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Review

Protein trafficking and maturation regulate intramembrane proteolysis ☆

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ABSTRACT

Intramembrane-cleaving proteases (I-CLiPs) are membrane embedded proteolytic enzymes. All substrates identified so far are also membrane proteins, involving a number of critical cellular signaling as well as human diseases. After synthesis and assembly at the endoplasmic reticulum, membrane proteins are exported to the Golgi apparatus and transported to their sites of action. A number of studies have revealed the importance of the intracellular membrane trafficking in i-CLiP-mediated intramembrane proteolysis, not only for limiting the unnecessary encounter between i-CLiPs and their substrate but also for their cleavage site preference. In this review, we will discuss recent advances in our understanding of how each i-CLiP proteolysis is regulated by intracellular vesicle trafficking. This article is part of a Special Issue entitled: Intramembrane Proteases.

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1. Introduction

Protease is an enzyme that catalyzes the hydrolysis of peptide bonds within proteins. Nearly 2% of the proteins encoded by the human genome are proteases, making up one of the largest classes of enzymes [1]. It is well known that proteases play critical roles in a plethora of biological processes such as development, differentiation, cell migration, immunity, wound healing and cell death.

Over the last decade or so, the new branch of proteases with unique character, called as intramembrane-cleaving proteases (i-CLiPs), has emerged [2]. To date, i-CLiPs are comprised of three major classes of proteases, rhomboid (serine protease), site-2 protease (S2P) (metalloprotease), presenilin-type proteases including γ-secretase and signal peptide peptidases (SPPs) (aspartyl protease). They are all multispanning membrane proteins and enzymatically active by themselves with the exception of γ-secretase, that is a complex of four proteins including catalytic subunit presenilin. All of their substrates identified so far are also membrane proteins, involving a number of critical cellular signaling as well as human diseases. Despite the

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hydrophobic nature of the lipid bilayer, these iCLiPs are somehow able to hydrolyze the peptide bond within the transmembrane segment of their substrate. The precise mechanism of action whereby these i-CLiPs catalyze proteolysis in a lipid bilayer has been a subject of intensive studies [3], and great strides have been made in understanding them especially since the first X-ray crystal structure of rhomboid family protease has been revealed. These are reviewed elsewhere in this special issue (see articles from Kroos and Akiyama; Wolfe; Lemieux) therefore we are not going into the details; instead we will focus on the other aspect of i-CLiP-mediated proteolysis.

Aberrant activation of proteases can be detrimental to the cells, therefore their activity must be tightly regulated to make sure that proteolysis occurs in the right place at the right time. Processing by i-CLiP is no exception considering that most of them seem to exist in an active state except for γ-secretase, which requires assembly of four subunits and endoproteolysis of catalytic subunit presenilin. It is widely known that there are several ways to control proteolytic reaction. For example, in the case of proteasome, substrates should be ubiquitinated so that only proteins destined for degradation are recognized. Another example is zymogen processing. Several proteases are first synthesized as a precursor in an inactive conformation. Once they reach to the site of action, the self-inactivating "prodomain" peptide is removed to put them into operation. In the case of i-CLiPs-mediated proteolytic processing, because both enzymes and substrates known to date are all membrane proteins as mentioned above, there is another way to control their reaction — membrane trafficking. The general theme of the regulation of intramembrane proteolysis by membrane trafficking is to limit the encounter between protease and its substrate until it is necessary as seen in the S2P and rhomboid-mediated processing. Moreover there is another important aspect of intramembrane proteolysis that could be affected by membrane trafficking. As for γ-secretase-mediated processing in particular, subcellular location where proteolysis takes place affects the cleavage site selection and consequently the property of proteolytic products, which is critical both in the physiological and pathological processes.

Membrane proteins are synthesized, properly folded and assembled in the endoplasmic reticulum (ER), exported to the Golgi apparatus and transported to their sites of action. A number of factors and signals/motifs that control trafficking or sorting of 'cargo' proteins to a certain organelle have been revealed so far, and cells seem to exploit these regulatory mechanisms to minimize the unwanted proteolytic reaction. In addition, especially for S2P and presenilin-type i-CLiPs, the 'priming' cleavage is required for making substrates available for the subsequent intramembrane proteolysis [2], and this priming cleavage also seems to be a subject of the regulation by membrane traffic. In this review, we will discuss recent advances in our understanding of how each i-CLiP proteolysis is regulated by intracellular vesicle trafficking. We start with the best studied example found in SREBP processing by S2P, and then go on to the trafficking regulation of the other iCLiP-mediated proteolysis.

2. Site-2 protease (S2P)

2.1. SREBP

When the cellular cholesterol level drops, a subset of genes required for lipid synthesis is upregulated by the controlled release of a membrane tethered transcription factor called sterol regulatory element binding protein (SREBP) [4]. SREBP is synthesized as a membrane bound precursor containing two transmembrane domains, the N-terminal transcription factor domain and the C-terminal regulatory domain. In 1994 [5], it was found that the proteolytic activities are required for the liberation of the N-terminal transcription activator domain — SREBP is first cleaved at "site-1" in its luminal loop region between two membrane-spanning helices then at "site-2", which is located within the first transmembrane domain. Site-2 protease (S2P) was identified in a screen to identify the protease responsible for site-2 cleavage [6]. S2P is a multispanning membrane protein and has an HEXXH zinc-binding motif common to the active site of metalloproteases, but in its hydrophobic region, fitting perfectly with the location of site-2 cleavage site in SREBP.

Proteolytic processing of SREBP is elegantly controlled by a feedback mechanism involving protein–protein interactions and membrane trafficking in response to the cellular content of cholesterol (Fig. 1). SREBP normally exists in complex with a protein called SCAP [7]. SCAP is a polytopic membrane protein and has the sterol-sensing domain (SSD) in the middle, and the C-terminal multiple WD repeats which interact with the regulatory domain of SREBP. When cells are with high cholesterol, cholesterol binds to SSD of SCAP to induce conformational change, allowing it to interact with Insig, the ER retention factor for SREBP–SCAP complex [8]. Interaction with Insig prevents SCAP from binding to Sec24, a component of COPII vesicle coat complex, thereby inhibiting the export of SREBP–SCAP complex from ER [9]. However, in sterol-depleted cells, conformation of SCAP without cholesterol is altered to reduce the affinity for Insig. Then SREBP–SCAP complex interacts with Sec24 to be exported to the Golgi, where S1P and S2P reside. Once SREBP–SCAP complex reaches the Golgi apparatus, SREBP is successively cleaved by these membrane-bound proteases.

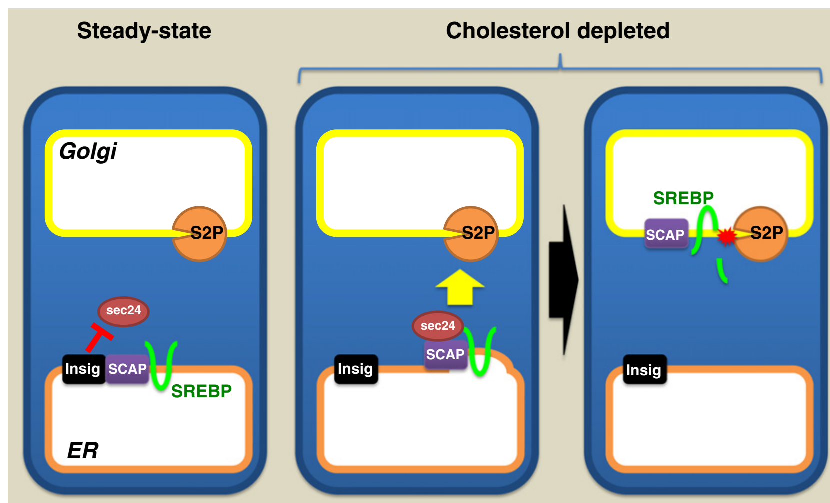

Fig. 1. SREBP processing by S2P.

2.2. Other S2P substrates

SREBP is not the sole substrate for S1P and S2P. Stress response transcription factors ATF6, OASIS and CREB-H are also cleaved by these proteases under stress-induced condition [10]. Remarkably, the regulatory mechanism of their proteolytic processing is very similar to the one used for SREBP processing. In the case of ATF6 for instance, ER chaperone BiP normally prevents ATF6 from being exported from the ER by acting as a retention factor, a role similar to the one Insig plays for SREBP, although BiP is a soluble luminal protein whereas Insig is a membrane spanning protein [11]. It is postulated that when unfolded proteins accumulate in the ER, ATF6 dissociates from BiP and the Golgi localization signals in its luminal region is exposed. As a result, ATF6 translocates from the ER to the Golgi where it is sequentially cleaved by S1P and S2P.

3. Rhomboid

3.1. Spitz

In *Drosophila*, Spitz (Spi) is expressed as a membrane tethered precursor at the ER. Export of Spi from the ER requires Star, a type II membrane protein associated with Spi to act as a chaperone [12,13]. In the absence of Star, Spi is retained in the ER via a PLCγ dependent mechanism [14]. Once exported from the ER, Spi reaches the Golgi or Rab4/Rab14 positive endosomes where Rhomboid-1 resides, and is cleaved to release soluble EGF ligand in the lumen [15,16] (Fig. 2). It has also been reported that Star itself can be a substrate for rhomboid, adding another layer of regulation for the trafficking and processing of Spi [17]. In addition, rhomboid-2 and -3 that are also capable of cleaving Spi are found in the ER [18]. Thus, Spi and Star processing might also occur in this organelle, although this ER-derived form seems to be retained there and does not actively participate in the signaling. Whether this “compartmentalization” mechanism can respond to the external stimuli — in other words, is Spi trafficking and subsequent processing by Rhomboid are accelerated in response to the demand for EGF ligand, as it has been seen in SREBP processing by S1P and S2P remain elusive. Interestingly, it has recently been shown that some of the catalytically inactive rhomboid-like proteins, or “iRhoms”, negatively regulate EGF signaling by enhancing ER-associated degradation (ERAD) of EGF precursor in *Drosophila* [19], serving as another layer of regulatory mechanism for rhomboid-mediated EGF signaling to limit the interaction between protease and its substrate (Fig. 2). Interestingly, one of the mammalian counterpart iRhom2 has been shown to enhance ER export of ADAM17/TNFα-converting enzyme (TACE) to promote TNFα production, suggesting that the functions of these rhomboid-like proteins are context-dependent [20].

3.2. Adhesins

In apicomplexan parasites, rhomboid-mediated intramembrane proteolysis seems regulated in a trafficking dependent manner. In *Toxoplasma gondii* and *Plasmodium falciparum*, some rhomboids such as TgROM4/5 and PfROM4, and their substrate adhesins/microneme proteins (MICs) have been shown to localize on the plasma membrane and in TGN/endosome related apical organelles called microneme, respectively. When these parasites invade host cell, adhesins/MICs translocate to the plasma membrane and bind to the cognate receptors on the surface of the host cell. During the invasion, these adhesins/MICs are transported by actomyosin contractile machinery to the posterior end of the parasite where TgROM5 or PfROM4 is localized and subsequently cleaved by them to complete the invasion [21].

4. γ-Secretase

γ-Secretase comprises four membrane protein subunits, presenilin, nicastrin, Aph-1 and Pen2 [22,23]. Presenilin contains two signature motifs, YD in TMD6 and GXGD in TMD7, containing aspartate residues critical for the catalysis. γ-Secretase cleaves a number of type I membrane proteins with relatively large extracellular domain. Interestingly, removal of this extracellular domain designated as “ectodomain shedding” has been shown to be the prerequisite and therefore rate-limiting regulatory step, for γ-secretase-mediated intramembrane cleavage. Of over 100 γ-secretase substrates reported so far, two have been extensively studied not only for their intramembrane proteolysis but also for their trafficking – APP and Notch. γ-Secretase has a unique character in terms of its cleavage site preference – it cuts the transmembrane domain of the substrate at several different positions. In the case of APP processing, γ-secretase processing yields two types of product, amyloid-β peptide (Aβ) secreted either extracellularly or into the lumen of the vesicle, and the intracellular domain (ICD) released into the cytosol and eventually entering into the nucleus. As for Notch processing, Notch ICD (NICD) goes into nucleus after its liberation and forms transcription activating complex, binds to the downstream target genes to drive its expression. Aβ-like fragments dubbed “Nβ” are also produced simultaneously and released into extracellular space; however their biological significance is not clear [24].

It has also been clearly demonstrated that presenilin needs to be assembled with other three subunits to exert its full proteolytic potential [23], while recombinant presenilin protein itself harbored the proteolytic activity in an in vitro assay [25]. Atypical ER-retention signals have been found in TMD4 and 9 in PS, TMD1 of Pen2 and TMD of Nct, some of which are recognized by an ER-retrieval factor Rer1 [26–29]. Once γ-secretase

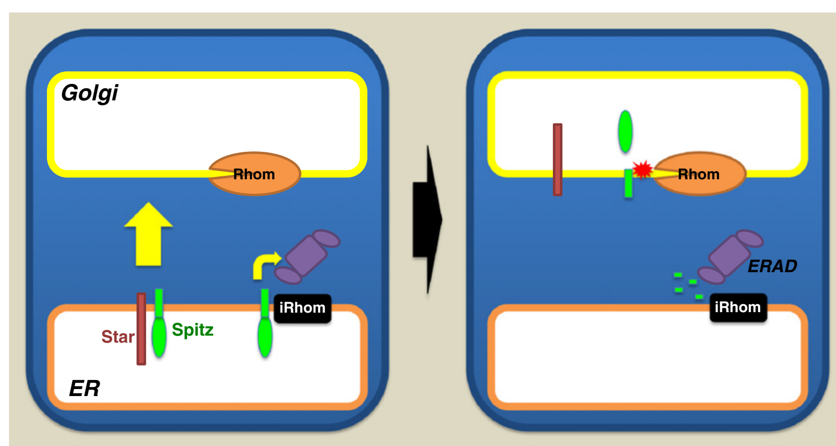

Fig. 2. Spitz processing by rhomboid.

complex is properly formed, these retention signals are masked and the enzyme is exported from ER to the cell surface along the constitutive õsecretory pathway [30,31]. However, the behavior of the active γ-secretase complex after reaching the plasma membrane and the mechanism that control its trafficking had been enigmatic basically due to the lack of probe that specifically detects fully-assembled complex. Previous studies (e.g. analyses of the sites where the substrates accumulate upon γ-secretase inhibition) suggest that γ-secretase activity might spread along the endocytic and recycling pathway such as TGN, plasma membrane, endosomes and lysosomes [32–38]. Notably, distinct cleavage site preference has been observed with different lipid compositions or under manipulation of endocytic activity suggesting that localization of γ-secretase is important to determine its proteolytic product repertoire [39,40]. Given the fact that aggregation propensity of Aβ as well as signaling competence of NICD critically depends on their cleavage sites, it is crucial to get a good grasp of the precise mechanism of γ-secretase trafficking. In this regard, we have recently found that γ-secretase is constitutively internalized via clathrin-mediated endocytosis and this pathway is regulated by CALM, which has been recently identified as a novel genetic risk factor for AD (Morohashi et al., unpublished data).

Here we focus on the two major γ-secretase substrates Notch and APP, and summarize the relationship between their trafficking and intramembrane proteolysis.

4.1. Notch

Notch receptor is normally expressed as heterodimeric single-pass membrane protein with a type I orientation at the cell surface [41]. This heterodimer is generated en route to the cell surface by furin-type convertase (S1 cleavage). When the ligand on the neighboring cells binds, a disintegrin and metalloprotease (ADAM) family protease cleaves at the short extracellular portion of the membrane-bound Notch C-terminal fragment (S2 cleavage). The resulting stub then becomes a substrate for γ-secretase and is cleaved at the cytoplasmic edge (S3) as well as multiple sites in the middle (S4) of its transmembrane domain (Fig. 3).

Although the importance of Notch receptor endocytosis has already been established in late 1990s, during the past several years the additional important clues came from a series of elegantly performed genetic studies in *Drosophila*. Bilder and colleagues reported that factors promoting endocytosis such as dynamin, rab5 and syntaxin 7 are required for ligand-dependent Notch signaling, while loss of some components of ESCRT complex which sort ubiquitinated endocytic cargo from early endosomes into the intraluminal vesicle (ILV) of multivesicular bodies (MVBs) resulted in an aberrant ectopic activation of Notch signaling [42,43], indicating that endocytosis and endosomal sorting of Notch are important for the γ-secretase-mediated intramembrane cleavage and signaling.

Factors that hold the key here are E3 ubiquitin ligases, as ubiquitination is known to be important not only for the proteasomal degradation but also for the endosomal sorting. Several E3 ligases have been implicated in the Notch signaling including Cbl, suppressor of Deltex/Itch/AIP4, Nedd4, Deltex and Sel-10/Fbxw7 [44]. The current model describes the trafficking and processing of Notch as follows: After ligand binding and subsequent ectodomain shedding, Notch is ubiquitinated and internalized via dynamin-dependent pathway. Endocytosed Notch traveled through endo-lysosomal compartment until sorting into ILV of MVB by ESCRT machinery and eventually get degraded in the lysosome. Deltex is involved in the lysosomal sorting and ligand-independent activation of Notch, which requires γ-secretase activity as well as endolysosomal trafficking regulators HOPS and AP-3 [45]. Signals can be sent to the nucleus while Notch C-terminal stub is on the limiting membrane but not anymore after it is incorporated into ILV as signal competent NICD would be released into its luminal space. Although key players have been identified from genetic studies, most of them are still waiting to be put into place to complete the picture outlined above. Further cell biological studies will clarify the mechanism whereby Notch endocytosis and sorting afterwards are regulated.

4.2. APP

APP is metabolized via two different routes — non-amyloidgenic and amyloidgenic pathways [46]. The difference between them is the protease that removes the extracellular domain of APP, where its processing occurs and at which position of extracellular domain it cleaves. In non-amyloidogenic pathway, ectodomain shedding is executed by ADAM family metalloproteases (called 'α-secretase'), most probably on the cell surface, that proteolyzes at a position very close to the membrane

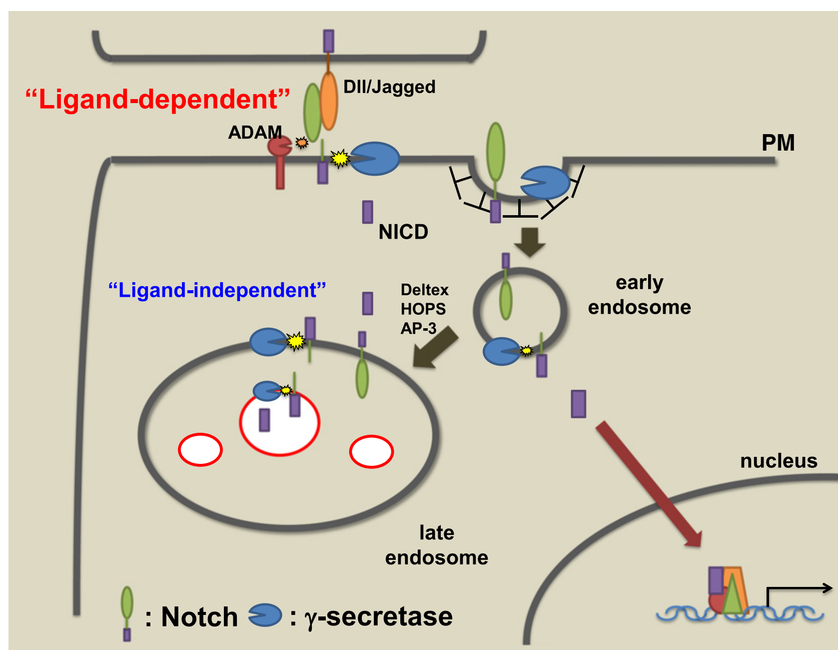

Fig. 3. Notch processing by γ-secretase.

boundary leaving 83-amino acid C-terminal fragment αCTF. In amyloidogenic pathway, a membrane-bound aspartyl protease called BACE1 (a.k.a., β-secretase) is responsible for this step on endosomes and/or TGN where luminal pH is relatively low. This BACE1 cleavage occurs at more distal position from membrane boundary, yielding slightly longer βCTF comprised of 99-amino acid residues. Both αCTF and βCTF become substrates for γ-secretase once they are generated. They could be cleaved wherever they come in contact with γ-secretase along the endocytic pathway and TGN in a similar manner to that in Notch cleavage. Thus, as mentioned above, the processing of APP seems mostly regulated at the level of ectodomain shedding, rather than the γ-secretase processing (Fig. 4).

APP has several known motifs that control intracellular trafficking in its cytosolic C-terminal tail. The most extensively studied one so far is NPTY sequence, a typical binding motif for PTB-domain containing clathrin adaptors [47]. Mutational study revealed that this motif is important for the internalization of APP and subsequent Aβ generation [48]. A number of adaptor proteins have also been found, including Fe65 family, X11 family, Dab1, Jip1b, ARH and Numb etc [49]. Disruption of their function, in many cases, leads to a prolonged residence at the cell surface and thus decreased Aβ generation. Although direct involvement of clathrin in APP internalization has not been tested so far, clathrin adaptor protein CALM, one of the recently identified genetic risk factors for AD, has been shown to regulate the endocytosis of APP and Aβ pathology in model mice, supporting the idea that APP is constitutively internalized via clathrin mediated pathway [50].

In addition to the NPTY motif, APP C-terminus has two other potential trafficking motifs, YTSI and YKFF, which fulfill the minimal consensus for YXXø signal for binding of clathrin adaptor AP complex μ subunit [47]. The Tyr residue in YTSI sequence has been shown to be critical for efficient basolateral sorting in polarized MDCK cells [51], whereas the latter YKFF has recently been shown to bind μ4 subunit of AP-4 [32]. Among five of AP complexes identified so far, AP-4 is known not to bind to clathrin. Instead, it regulates TGN-endosome transport. In the case of APP, disruption of the interaction with AP-4 causes its accumulation in the TGN and increased Aβ and AICD generation as a result of enhanced γ-secretase processing.

During the past several years, the TGN-endosome transport has attracted much attention from the field for another reason. The Vps10p-related type I transmembrane protein sorLA/LR11, genetically associated with AD, is also implicated in TGN-endosome trafficking of APP by regulating its endosomal sorting [52,53]. The expression level of SorLA/LR11 correlates with the level of Aβ in the brains of AD patients as well as in the cultured cells. Loss of SorLA/LR11 expression leads to endosomal accumulation of APP as well as increased Aβ generation. However, functional interaction between sorLA/LR11 and AP-4 remains to be determined.

Regarding the regulatory role of APP cytoplasmic domain, it remains an important and unanswered question whether the trafficking of APP holoprotein and CTFs is regulated by common mechanisms. It has been suggested that once the extracellular domain of APP is removed by α- or β-secretase, CTF is destined for lysosomal degradation [54]. Future studies will clarify if the existence of APP extracellular domain affects its cellular itinerary. Nevertheless, these results suggest that APP is constitutively cycles between plasma membrane, endosomes and TGN, and alteration in this recycling machinery would result in the change in the production of Aβ, thus critical for the etiology of AD.

It is important to note that Aβ metabolism has been shown to be affected by neuronal activity [55]. Pharmacological manipulation in hippocampal slice cultures revealed that the level of activity correlates with the amount of Aβ secreted by affecting β-secretase cleavage, and later it has been shown that this activity-induced Aβ generation requires dynamin-dependent endocytosis [56]. In addition, it has recently been shown that neuronal activity also affects the conformation of γ-secretase catalytic subunit presenilin and Aβ40/42 ratio through exocytic trafficking activity that can be blocked by tetanus toxin treatment [57]. Whether this is a regulation in a narrow sense – in other words whether Aβ forms a part of feedback loop – or is it just the result of a coincidental enhanced/decreased cleavage by β- and γ-secretases due to the change in endocytic/exocytic rate caused by neuronal activity, remains unknown.

5. Signal peptide peptidase (SPP)

SPP is a member of presenilin-like intramembrane aspartyl proteases. It has two signature motifs containing catalytic pair of aspartates YD and GXGD within transmembrane domains, similar but with an opposite orientation to that of presenilin [58]. Therefore it is not surprising that SPP cleaves type II transmembrane proteins which have opposite membrane topology to the substrates of γ-secretase. In human SPP subfamily consists of five members, SPP, SPPL2a, SPPL2b, SPPL2c and SPPL3.

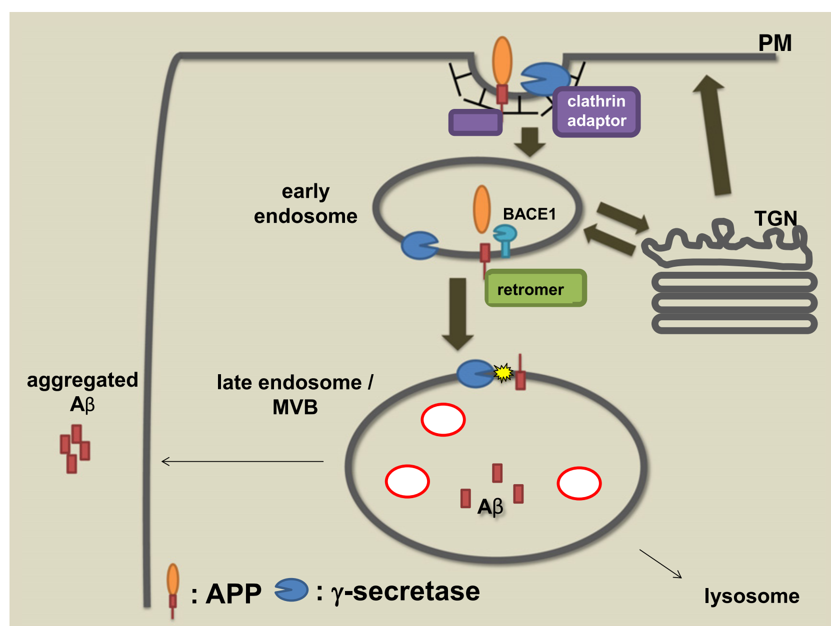

Fig. 4. APP processing by γ-secretase.

Previous reports have revealed that each member has distinct subcellular localization. For example, SPP mainly localizes at the ER whereas SPPL2a at the late endosome/lysosome, SPPL2b at the plasma membrane, SPPL3 at ER and/or Golgi [59–63] (and there is a possibility that SPPL2c is a pseudogene [58]). As for SPP, it has been shown to cleave MHC class I signal peptide in the ER, so in this case membrane trafficking regulation does not seem to be required.

5.1. TNFα

In remarkable resemblance to the γ-secretase mediated Notch processing, SPPL2a and SPPL2b have been shown to cleave type II membrane protein tumor necrosis factor-α (TNFα) to liberate intracellular domain, which transmits signal to the nucleus to induce IL-12 expression [60]. Interestingly, depletion of either SPPL2a or b almost completely abolished IL-12 production, suggesting that they somehow act in concert for this signal transduction, while they show distinct subcellular localization. Whether TNFα processing occurs constitutively or in a regulated manner by compartmentalization, and how cells distinguish SPPL2a- and SPPL2b-mediated processing of TNFα, remain elusive. What determines the late endosomal/lysosomal sorting of SPPL2a through its C-terminus? Further identification of substrates as well as retention/sorting factors for SPP subfamily proteases will help to gain more insights into the regulatory mechanism for their proteolysis.

6. Perspective

Over a decade, the power of the genetic and cell biological studies has greatly helped us to understand how i-CLiP processing is regulated by membrane protein traffic. GFP and live cell imaging technologies have also expand our knowledge regarding functional relationships between the vesicle trafficking and the atypical cleavage reaction. However, the molecular details still remain elusive especially as to what kind of small GTPases, adaptors, vesicle tethering factors and motor proteins are involved in these processes. Recent advances in mass spectrometric analyses have provided a chance to clarify the whole picture of protein–protein/protein–lipid interactions involved in i-CLiP-mediated cleavage [64]. Development of tools to detect endogenous i-CLiPs and their substrates in immunofluorescence analysis is also crucial to answering these fundamental questions. In addition, several small chemicals have been identified as regulatory compounds for vesicular trafficking by chemical biology studies [65]. Identification of regulatory mechanisms for i-CLiP processing using such innovative methods will shed light on the ways these proteases regulate crucial biological processes, and open the possibility for the novel therapeutics against several diseases, such as AD.

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