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Minireview

On the mechanism of SPP-catalysed intramembrane proteolysis; conformational control of peptide bond hydrolysis in the plane of the membrane

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Abstract Intramembrane-cleaving proteases are members of a novel type of enzyme that hydrolyse substrate proteins within transmembrane regions. The presently known proteases that catalyse such cleavage reactions are membrane proteins of high hydrophobicity and multiple predicted transmembrane regions. A key feature is the positioning of active site residues in hydrophobic segments implying that the catalytic centre is assembled within the plane of the membrane. Nevertheless, all these proteases appear to utilise catalytic mechanisms similar to classic proteases that expose their active site domains in aqueous compartments. In the present review, we will address the mechanism of intramembrane proteolysis on the example of the signal peptide peptidase, and discuss how enzyme-catalysed hydrolysis of peptide bonds within the plane of a cellular membrane might occur.

Key words: Integral membrane protein; Lipid bilayer; Presenilin; Protease; Signal peptide peptidase

1. Introduction

Proteases are the largest class of enzymes [1,2]. They act as modulators of many vital processes in all organisms. The proteases, also known as peptidases or proteinases, catalyse the hydrolytic cleavage of peptides and proteins at distinct peptide bonds. Until recently, proteases had been shown to expose their catalytic domain exclusively in an aqueous compartment such as the cytosol or extracellular space, with the active site generally located in a groove on the surface of the protease. During the past few years, a novel group of proteases has been discovered that appear active within cellular membranes (Fig. 1), that is, they cleave peptide bonds in the plane of lipid bilayers and promote so-called intramembrane proteolysis.

At present, three families of proteases that promote intramembrane cleavage are known. The first family comprises a group of metalloproteases. Its prototypic member, the human site 2 protease (S2P), has been discovered as the protease activity that activates the membrane-bound transcription factor SREBP (for sterol element-binding protein), which in turn regulates cholesterol and lipid homeostasis [3,4]. The second family is a group of aspartic proteases with the presenilins (PS) as the prototypic members. The PSs were first discovered through genetic linkage analysis in families with early onset forms of Alzheimer's disease [5,6]. They are involved in the cleavage of β-amyloid precursor protein (β-APP) and the production of amyloidogenic Aβ peptides, which are a major risk factor of Alzheimer's disease [7,8]. They also play a role in the liberation of signalling molecules such as NOTCH [9]. Another member of this aspartic protease family is the signal peptide peptidase (SPP), which catalyses intramembrane proteolysis of signal sequence remnants and possibly also membrane proteins in the endoplasmic reticulum (ER) membrane of animal and plant cells [10]. In humans, SPP is required for the generation of HLA-E (for human lymphocytic antigen-E) epitopes, which play a key role in immune surveillance [11,12]. The third family comprises the rhomboids, a group of serine proteases. Their proteolytic function was first revealed for the *Drosophila melanogaster* rhomboid-1, which liberates several membrane-bound epidermal growth factor ligands [13]. It appears though that a common theme of all these intramembrane-cleaving proteases is that they liberate signaling or regulatory molecules from membrane-bound precursors (for review see [14–16]).

The biochemical characterisation of intramembrane-cleaving proteases is just at the beginning. Biochemical assays, such as are available for many classic proteases, are not established yet, mostly because the purification of any of those proteins has not been achieved so far. Nevertheless, cellular assays (for S2P, PS, SPP and rhomboid) [3,7,17,18] and cell-free assays using detergent-solubilised membrane preparations (for PS and SPP) have been developed [19,20]. On the basis of these assays, substrates and inhibitors have been identified, and first insights into the requirements for intramembrane cleavage have been obtained. However, the mechanism of intramembrane cleavage is poorly understood. In fact, we have presently no formal proof demonstrating that hydrolysis of peptide bonds does indeed take place within the membrane. Confusion may arise because peptide bonds appear to be hydrolysed within the lipid bilayer, a notably hydrophobic environment that largely excludes water. This view may, however, not be correct. Intramembrane proteolysis refers to *enzyme-catalysed hydrolysis* in cellular membranes. Actually, this type of reaction is not new; there are already enzymes that promote hydrolysis in the membrane. One example is rhodopsin,

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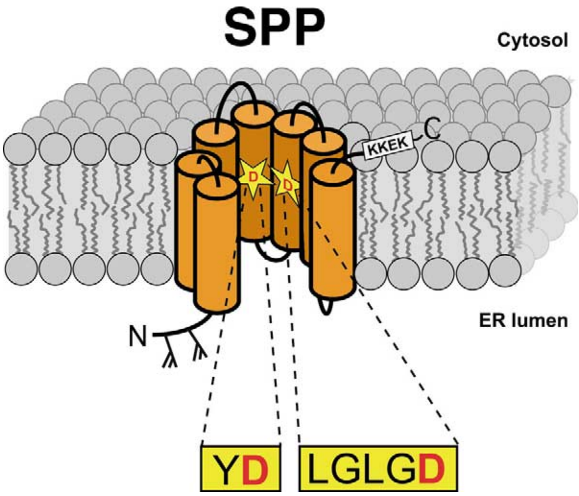

Fig. 1. Model of an intramembrane-cleaving protease. The illustration shows the predicted topology of SPP, which contains seven to nine putative transmembrane regions, two N-glycans near the N-terminus, and an ER retrieval signal (KKEK) at the C-terminus. Typical for all intramembrane-cleaving proteases, SPP has the active site residues embedded within the centre of transmembrane regions, here the two aspartates in the motifs YD and LGLGD. The multiple transmembrane regions may assemble a proteolytic domain within the plane of the membrane consistent with the observed cleavage of substrates in the centre of their transmembrane portion.

which can hydrolyse the Schiff base to the retinol buried within the membrane-embedded domain [21]. Although the presently available evidence for intramembrane proteolysis is all indirect – substrate membrane proteins are cleaved within a transmembrane region and cleavage products are released from the membrane, and proteases have multiple transmembrane regions some of which contain the active site residues – it strongly suggest that intramembrane-cleaving proteases can bind transmembrane segments and catalyse their hydrolysis within membranes. In this review, we will discuss implications of recent findings on the mechanism of SPP and related proteases that allow us to speculate on how intramembrane proteolysis may proceed.

2. Selection of substrates for SPP-catalysed intramembrane proteolysis

Like for all proteases, the activity of intramembrane-cleaving proteases has to be controlled such that they do not attack proteins at random. Proteases typically display a high degree of specificity, which may be achieved by e.g. specific recognition of a distinct consensus sequence or a tight regulation of the protease's activity. Based on present knowledge, no such principal modes of control seem to be valid for SPP and presumably also not for most other intramembrane-cleaving proteases [22–26]. But what are then the guidelines that rule SPP activity? What do SPP substrates look like, and how, if at all, is the attack of SPP on these proteins controlled? Systematic investigations of substrates of SPP provided first insights into how intramembrane proteolysis might be controlled.

2.1. First step: preparing the substrate for intramembrane cleavage

From the presently known substrates of SPP – a number of signal peptides derived from secretory and membrane proteins – we may envision a rather broad substrate spectrum [25]. Many signal peptides seem potential substrates for SPP but also many membrane proteins of the ER have short transmembrane regions with the same topology as signal peptides and may likewise be substrates for SPP. However, they are apparently not readily attacked by the protease. One prerequisite for cleavage by SPP is the preceding cleavage of the substrate protein by signal peptidase (SPase) (Fig. 2) [25]. Similarly, substrates for PS and S2P are cleaved by another protease before intramembrane cleavage can actually occur [23,27–29]. It appears though that substrates have to be tailored first for intramembrane proteolysis by these proteases.

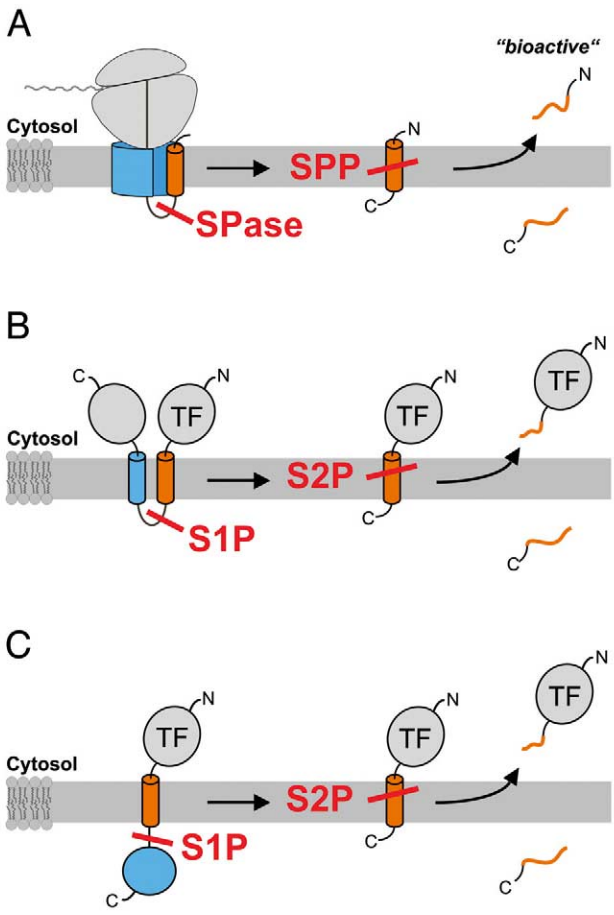

Fig. 2. Tailoring substrates for intramembrane proteolysis. A: Signal peptides have to be liberated from the precursor protein by cleavage with SPase in order to become substrates for SPP cleavage. Without first cleavage, association of the scissile transmembrane region with other membrane proteins such as the translocation pore (blue) or a bulk ectodomain (similar to C) of the precursor protein may prevent access of SPP and interfere with intramembrane cleavage and liberation of signal peptide-derived bioactive peptides. B: For activation, the transcription factor SREBP has to be cleaved first in the luminal loop of the 'helical hairpin' by the site-1 protease (S1P). In the dormant state, the first transmembrane region (blue) of the helical hairpin may shield the scissile transmembrane region. The first cleavage separates the two membrane-bound domains such that the N-terminal transcription factor (TF) domain gets free to be attacked by S2P. C: Like SREBP, the transcription factor ATF6 has to be cleaved first by S1P to become a substrate for S2P. Here, the bulk ectodomain (blue) may block access of S2P and prevent intramembrane cleavage and activation of the transcription factor.

Such a two-step proteolytic process, first revealed for the activation of SREBP, may reflect how intramembrane-cleaving proteases could be kept under control [14].

The initial cleavage reactions preceding intramembrane proteolysis by the proteases S2P, PS and SPP yield peptides with a single transmembrane region containing only a short exoplasmic extension. Such peptides may have higher lateral mobility in the lipid bilayer and bind to the proteases more readily than without such first cleavage. One possibility though to escape binding to the protease active site could be the association with another protein or transmembrane region in a multipass membrane protein or oligomeric complex such that the potential substrate transmembrane region is covered and not freely accessible by the protease (Fig. 2). In addition to that, bulk domains or distinct residues flanking a transmembrane region [25] may affect the interaction with the protease.

2.2. *Key feature: flexibility in the scissile transmembrane region*

SPP cleaves signal peptides in the centre of their transmembrane region [20]. At this position many signal peptides contain one or more helix-destabilising residues. Structure analysis of synthetic signal peptides by nuclear magnetic resonance in a membrane-mimicking environment has revealed that this apparently common feature forces signal peptides to adopt a dynamic helix-break-helix structure [30]. It has been speculated that this structural property facilitates the loop-like insertion of signal sequences into the translocation machinery of the ER membrane during the early steps of protein secretion [31]. More recent studies have now revealed that helix-destabilising residues in the membrane-spanning portion of signal peptides are also critical for intramembrane cleavage by SPP [25]. These residues do not necessarily have to be located at the site of SPP cleavage. In fact, many residues are tolerated at that position pointing to a rather broad substrate spectrum of SPP. Therefore it is apparently not the nature of helix-destabilising residues per se that is important for cleavage, e.g. for substrate recognition by SPP, but rather the impact on the structure of the transmembrane region. Because most signal peptides contain helix-destabilising residues in that region, the prediction would be that they are principal substrates for SPP. A similar but not necessarily sole requirement for helix-destabilising residues seems to be critical for the intramembrane cleavage by the proteases S2P and rhomboid-1 [24,26].

Why could there be a need for helix-destabilising residues for intramembrane cleavage? Typically single transmembrane peptides adopt an α-helical conformation [32]. In such a structure, the peptide backbone is not accessible for proteolysis unless the peptide is unfolded to a certain extent [33]. In the case of signal peptides, the rather short hydrophobic region (7–15 residues) is not long enough to span the hydrophobic interior of a lipid bilayer in a helical conformation. Nevertheless, due to their strong propensity to adopt an α-helical conformation in a hydrophobic environment, which in turn may generate a sufficiently hydrophobic structure to promote the first steps of membrane insertion, it is likely that upon cleavage by SPase signal peptides are initially liberated into the ER membrane in a helical conformation. Later then, as a result of hydrophobic mismatch, signal peptides may be forced to extend and adopt a β-structure [34]. Such a conformational change, however, can only be achieved when the previously intra-chain hydrogen bonds are compensated by e.g. inter-chain hydrogen bonds with other membrane proteins, or peptide aggregation. Alternatively, polar residues flanking the hydrophobic portion may be dragged into the membrane in order to form a helix sufficiently long to span the lipid bilayer.

The structural basis ruling helix formation in transmembrane regions has been addressed by circular dichroism spectroscopy in micellar/vesicular environment using model transmembrane segments hosting guest residues [35]. The helix propensities determined were: Ile > Leu > Val > Met > Phe > Ala > Gln > Tyr > Thr > Ser > Asn > Gly > Pro. This transmembrane helicity scale supports our model that the helix stability determines substrate cleavability by SPP. The aliphatic residues valine and leucine, which have a high helicity, make a signal peptide transmembrane region a poor SPP substrate, whereas polar and small residues, such as serine, asparagine and glycine, increase the efficiency of SPP-catalysed cleavage. Even in the presence of helix-destabilising residues, a transmembrane segment is nevertheless considered to adopt a helical conformation in order to satisfy intra-chain hydrogen bonds [36] and to minimise the cost of burying the polar amide and carbonyl groups of the peptide backbone within the apolar region of the lipid bilayer [37]. For example, proline and glycine residues, which have the weakest tendency to form helices, are often found in native transmembrane helices [38]. Such residues certainly reduce the stability of a helix and facilitate unwinding towards an open conformation. Unwinding may actually be induced or actively promoted upon interaction with another protein, for example SPP. We may thus speculate that SPP substrates require a certain threshold of structural instability of the transmembrane region to allow unwinding and disclosure of a peptide bond for intramembrane proteolysis.

3. Conformational control of membrane-associated proteolysis

Selectivity of proteases commonly arises as a consequence of transition state stabilisation of a cognate but not of a non-cognate substrate. Binding to a protease, however, is not solely defined by the nature of the active site. A series of binding sites usually favour the interaction with particular amino acid residues N- and/or C-terminal to the scissile peptide bond, thereby defining the specificity of the respective protease. The prototype protease for which the substrate-binding site has been described was trypsin. Its substrate-binding pocket close to the active site preferentially binds basic amino acid residues resulting in cleavage of peptide bonds C-terminal to lysine or arginine residues [39]. In contrast, the intramembrane-cleaving proteases characterised so far do not recognise distinct consensus sequences in substrate transmembrane regions [22–26]. This raises the question how proteases such as SPP recognise their hydrophobic substrates within the membrane.

As described above, substrates of SPP and other intramembrane-cleaving proteases are single transmembrane peptides, which typically adopt a helical conformation with all the peptide bonds engaged in hydrogen bonding. Since the binding of the substrate occurs in the hydrophobic environment of a lipid bilayer, the scissile transmembrane region has to partition from the lipid bilayer into the protease active site and there-

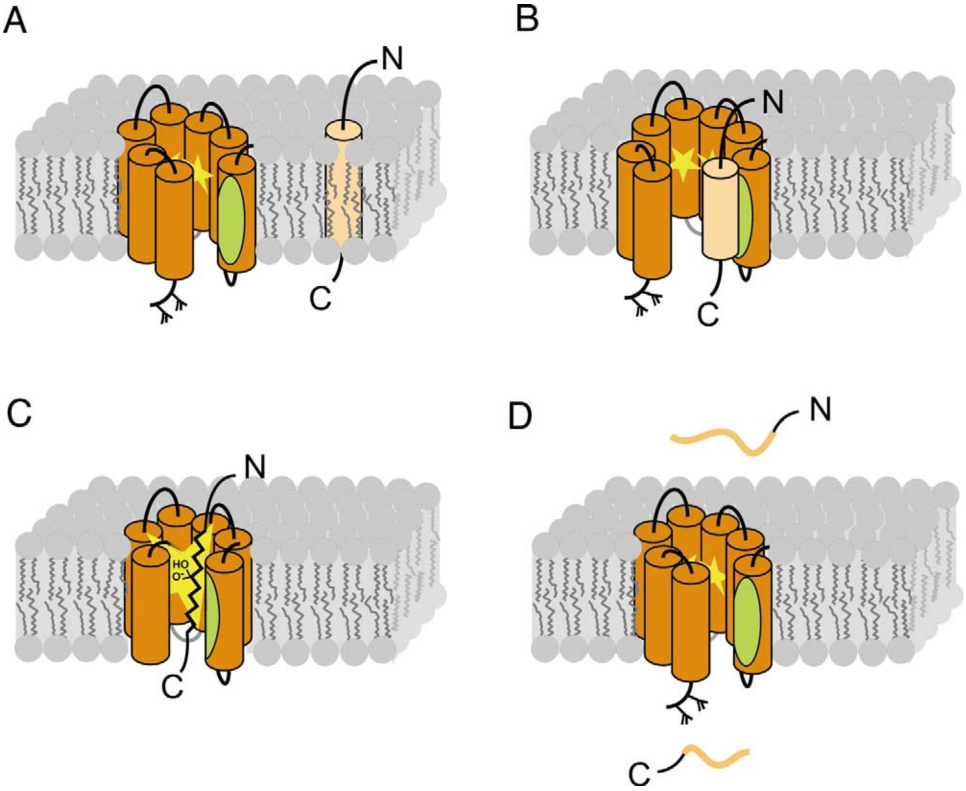

Fig. 3. Model for substrate binding and cleavage by SPP. The substrate transmembrane helix (yellow) may partition from the lipid bilayer (A), to a putative substrate-binding site (green) on the lipid-exposed surface of SPP (B). Subsequently, the substrate peptide may translocate into the catalytic cavity formed around the substrate peptide. Local unfolding of the helical conformation may establish tight binding in a more polar environment and formation of the transition state (C; for clarity, the N-terminal 'front' helix is not shown). Upon cleavage, products are released out of the membrane, and SPP assumes its initial conformation (D).

fore must interact with transmembrane regions of the protease (Fig. 3). Specific interactions between transmembrane helices are mainly due to van der Waals-driven 'knob-into-whole' or 'ridge-into-groove' packing [40]. This, however, requires distinct residues, the 'knob' or 'ridge', on the one helix, and a cognate 'whole' or 'groove' formed by small residues on the other helix [41]. Since intramembrane-cleaving proteases do not appear to recognise distinct consensus sequences, such a specific binding of substrate transmembrane regions is rather unlikely. Non-specific interactions of transmembrane helices, in contrast, are generally weak [40] and cannot be discriminatory. These considerations, the broad substrate spectrum of many intramembrane-cleaving proteases and the finding that helix-destabilising residues in the scissile transmembrane region support efficient cleavage [24–26] argue that it is rather the intrinsic propensity of substrates to (locally) unfold but not a specific recognition that determines which peptides are susceptible to intramembrane cleavage.

Although no structural data of intramembrane-cleaving proteases exist, the requirement for helix-breaking features within a cognate substrate described above suggests that they bind the scissile peptide in the catalytic site in an open, unfolded conformation (Fig. 3C). In order to compensate the intra-chain hydrogen bonds that have to be broken during unwinding of the substrate transmembrane helix, new hydrogen bonds with the protease may be formed. To minimise the energetic cost of exposing the hydrophilic peptide backbone, the protease may form a polar pocket. Substrates with low helix stability could readily unfold and slip into the protease's polar pocket whereas stable helices cannot (Fig. 4). Such an induced fit mechanism could account for specificity when the conformational change is rate-limiting [42]. Binding of substrates in an unfolded conformation through hydrogen bonds to polar residues of the protease may also facilitate release of cleavage products into the aqueous phase.

Insights into the mechanism of intramembrane-cleaving proteases have recently been provided for PS, which has an active site identical to that of SPP. Using an immobilised active site-directed inhibitor, PS was co-purified with the substrate βCTF, which results upon ectodomain shedding of β-APP [43]. This result indicated that the substrate of the aspartic intramembrane proteases is initially bound to the protease elsewhere but not to the catalytic site (Fig. 3). Interestingly, full-length APP was not co-purified by that procedure, suggesting that initial substrate binding requires preceding ectodomain shedding. The observation that substrate binding and catalysis may be independent is in accordance with other inhibitor studies, which showed that active site-directed aspartic protease transition state analogues act in a non-competitive manner on PS [44,45]. Again these results indicate that substrate binding is physically separated from the catalytic centre, and, as a consequence, the substrate has to be transferred from an initial binding site into the catalytic centre.

Other membrane-associated proteases with a low sequence preference are SPase [46] and 'shedding' proteases of the ADAM family such as α-secretase [47] (Fig. 4). SPase binds substrates via β-sheet-type hydrogen bonds [48]. Cleavage is

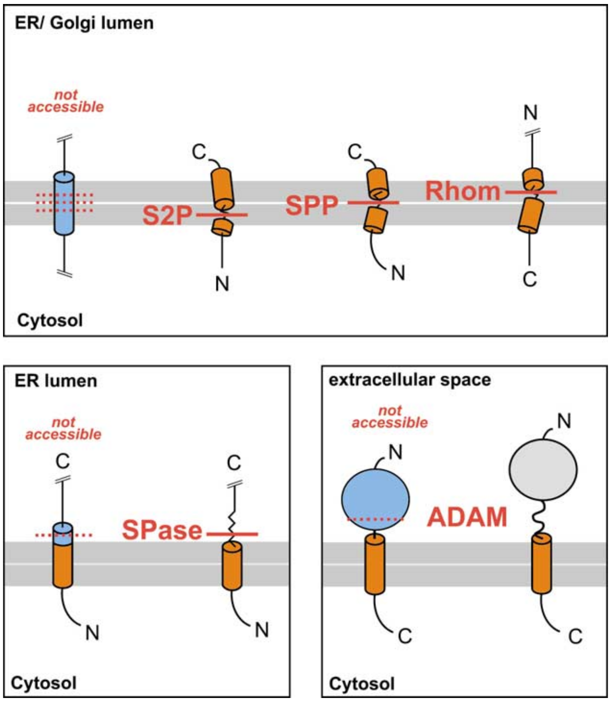

Fig. 4. Conformational control of membrane-associated proteolysis. Intramembrane cleavage by S2P, SPP and rhomboid-1 (Rhom) and juxtamembrane cleavage by SPase and ADAM 'sheddases' requires an open and disordered conformation of the substrate. Specificity is mainly determined by the relative position of the protease's active site with respect to the plane of the membrane, and by shielding the peptide bonds of non-substrates within stable protein folds (highlighted in blue).

mainly affected by the primary and secondary structure of the juxtamembrane exoplasmic region of the signal sequence. For example, a helix break, frequently formed by a proline residue, is required to form a transition between the helical transmembrane region and the unfolded C-terminal portion, which has to fold into an extended β-strand conformation to fit into the SPase active site [48]. Likewise, ADAM proteases cleave a heterogeneous group of membrane proteins in a rather unselective manner within a structurally disordered region close to the membrane anchor [49–53]. More recently, a low sequence preference has also been reported for the shedding protease BACE-1/β-secretase [54]. Taken together, these findings suggest that selectivity of membrane-associated proteases may generally be low and specificity may be accomplished by shielding peptide bonds of non-substrates within stable protein folds rather than specific consensus cleavage motifs.

4. Concluding remarks

During the past couple of years, intramembrane proteolysis has become an established phenomenon. Proteases have been discovered that – although they use water to cleave peptide bonds – seem to catalyse hydrolysis in the water-excluding environment of cellular membranes. How these enzymes make use of water and promote intramembrane proteolysis is presently a matter of speculation and may only be conclusively answered when structural information on one of these proteases becomes available. Another matter of discussion is how substrates are recognised. Intramembrane-cleaving proteases seem to attack polypeptides with single transmembrane regions only, which in addition contain helix-destabilising residues. This latter requirement may facilitate a conformational change upon interaction with the protease such that peptide bonds become accessible for proteolysis, as discussed herein. Furthermore, the protease themselves may undergo conformational rearrangements in order to transfer a substrate peptide from an initial lipid-exposed recognition site to a catalytic cavity for hydrolysis and subsequent release of cleavage products out of the membrane. To address such mechanistic questions, there is urgent need for purified intramembrane-cleaving proteases, something which has not been achieved so far. Intramembrane proteases have important roles in cell signaling and regulation such as control of cholesterol and lipid homeostasis [3,4], cell development [9,55], immune surveillance [11,12], and intercellular communication [13,18]. It is evident that these proteases are of potential value as therapeutic targets, particularly those that have a proven link to human diseases such as Alzheimer's disease (PS) [5–7] and hepatitis C virus infection (SPP) [56]. Exploring their therapeutic value, however, clearly requires a detailed understanding of the mechanism of intramembrane proteolysis, which will challenge structural biologists and biochemists in the future.

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