SIGNAL PEPTIDE PEPTIDASES AND γ-SECRETASE: COUSINS OF THE SAME PROTEASE FAMILY?

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Running title: Common cleavage mechanism of GxGD type proteases

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Signal peptide peptidase (SPP) is an unusual aspartyl protease, which mediates clearance of signal peptides by proteolysis within the endoplasmic reticulum (ER). Like presenilins, which provide the proteolytically active subunit of the γ-secretase complex, SPP contains a conserved GxGD motif in its C-terminal domain which is critical for its activity. While SPP is known to be an aspartyl protease of the GxGD type, several presenilin homologues / SPP-like proteins (PSHs/SPPL) of unknown function have been identified by database searches. In contrast to SPP and SPPL3, which are both restricted to the endoplasmic reticulum, SPPL2b is targeted through the secretory pathway to endosomes /lysosomes. As suggested by the differential subcellular localization of SPPL2b and SPPL3 distinct phenotypes were found upon antisense gripNA mediated knockdown in zebrafish. *spp* and *sppl3* knock downs in zebrafish result in cell death within the central nervous system, whereas reduction of *sppl2b* expression causes erythrocyte accumulation in an enlarged caudal vein. Moreover, expression of D/A mutants of the putative C-terminal active sites of *spp*, *sppl2*, and *sppl3* produced phenocopies of the respective knockdown phenotypes. These data suggest that all investigated PSHs/SPPLs are members of the novel family of GxGD aspartyl proteases. More recently it was shown that SPPL2b utilizes multiple intramembrane cleavages to liberate the TNFα intracellular domain into the cytosol and to release the C-terminal counterpart into the lumen. These findings suggest common principles of intramembrane proteolysis by GxGD type aspartyl proteases. In this article, we will review the similarities of SPPs and γ-secretase based on recent findings by us and others.

INTRODUCTION

Intramembrane proteolysis is mediated by a class of novel polytopic proteases, which have their active centers located within the hydrophobic transmembrane domains [1,2]. Members of these proteases include the site-two-protease (S2P) [1], rhomboids [3,4], γ-secretase [5], and signal peptide peptidase (SPP) [6,7]. While S2P and rhomboids belong to the class of metallo- and serine proteases respectively, γ-secretase and SPP are aspartyl proteases [2,8,9].

The catalytic core of γ-secretase is provided by either of the two homologous presenilins (PS1 or PS2). Co-factors including APH-1, PEN-2, and Nicastrin are absolutely required to generate a functional γ-secretase complex [10-14]. PS1 or PS2 containing γ-secretase complexes can both mediate the intramembrane cleavage of the ß-Amyloid precursor protein (APP), Notch and probably many other type-I oriented substrates as well [8,15-18] suggesting functional redundancy. The C-terminal critical aspartate of PSs is located within a conserved GxGD motif [19], whereas the N-terminal aspartate is embedded within a YD sequence segment. The GxGD signature motif is highly conserved in SPP, an unrelated polytopic aspartyl protease [2,7,20,21] as well as in the type-four prepilin peptidases (TFPP) [19,22]. Moreover, mutagenesis of the corresponding aspartate residue completely blocks proteolytic activity of SPP [7], PS1 [5], PS2 [15,23], and TFPP [22].

The facts that active site inhibitors of γ-secretase can be cross-linked to presenilins [24,25] and bind to presenilins in dependence of the critical aspartate [26], suggest that the aspartate within the GxGD motif comprises the C-terminal active site of these proteases. Besides this highly conserved active site, further similarities are observed between PSs, SPP, and TFPPs, which for example include a PxL motif within the C-terminal domain.

SPP is required for the removal of signal peptides after their liberation by signal peptidase during translocation of proteins into the endoplasmic reticulum [2]. In addition, SPP is also involved in

immune surveillance and processing of the Hepatitis viral core protein [7] suggesting a more general role of SPP in the liberation of bioactive peptides [2,7,20,21]. Besides SPP a family of homologous proteins was identified by database searches [2,7,20,27,28]. These proteins were named SPPL (SPP-like) 2(a,b,c), and 3 (in yeast an additional SPPL, SPPL4, exists) [7], PSHs 1-5 [27] or IMPASes [28]. For clarity, we will use the term SPPL throughout this manuscript. Although SPPLs share some homology with SPP, it is not known if they exhibit any proteolytic activity [21]. Here we will briefly summarize the recent findings on the cellular function of SPP family members and then compare these data to the well defined γ-secretase biology.

All SPPs are members of the GxGD family of aspartyl proteases

The catalytically critical aspartate residue of SPP, TFPPs, and PS1 and PS2 [2,9,19,22] is embedded within a GxGD motif, which is also fully conserved in all SPPLs. Together with the aspartate of the equally critical N-terminal YD motif [5,29], these are the only aspartate residues, which are fully conserved throughout the SPPs, SPPLs, PSs, and TFPPs. Moreover, Weihofen et al. mutagenized the conserved aspartate residue 265 within the GxGD motif of SPP and found that SPP D265A lost its proteolytic activity [7]. As shown for PS1, a transition state inhibitor, known to block γ-secretase activity via binding to its active site also blocks SPP function [30]. This fits well with the mutagenesis of the corresponding aspartate residues within the GxGD motif of PS1, PS2 [5,15,23] and TFPP [22], which all lost their entire proteolytic activity upon mutagenesis of the aspartate. Thus the corresponding aspartate residues within the GxGD motif of SPPLs may likely be responsible for their catalytic function as putative aspartyl proteases. To investigate the functional significance of the corresponding aspartate in SPPLs *in vivo* and to provide evidence that SPPLs are aspartyl proteases, this amino acid was mutated in SPP and its homologues to alanine and the resultant phenotype was compared to that of corresponding

knockdowns in zebrafish [31]. *spp* and *sppl3* knock downs in zebrafish result in cell death within the central nervous system, whereas reduction of *sppl2b* expression causes erythrocyte accumulation in an enlarged caudal vein. Strikingly, expression of D/A mutations of the putative C-terminal active sites of *spp*, *sppl2*, and *sppl3* produced phenocopies of the respective knockdown phenotypes. This strongly suggests a functional role of the GxGD domain in SPPLs, and makes it very likely that SPPLs also belong to the family of GxGD aspartyl proteases.

Finding substrates for the SPPL-Protease family

To finally prove that SPPLs are indeed proteases, it is essential to find substrates being turned over by the proteases. For SPP it has been suggested that only type-2 oriented transmembrane proteins may be accepted as substrates [7,32,33]. Furthermore Martoglio and coworkers postulated, that the critical aspartates of the active site in SPP/SPPLs are oriented in the opposite direction to those of PSs in the γ-secretase complex [2,7], which only cleave type-1 transmembrane proteins [10]. Consequently a type-2 transmembrane protein located to late endosomale compartments could be a good candidate substrate for SPPL2a and SPPL2b. And indeed the type-2 transmembrane protein TNFα was identified as a substrate for SPPL2a and SPPL2b [34,35]. To elucidate whether GxGD proteases in general use a common mechanism to cleave their substrates within the hydrophobic transmembrane domains, Fluhrer et al. closer investigated the cleavage of TNFα by SPPL2b, using mass spec analysis and radiosequencing. Two cleavage products were identified. An intracellular peptide (TNFα ICD) in the cell lysate and a secreted peptide (TNFα C-domain) (Fig. 1) in the cell culture medium were detected [34]. Strikingly the two cleavage products were not the result of a single cleavage, but were rather separated by a few amino acids, consequently suggesting a dual or even multiple cleavage event used by SPPL2b to release the transmembrane domain of TNFα. Interestingly, the TNFα ICD,

generated by SPPL2b, is involved in the transcriptional regulation of IL-12 [35]. Thus SPPL2b is not only a novel intramembrane protease of the GxGD-type, but is also critically required for a so far unknown signaling pathway.

γ-secretase versus SPPLs: Relatives with similarities and differences

The SPPLs have been identified as an additional family member of the GxGD proteases. As for the PSs mutation of the critical aspartate within the GxGD motive of SPPLs leads to a complete loss of proteolytic activity [31,34]. SPP/SPPL activity is massively increased upon overexpression of a single cDNA, suggesting that these proteases are active as monomers or homodimers [30], which do not require additional binding proteins for their activity. Sole overexpression of PSs on the other hand does not cause an increased proteolytic activity of γ-secretase, because γ-secretase requires complex formation of PS, Aph-1, Nct, and Pen-2 to be active [11].

Although both protease families show fundamental differences with respect to complex formation and primary structure, they exhibit surprising similarity in the cleavage pattern of their substrates. SPPL2b, like γ-secretase, performs multiple intramembrane cleavages separated by a number of amino acids. In analogy to γ-secretase cleavage products, the cleavage of TNFα by SPPL2b leads to an intracellular domain (TNFα ICD) and to secreted peptide (TNFα C-domain) (Fig. 1). These fragments correspond to the APP intracellular domain (ICD) and Amyloid ß-peptide respectively (Fig. 2). Theses findings may suggest a common cleavage mechanism for intramembrane proteolysis of GxGD type aspartyl proteases.

PS1 only cleaves type I transmembrane proteins while SPPL2b seems to cleave exclusively type II oriented transmembrane proteins. Accordingly, the membrane topology of SPPL2b compared to PS1 is reversed. It is therefore tempting to speculate that the cleavage pattern of

SPPL2b may include a γ- cleavage (after amino acid 34 and 39) and an ε-like cut [36] (after amino acid 49) as well as a ζ-like cleavage [37] (after amino acid 51) at the luminal side of the membrane. Moreover intramembrane proteolysis of TNFα by SPPL2 generates a cytoplasmic cleavage product, which is required for cellular signaling [35]. A similar cellular signaling function is well established for the cleavage of Notch by γ-secretase [38].

The question arises of how one protease activity can cut its substrate at several sites. Like presenilin [39], the catalytically active component of the γ-secretase complex, SPP and all SPPLs appear to occur as homodimers [30,31]. Moreover, at least for SPP, it has been shown that dimerization facilitates the binding of an active-site directed photoaffinity labeled γ-secretase inhibitor, suggesting that dimerization is required to form the fully active catalytic site of SPP [30].

If SPP or SPPL3 process their substrates by a similar mechanism *in vivo* remains to be demonstrated. However, recent in vitro experiments using an artificial substrate suggest that SPP may cleave at predominantly one side within the membrane, although additional cleavages could not be excluded [29].

Taken together, the remarkable similarities of SPPL2b and γ-secretase regarding their cleavage mechanisms together with their role in signaling and gene regulation suggest a common concept of intramembrane proteolysis. It is now important find out why γ-secretase requires additional co-factors for its biological function, while all SPP family members apparently do not require such proteins.

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Figure legends:

Fig.: 1
Schematic representation of the TNFα processing by SPPL2b

Fig.: 2
APP processing by γ-secretase

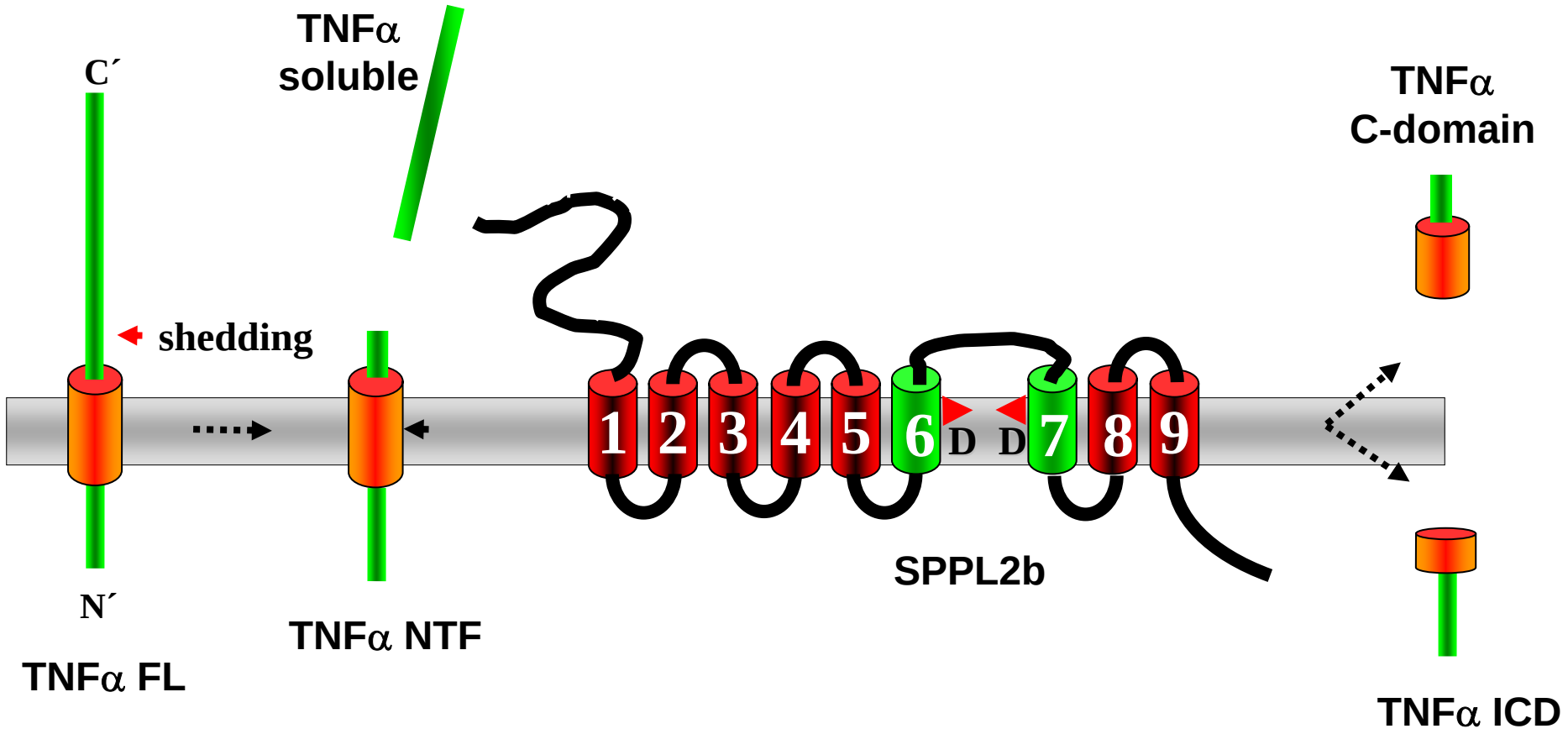

Fig.: 1 Fluhrer & Haass

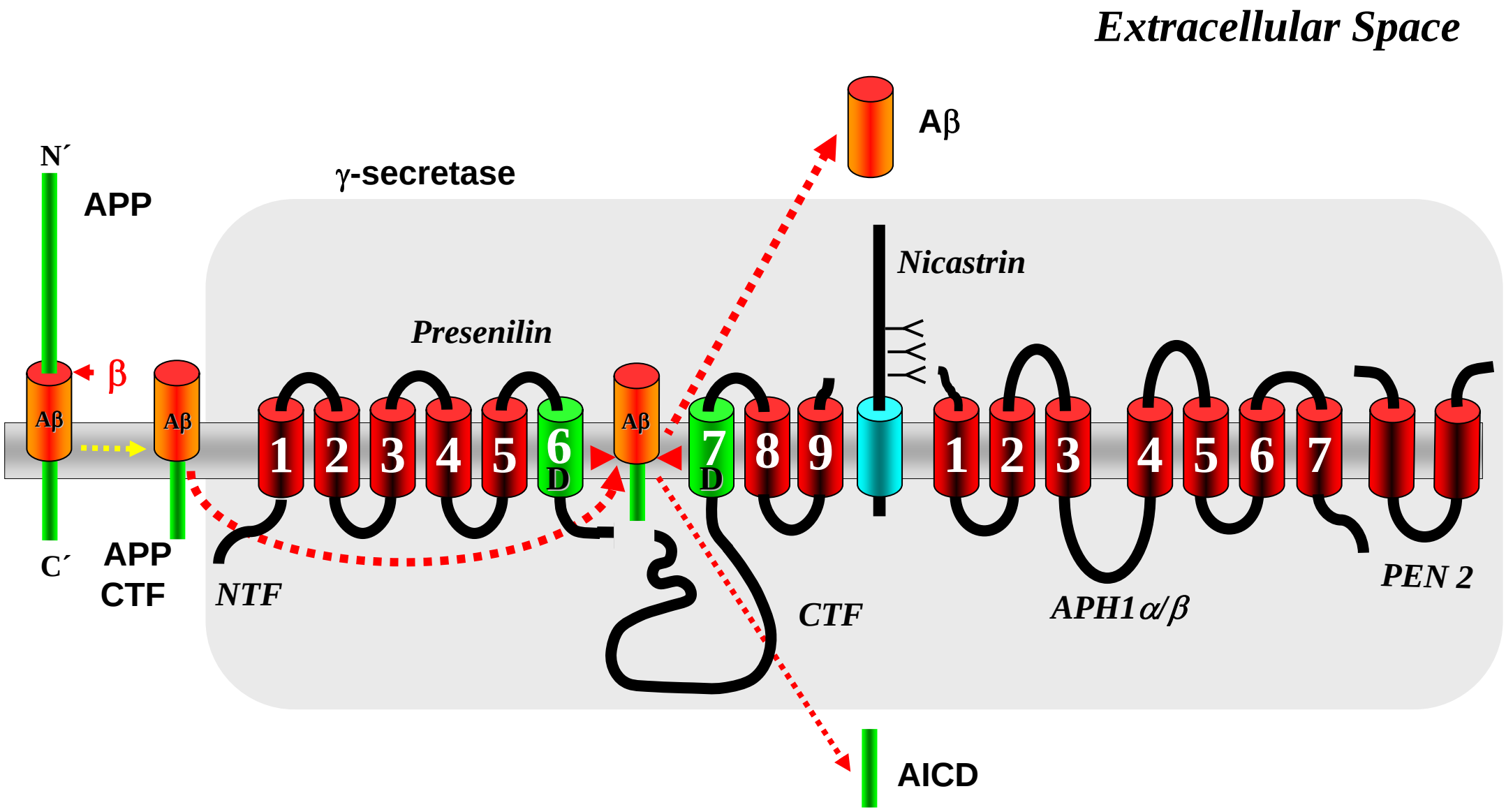

Fig.: 2 Fluhrer & Haass