alzheimer's research&therapy

REVIEW

Protein quality control by Rer1p in the early secretory pathway: from mechanism to implication in Alzheimer's disease

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Abstract

γ-Secretase-mediated production of amyloid β from the amyloid precursor protein is recognized as a central player in the neuropathogenesis of Alzheimer's disease (AD). One of the most peculiar features of this enzymatic activity is the fact that it targets transmembrane domains of mostly type I integral membrane proteins and thus manages to proteolyse peptide bonds within the hydrophobic lipid bilayers. In addition, γ-secretase does not exert its activity solely towards amyloid precursor protein, but to an increasing number of membrane proteins, including Notch, cadherins, syndecans, and so on. Because of the requirement of intramembrane proteolysis for a plethora of signaling pathways and cellular processes during embryonic development and organ physiology, this enzyme has drawn a lot of attention in the past 20 years. γ-Secretase is a multimeric transmembrane complex consisting of the catalytic presenilin, nicastrin, presenilin enhancer 2 (PEN2) and anterior-pharynx defective-1 (APH1) subunits. Proper assembly into functional complexes requires quality control mechanisms associated with the early biosynthetic compartments and allows mature complexes to transit to distal compartments where its activity is required. We previously identified Retrieval to ER protein 1 (Rer1p) as the first negative regulator of the stepwise assembly of γ-secretase during endoplasmic reticulum-to-Golgi transport. We review here the state of the art on how Rer1p regulates complex assembly, particularly γ-secretase, and evaluate the therapeutic potential of such regulatory processes in the context of AD.

Proteins at the plasma membrane constitute a major communication platform between a cell and its environment. In this context, the number of receptors, channels, adhesion molecules or transmembrane proteases at the cell surface critically regulates the cellular and signaling properties of a cell. γ-Secretase is a multimeric protease complex that localizes at the plasma membrane and endosomes and generates the amyloid β (Aβ) peptide, the principal component of amyloid plaques in Alzheimer's disease (AD) [1,2]. The complex is composed of four highly hydrophobic subunits - presenilin (PSEN), nicastrin (NCT), anterior-pharynx defective-1 (APH1) and PSEN enhancer 2 (PEN2) - and contains 19 transmembrane domains. In order to be active in its intended compartments, it requires strictly controlled folding, assembly, maturation and routing that are jointly regulated by the secretory and endocytic pathways [3].

Protein quality control and multiprotein complex assembly in the early secretory pathway: the identification of Rer1p

Proteins destined to function outside the cell or in membrane compartments, including the Golgi, the plasma membrane and the endosomal system, are first synthesized by endoplasmic reticulum (ER)-bound ribosomes and co-translationally translocated in the ER. Here, newly synthesized proteins fold under strict quality control scrutiny into their native structure, which corresponds to the most energetically favorable state [4]. Only properly folded and assembled proteins can reach the Golgi, from where they are transported to their final destination. Misfolded proteins, on the other hand, are recognized, retained and refolded in the ER with the help of chaperones and folding enzymes. If none of the folding

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strategies work, defective proteins will be sent for degradation by ER-associated degradation or autophagy. These primary quality control mechanisms apply to all proteins despite their individual characteristics. Indeed, the recognition of non-native conformation relies on biophysical properties of proteins and includes exposure of hydrophobic regions, unpaired cysteine residues, mobile loops, lack of compactness and the tendency to aggregate.

Multiprotein complexes (for instance, ion channels and immune receptors but also enzymatic activities such as γ-secretase) require additional secondary quality control that ensures proper assembly of individual subunits and functionality of the complex [5,6]. This is needed to prevent single subunits or incompletely assembled complexes being trafficked to distal compartments where they could potentially exert aberrant functions. Such mechanisms often rely on short peptide sequences in cytosolic and transmembrane domains (TMDs) of individual subunits that determine their retention, retrieval or degradation until correct assembly has taken place. Apart from ensuring correct multimeric organization (addressed in more detail later), retrieval from the Golgi apparatus is also important for many ER proteins to retain their strict localization in the ER [4].

Retrieval is often mediated through luminal or cytosolic motifs such as KDEL (lysine-aspartic acid-glutamic acid-leucine) and di-arginine or di-lysine based motifs (for example, RXR, KKXX or KXKXX, where R is arginine, K lysine and X any amino acid), respectively. The KDEL sequence is found in the carboxyl termini of several soluble ER-localized folding chaperones, like grp78 and grp94 [7]. On the other hand, the di-arginine or di-lysine motifs are found in a broad range of transmembrane ER-localized proteins and membrane protein complexes destined to traffic out of the ER [5]. Cytosolic and luminal motif-based retrieval works by recruiting the coat protein complex I (COPI) in the cis-Golgi directly or via the KDEL receptor when the KDEL sequence is present [8-10]. This interaction eventually retrieves the escaped or unassembled protein via retrograde transport to the ER. As such, the exposure of the motif enables the protein to return to the ER and masking this signal thus allows the protein to escape retrieval mechanisms and further traffic along the secretory pathway. In the light of multiprotein complex assembly, and following this strategy, such motifs allow only correctly oligomerized complexes that have attained the proper quaternary structure and hidden their retention signals to pass to the Golgi. This has been well described for the ATP-dependent K channel [11], the G-protein-coupled γ-aminobutyric acid B neurotransmitter receptor [12] and the receptor for immunoglobulin E [6].

Multiproteins that have no such 'classical' ER retrieval motifs in their individual subunits rely on other membrane chaperones that bear signals to aid with assembly. One such example is the Retrieval to ER protein 1 (Rer1p), which is the main focus of this review. Rer1p is a relatively small, highly hydrophobic protein (196 amino acids for the human sequence) composed of four TMDs. Both the amino and carboxyl termini and the loop domains are exposed to the cytosol (the predicted structure is shown in Figure 1A) [13]. The carboxyl terminus bears a positively charged di-lysine or arginine motif (GKKKY in yeast; Figure 1B) required for retrieval of Rer1p from the intermediate compartment/cis-Golgi to the ER [14]. Rer1p was originally identified in a yeast screen for mutants defective in the retention of Sec12p [15]. Yeast Sec12p is an ER-resident type II transmembrane glycoprotein required for the formation of transport vesicles from the ER that does not harbor any cytosolic/luminal ER retention motifs (for example, KDEL, di-lysine/arginine, described above) but rather utilizes Rer1p to maintain a specific ER localization [15,16]. Since its original discovery, many additional yeast proteins binding to Rer1p for ER retrieval have been discovered and, interestingly, they have very different topologies and numbers of TMDs: examples include Sec71p and Sec63p (subunits of the translocation machinery), iron transporter machinery subunit Fet3p, Sed4p (which controls the formation of the COPII vesicle), and the ER α1,2-mannosidase Mns1p (Figure 1C) [17-19]. As opposed to cytosolic and luminal motifs, Rer1p utilizes TMD-TMD interactions with cognate subunits for retrieval to the ER. More particularly, original studies in yeast demonstrated that Rer1p binds polar residues within the interactor's TMD that are exposed in the hydrophobic lipid bilayer [17]. However, within Rer1p the interaction requirements are not yet fully understood and appear to be multimodal. For instance, the highly conserved Tyr152 in the fourth TMD is important for the recognition of Sec12p but not Sec71p [17]. Interaction between Rer1p and its partner probably occurs in the intermediate compartment or cis-Golgi. Later, binding of the Rer1p-interactor complex to COPI retrieves the proteins to the ER [16]. As long as the TMD motif is accessible to Rer1p, the interacting subunit is efficiently retrieved to the ER in a COPI-dependent manner; masking this signal upon oligomerization with its partner subunits is required to proceed further along the secretory pathway. Hence, the Rer1p-binding motif uses a similar 'hide and run' strategy to support multiprotein complex assembly.

Oligomerization may also rely on alternative mechanisms that are based on the presence of ER export signals on only a few specific subunits of the complex. This is the case for the potassium channel Kir3, where four members can assemble in different combinations to generate active channels [20]. The Kir 3.1 homotetramer is not functional because it lacks an ER export signal and is retained in the ER. Kir 3.1 needs to co-assemble with

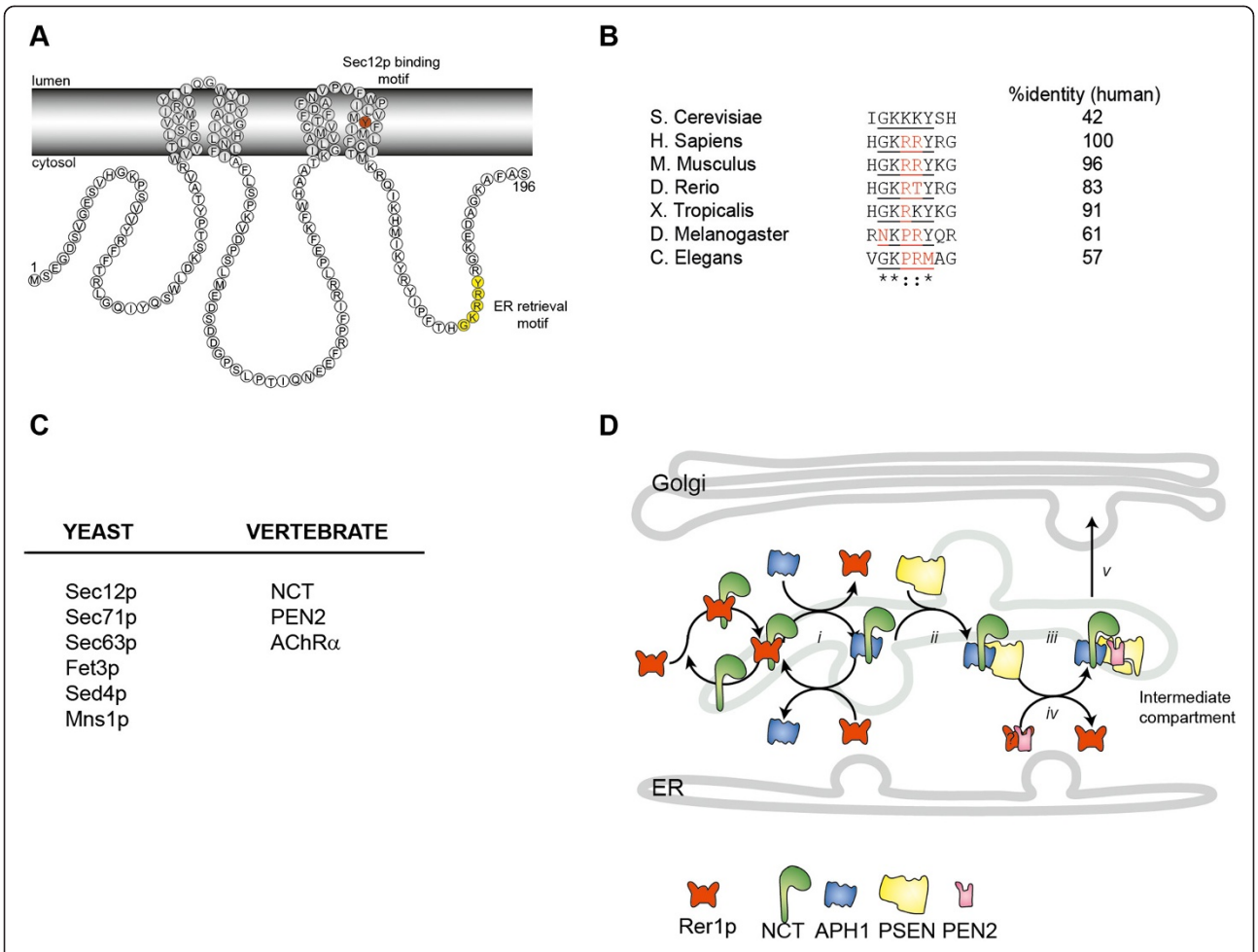

Figure 1 Rer1p regulates complex assembly in the early secretory pathway. (A) Predicted structure of human Rer1p. Rer1p is composed of 196 amino acids and four transmembrane domains. The amino/carboxyl termini and the loop domains are located in the cytosol. Highlighted are the conserved Y152 residue described for the yeast Sec12p-Rer1p interaction [17] and the di-lysine/di-arginine endoplasmic reticulum (ER) retrieval motif located in the carboxyl terminus [14]. **(B)** Homology of the ER retrieval motif of Rer1p between various clades and species. The sequence is not identical but the positive charge of the motif is conserved throughout evolution. On the right is indicated the sequence identity of the ortholog proteins compared to human. **(C)** List of Rer1p interactors identified in yeast and vertebrates so far. **(D)** Assembly of the γ-secretase complex is superimposed on ER-Golgi trafficking. (i) By competing with anterior pharynx-defective 1 (Aph1) for binding to nicastrin (NCT), Rer1p acts as a limiting factor in the formation of the initial NCT-APH1 subcomplex. Later (ii) presenilin (PSEN) and (iii) PSEN enhancer 2 (PEN2) are incorporated in the complex. Whether PSEN and PEN2 join successively or simultaneously is still controversial. (iv) Rer1p may regulate the integration of PEN2 into the complex. Endoproteolysis of PSEN happens after PEN2 assembly. (v) When the complex is fully assembled, it is trafficked to the Golgi where γ-secretase components are glycosylated. AChR, acetylcholine receptor.

other partners, such as Kir3.2A or Kir3.4, that harbor such signals to efficiently exit the ER.

A combination of the aforementioned strategies brings in additional layers of quality control where only properly assembled complexes with a specific subunit composition are selected for forward transport, leaving unassembled monomers behind to search for an appropriate partner.

From yeast to mammalian cells: Rer1p controls γ-secretase assembly

The first mammalian protein discovered to be retrieved to the ER by Rer1p was NCT [21], followed by PEN2 [22], two components of the multiprotein γ-secretase complex, known for its contribution to AD pathology (Figure 1C). γ-Secretase is a di-aspartyl protease responsible for the cleavage of an increasing number of (mostly) type I integral membrane proteins. Among the 90 proteins identified as substrates, the most studied are Notch, for its determining role in development and cancer, and amyloid precursor protein (APP), for its involvement in AD (reviewed in [1,2,23]). Sequential cleavage of APP by β-secretase and γ-secretase generates Aβ peptides, aggregated forms of which are major constituents of amyloid plaques, one of the neuropathological hallmarks of AD. γ-Secretase cleaves its substrate within the

plane of the lipid bilayer in a process termed regulated intramembrane proteolysis that is unique to a small family of intramembrane cleaving proteases; besides γ-secretase, this family also contains the related signal peptide peptidases, the zinc metalloprotease site-2 protease, and rhomboid proteases [24,25]. γ-Secretase -mediated regulated intramembrane proteolysis may activate, turn off or switch the signaling properties of the transmembrane protein involved depending on whether the full-length, membrane-associated carboxy-terminal fragment or intracellular domain is responsible for its function. Most, if not all, of its substrates have signaling properties and regulate cellular events such as cell fate determination, adhesion, migration, neurite outgrowth, axon guidance or formation and maintenance of synapses. It is important to note here that because of the growing number and versatility of its substrates, γ-secretase is associated with a wide range of physiological processes; therefore, alterations of tissue or cellular expression and activity levels of γ-secretase are increasingly associated with human pathologies, ranging from neurodegeneration to cancer (reviewed in [1]).

The γ-secretase complex is composed of four different transmembrane proteins. PSENs, which harbor the catalytic activity of the complex, were first recognized by geneticists as the protein products of genes mutated in familial AD. Among the 180 mutations in PSEN1 and 13 in PSEN2 identified so far, some have been studied in detail and shown to directly affect the processing of APP by increasing either the production of Aβ or the ratio of Aβ42/40. But at the same time, it is becoming clear that the molecular mechanisms resulting in familial AD pathology differ a lot between the different mutations [26]. Moreover, PSEN absolutely requires the co-factors NCT, APH1 and PEN2 to form a functional complex [2,3]. Genetic ablation of only one component results in mislocalization (notably retention in the ER), incomplete maturation and destabilization of the remaining components. This clearly indicates that inter- and intramolecular interactions are crucial in the course of assembly, transport and activation of the γ-secretase complex.

γ-Secretase assembly occurs in a sequential and stoichiometric way and is superimposed with transport regulation to ensure cell- and tissue-specific levels of activity [3] (Figure 1D). As a first step, an NCT-APH1 subcomplex forms, which is stable in the absence of PSEN and PEN2. Later, the formation of a mature PSEN complex may involve direct binding of APH1-NCT to PSEN followed by the incorporation of PEN2. Or, alternatively, the APH1-NCT pre-complex may bind directly to a preformed PSEN1-PEN2 structure to generate the mature active γ-secretase complex. Incorporation of PEN2 promotes endoproteolysis of the full-length PSEN in stable heterodimers [27,28].

The slow maturation (that is, complex N-glycosylation) of NCT in the Golgi and long turnover of γ-secretase components incorporated in the complex suggest that the components are actively recycling from the Golgi or retained in the ER [21,29]. However, no typical retrieval motifs are present in individual γ-secretase components. A potential ER retention motif in the PSEN1 carboxyl terminus [30] has been documented but this has to be scrutinized based on the re-visited nine TMD topology of PSENs [31-34].

We originally discovered that initial γ-secretase complex assembly is controlled by the retrieval protein Rer1p through its competitive interaction with NCT, implementing active Golgi-to-ER recycling in titrating γ-secretase complexes [21]. Shortly after our findings, PEN2 was shown also to bind to Rer1p [22]. In support of our work, Park and colleagues [35] demonstrated an interaction between Rer1p and the immature γ-secretase complex. In particular, they confirmed the interaction with the immature NCT and, importantly, the functional implication of the Rer1p interaction in negatively regulating the assembly, trafficking and activity of the complex [21,35]. We furthermore demonstrated that Rer1p binds to the single NCT TMD via centrally positioned polar residues that most likely form a hydrophilic patch on one side of the TMD α-helix, similarly to known yeast interactors. Since the same residues are crucial for NCT-APH1 interaction [36], it strongly suggests that both Rer1p and APH1 proteins compete for binding to NCT. In this way, the binding of Rer1p to NCT may decrease its availability to bind APH1 and form the initial subcomplex, a prerequisite for full complex assembly. Conversely, the binding of APH1 to the NCT TMD likely masks Rer1p interaction and allows complex assembly to proceed and escape from ER retrieval mechanisms. Thus, by controlling the assembly of the complex as early as during recycling between the Golgi and ER, Rer1p affects the overall levels and activity of the enzyme in post-Golgi compartments. This becomes evident when looking at APP processing, where more Aβ is secreted from Rer1p downregulated cells, while the reverse effect could be observed in overexpressing cells [21,35]. This makes Rer1p the first *bona fide* negative regulator linking transport regulation to γ-secretase assembly. In summary, this example of secondary quality control provides the cell with a mechanism to tightly regulate the quantitative levels of γ-secretase complex and activity in post-Golgi compartments. This is critically important given the high number of substrates and downstream signaling cascades.

While we have focused here on how Rer1p regulates ER retrieval of NCT, it is important to stress that γ-secretase assembly and retrieval may also be regulated by Rer1p-independent mechanisms (which are

currently less well defined molecularly) mediated through the NCT ecto- or luminal domain, the PSEN TMD and carboxy-terminal domains [3,21,30,37,38]. More studies are required, however, to evaluate their true contributions to the assembly processes.

Function of Rer1p *in vivo*

While most studies have been performed in cell culture or yeast, so far only two reports have evaluated the *in vivo* function of Rer1p, in mouse and zebrafish, respectively.

First, using muscle cell lines and mouse, Valkova and colleagues [39] showed that Rer1p controls the assembly of another protein complex, the muscle acetylcholine receptors (AChRs), and consequently the neuromuscular junction. By direct interaction with the α subunit of the AChR, Rer1p retrieves unassembled AChRα and prevents it from degradation. As a consequence of Rer1p loss (using short hairpin-mediated knockdown or heterozygous gene-trap mice), escaped AChRα fails to assemble into a functional receptor, resulting in a reduction of cell-surface-localized receptor and a reduction in neuromuscular junction size. Thus, Rer1p also functions here as a negative regulator of the multisubunit assembly of the AChR, confirming and extending seminal findings on the Rer1p-NCT interaction [21].

Using zebrafish as a small model organism for embryonic development, we recently identified an unprecedented function of Rer1p and γ-secretase in the control of ciliary length and function *in vivo* [40]. Cilia are microtubule-based projections emanating from almost all cells of the vertebrate body [41]. Their function is to either generate fluid flow and motion in the case of motile cilia (for example, sperm, lung epithelium) or orchestrate signaling pathways, including hedgehog, wnt and platelet-derived growth factor receptor A, in the case of primary cilia [42,43]. Genetic defects in genes encoding ciliary proteins give rise to an expanding class of human syndromes termed ciliopathies [44], where they interfere with the crucial role of cilia in development and organ function. Because of the selective high expression of Rer1p in ciliated cells and organs, morpholino-mediated downregulation in zebrafish results in developmental defects reminiscent of cilia shortening [40]. More particularly, ciliary defects were observed in the left-right asymmetry organ known as Kupffer's vesicle, the olfactory epithelium, the pronephros and the photoreceptor of the retina as well as the hair cells of the inner ear or the lateral line that detect movement and vibration in the surrounding water. This resulted in impaired organ physiology as reflected in hearing, vision and left-right asymmetry defects, which are hallmarks of ciliopathies [45]. Rer1p plays a conserved role in the maintenance of ciliary length and function in cell culture as well, where loss of Rer1p reduced ciliary length and secondarily decreased Hedgehog signaling. Mechanistically, some of the cilia defects clearly result from the increased γ-secretase activity and Notch signaling upon suppression of Rer1p, whereas reduced expression of the master regulator of ciliogenesis, Foxj1a [46], in the absence of Rer1p appears to be affected by another mechanism that may involve other γ-secretase substrates or another Rer1p interactor. This work highlights unexpected/unprecedented *in vivo* consequences of increased γ-secretase and, thus, the critical importance of balanced γ-secretase activity during development, and likely adulthood as well.

Is targeting the assembly of γ-secretase a sound therapeutical strategy against Alzheimer's disease?

Reported data have so far established Rer1p as a limiting factor in initial complex assembly of γ-secretase. Increasing Rer1p expression levels thus results in lower γ-secretase levels and therefore less proteolytic fragments like Aβ or Notch intracellular domain, in the case of Notch processing, to name only two prominent substrates. Since controlling the production of Aβ via modulation of γ-secretase is an important therapeutic strategy in AD, the question arises whether targeting Rer1p levels is a sound strategy to tackle AD. *In vivo* data are clearly revealing that neither a loss nor a gain of γ-secretase activity is safe, particularly during embryonic development but also in adulthood [1,40]. Although this has been shown in several cases to arise from a gain or loss of function of the Notch signaling pathway, it is also likely that other signaling pathways originating from the plethora of substrates contribute to it. As such, interfering with γ-secretase assembly by targeting Rer1p will affect the processing of many, if not all, of its substrates and therefore may hamper its applicability as a safe strategy. Also, in order to diminish γ-secretase activity, one would need to increase the levels of Rer1p. It has been proven very challenging to develop small molecules that specifically increase the expression of one protein without inducing side effects. In this regard, one would have to consider gene therapy, which is still at an early stage of clinical development. A second challenge is the heterogeneity of γ-secretase. The existence in the human genome of two genes encoding PSEN (*PSEN1* and *PSEN2*) and two genes encoding APH1 (*APH1a* and *APH1b*) means at least four different combinations of the complex can be made and these have been shown to co-exist in the same cells and/or tissues [47]. As it is becoming clear that the distinct complexes have different biochemical/physiological properties and selectivity for specific Aβ peptides [48], understanding the nature of each of these complexes is becoming highly significant

and could lead to the discovery of specific inhibitors for AD research.

However, Rer1p mainly targets NCT, a subunit common to all different γ-secretase complexes: thus, altering Rer1p levels as a therapeutic strategy will affect levels of all complexes rather than exerting selective inhibition. This contrasts with the accepted view that specifically targeting APP processing without affecting the cleavage of other substrates is a preferred therapeutic approach. In order to study eventual beneficial effects, crosses of existing transgenic AD models with transgenic mice overexpressing Rer1p may be considered. Nevertheless, our findings on Rer1p being an unselective early regulator of γ-secretase assembly does not exclude the existence of other subunit-specific or tissue-specific modulators that are yet to be discovered. Many factors influencing γ-secretase activity have been postulated to affect its trafficking, but more detailed mechanisms still need to be deciphered [3]. To achieve this goal, it is highly relevant to continue our efforts to increase our fundamental understanding of γ-secretase assembly and transport regulation in the light of its heterogeneity as well as the selectivity towards different substrates [23,49].

Conclusion

Since the discovery of PSENs almost 20 years ago and its direct involvement in γ-secretase [50,51], tremendous progress has been made in terms of understanding γ-secretase biogenesis, function and implications in diseases. Due to its large size and multimeric composition, it is becoming well-accepted that γ-secretase relies on a strictly controlled assembly during the early secretory pathway. One of the important early steps is regulated by Rer1p and consists of retrieving unassembled NCT to the ER until it is competed out by APH1. This is a critical prerequisite to achieve full complex formation. Disturbed Rer1p levels affect γ-secretase activity in post-Golgi compartments and thus contribute to the generation of the toxic Aβ. Targeting Rer1p in a therapeutic approach may be extremely challenging, mainly because of the danger of creating unexpected side effects through the Notch pathway, other Rer1p interactors and indirectly ciliogenesis. So far, only two protein complexes have been shown to be regulated by Rer1p in vertebrates, but we expect this list to grow considerably in the future. Rather, we should pursue our work and gather a better understanding of the basic cell biology of γ-secretase in order to develop substrate-specific inhibitors or modulators that produce, for instance, shorter Aβ peptides without affecting the Notch pathway.

Abbreviations

AChR: Acetylcholine receptor; AD: Alzheimer's disease; APH1: Anterior pharynx-defective 1; APP: Amyloid precursor protein; Aβ: Amyloid β; COP: Coat protein complex; ER: Endoplasmic reticulum; KDEL: Lysine-aspartic acid-glutamic acid-leucine; NCT: Nicastrin; PEN2: PSEN enhancer 2; PSEN: Presenilin; Rer1p: Retrieval to ER protein 1; TMD: Transmembrane domain.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The research of WA is financially supported by VIB and KU Leuven (GOA/11/009) and grants from the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (G.0.708.10, G.0754.10 and G.A091.11), the federal government (IAP P7/16), the Hercules foundation (AKUL/08/058, AKUL/09/037, AKUL/11/30) and the SAO/FRA (grant S#12012).

Published: 05 Dec 2013

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10.1186/alzrt227
Cite this article as: Jurisch-Yaksi and Annaert: **Protein quality control by Rer1p in the early secretory pathway: from mechanism to implication in Alzheimer's disease.** *Alzheimer's Research & Therapy* 2013, 5:61