Accession: Protein AAH25401, DNA AJ420898 (EMBL Database)
- Chromosome: 17
- Calculated molecular weight: 7447.65 Da; Estimated pI: 6.24
- Predicted Transmembrane segments (THMM2.0) (underlined):
- According to THMM prediction, the N-terminal sequence is outside (active site orientation as in PS), but on the other hand SignalP predicts a N-terminal signal sequence (VAG-GK) (active site as in SPP).
- Signal peptide has helix-breaking residues (potential substrate for SPP/SPPL proteins).
- According to prediction the QPALLY motif is not within TM!!
- Potential N-Glycosylation sites at 33, 100, 374. Assuming that SPPL2c has a signal sequence it is glycosylated at position 33 and 100.

```
10  20  30  40  50
MACIGPLLPGFPALLSISTVAGKYGAVHVSSENKDYCILFSSDFVTLP
60  70  80  90  100
RDHHPALLPGPDDOTKAPFCEGDSPHQAOQSPQRPQLQTTANVMQGN
110 120 130 140 150
CSFTHKGMLAQOOGAHGLLIVSVSDQCSSUTTLPQDRQPLADLTIPV
160 170 180 190 200
AMITYAHMLDIILSHTGAEAVVRFAMYAPPEPIDIYNYLVIFILAVGTVAA
210 220 230 240 250
QGYNAGLTRANRLQRRRARGGSGHMHQLQKAAAGAQKEKDNEDIPVD
260 270 280 290 300
FTPAMTCQVTLLSCLSMLLLYFYDYTETGIFGLOGAGIGLYSCSLAPE
310 320 330 340 350
VCHLSLQDQCQPAMPSHMLAGSLPLLILLASLGATVIFKWAYFNERDWHDL
360 370 380 390 400
LQDTLRISYCLFVLHRVPLKCMSSFLALLAIFVVTYTFTTFTNYG
410 420 430 440 450
KSAQAMVAGLPASESHRELPENVKPRRLVSALTICQPPSILQGPDIV
460 470 480 490 500
VPGFILAYCCRFVQVCSSQYFYACTVAYAVGLLYVTMAMVLMQGQPA
510 520 530 540 550
LLYLVSTLTSILAVCAQSELFLTFQGGRKMCGLCPSAGSBRQKQG
560 570 580 590 600
GAADNTASTLQEGTSRGAGCGDSLNSDYTTTIVSIKNEATNPEDRDGS
610 620 630 640 650
SKQWSDALDPRFFLPPAGSEELVPMHMAMLFIMPMPPPSELGHV
660 670 680
HAQQAAMETGLPAGLHRKFRKGLFGRFQSMSTQAPL
```
7.3. Appendix III: Phylogenetic tree of SPP/SPPL family

SPP/SPPL protein subfamilies are shown in green, representatives of PS and TFPP in red and blue, respectively.

**SPP/SPPL family:**

**SPP subfamily:**
Found in all higher eukaryotes. Based on this phylogenetic tree the SPPL1 Sp (Result Part II) belongs well to the SPP subfamily.

**SPPL2 subfamily:**
Found likely only in mammals!

**SPPL3 subfamily:**
Found in all higher eukaryotes.

**SPPLP subfamily:**
Plant family

**SPPL subfamily:**
Archaea family

SPPL4 Sc and SPPL2 Ce were not assigned to a subfamily.

**PS family:**
Animals, plants

**TFPP family:**
Bacteria

Hs., Homo sapiens; Mm., Mus musculus; Dm., Drosophila melanogaster; Ce., Caenorhabditis elegans; At., Arabidopsis thaliana; Sp., S. pombe; Sc., S. cerevisiae; Ta., Thermoplasma acidophilum; Ts., Thermoplasma volcanium; MYXXA, Mycococcus xanthus; XANCP, Xanthomonas campestris; VIBVU, Vibrio vulnificus; PSEST, Pseudomonas stutzeri

SPP, Signal peptide peptidase; SPPL, SPP-like; PS, Presenilin; TFPP, Type IV prepilin peptidase
Method: UPGMA; Best Tree; tie breaking = Systematic
Distance: Uncorrected ("p")
Gaps distributed proportionally
### 8. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AICD</td>
<td>amyloid intracellular domain</td>
</tr>
<tr>
<td>APP</td>
<td>β-amyloid precursor protein</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor-6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid β peptide</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-chlolamidopropyl)dimethylammonio]-1-propansulfonic acid</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavilan A</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EndoH</td>
<td>endo-β-N-glucosaminidase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>FAD</td>
<td>familial early-onset Alzheimers's disease</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA-E,-A</td>
<td>human lymphocyte antigen-E, -A</td>
</tr>
<tr>
<td>I-CLIP</td>
<td>intramembrane cleaving protease</td>
</tr>
<tr>
<td>II</td>
<td>invariant chain</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTF</td>
<td>N-terminal fragment</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>p-Prl</td>
<td>preprolactin</td>
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<tr>
<td>RIP</td>
<td>regulated Intramembrane proteolysis</td>
</tr>
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<td>RM</td>
<td>rough microsomes</td>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>S1P</td>
<td>site-1 protease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S2P</td>
<td>site-2 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleaving activating protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sol.</td>
<td>solubilised</td>
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<tr>
<td>SPase</td>
<td>signal Peptidase</td>
</tr>
<tr>
<td>SPC</td>
<td>signal peptidase complex</td>
</tr>
<tr>
<td>SPF</td>
<td>signal peptide fragment</td>
</tr>
<tr>
<td>SPP</td>
<td>signal peptide peptidase</td>
</tr>
<tr>
<td>SPPL</td>
<td>SPP-like</td>
</tr>
<tr>
<td>SPPase</td>
<td>signal peptide peptidase</td>
</tr>
<tr>
<td>SR</td>
<td>SRP receptor</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
</tr>
<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>(ZLL)₂-ketone</td>
<td>1,3-di-(N-carboxybenzoyl-L-leucyl-L-leucyl) amino acetone</td>
</tr>
</tbody>
</table>
9. List of Publications


10. Curriculum Vitae

Name: Weihofen
Surname: Andreas
Address: Sihlfeldstr. 58, 8003 Zürich
Date of birth: April 28, 1973
Place of birth: Solothurn (CH)
Citizen: Giffers (FR), Switzerland
Marital status: Single

Education:

1980-1985 Primarschule (Primary school), Zuchwil (CH)
1985-1993 Gymnasium (Grammar school), Solothurn (CH)
Jan 1993 Matura Typus B (Latin)
1993-1998 Biology II studies at the University of Basel (CH)
1998-1999 Diploma thesis at the Biocentre of the University of Basel, Division of Molecular Microbiology
(Prof. Charles Thompson): Characterisation of two members of the SigB-family in Streptomyces coelicolor A3(2).
Degree: dipl. phil II, Molecular Genetics

Sep 1999-present Graduate studies at the Institute of Biochemistry, Swiss Federal Institute of Technology, Zürich (Dr. Bruno Martoglio, Prof. A. Helenius): Signal Peptide Peptidase – Identification of a Presenilin-type Aspartic Protease
11. Acknowledgments

My first thanks go to Bruno Martoglio, for making it possible to perform my thesis in his upcoming lab. His continuous support and interest was indispensable for the progress of my work. Thanks for the patience, trust and discussions. His belief in SPP always encouraged me to go on! Second, I'm also very grateful to Ari Helenius for being my referee. I enjoyed the inclusion in his lab activities and certainly took benefit from his lab. Further I would like to thank Ulrike Kutay for her spontaneous agreement to be my co-referee. My special thanks go also to Marius Lemberg for helpful discussions and support. His work contributed much to the progress of my work, thanks. In addition, I'm grateful to all our collaborators that made it possible to identify SPP. Many thanks as well to Lukas for the scientific discussions affected by Gin Tonic. Furthermore I would like to thank especially the smokers Anna and Kowi who helped me to satisfy my addiction. Justine, thank you for the diverting breaks, and Eva, thanks for critical reading of the thesis manuscript. Many thanks as well to all the other present and former members of the Institute of Biochemistry for their suggestions and support. Finally, I would like to thank my parents and my sister Karin for their continuous mental and financial support during my education.