THE
EMBO
JOURNAL

New EMBO Member's Review

Take five—BACE and the γ-secretase quartet conduct Alzheimer's amyloid β-peptide generation

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In 1959, Dave Brubeck and Paul Desmond revolutionized modern jazz music by composing their unforgettable *Take Five* in 5/4, one of the most defiant time signatures in all music. Of similar revolutionary importance for biomedical and basic biochemical research is the identification of the minimal set of genes required to obtain a deadly time bomb ticking in all of us: Alzheimer's disease. It now appears that one needs to *Take Five* genes to produce a deadly peptide by a proteolytic mechanism, which paradoxically is otherwise of pivotal importance for development and cell fate decisions.

A 100 years anniversary of Alzheimer's disease research

It is almost precisely 100 years ago that *Auguste D.* reported to a German psychiatrist in Frankfurt with the words: 'I lost myself'. The psychiatrist was non other than Alois Alzheimer, and this day should mark the beginning of Alzheimer's disease (AD) research. By that time Auguste D. was the one and only patient identified—now we have millions worldwide. As the major risk factor for AD is aging, one can only expect a further sharp increase in the number of patients in the near future. Therapeutic treatment against this devastating disease is desperately required. Fortunately, major progress has been made in the last years, which has led to the first human trials with drugs aimed to lower the burden of the major culprit, the neurotoxic amyloid β-peptide (Aβ) (Hock *et al*, 2003). Aβ is a highly hydrophobic peptide, which aggregates to form oligomers. If these oligomers aggregate further, they start forming fibers, which eventually precipitate and accumulate in the disease defining amyloid plaques

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(Figure 1). While the fibers are deposited in the brain parenchyma, additional toxic aggregation events appear to be induced within the cell. Tau, a protein that stabilizes microtubules, dissociates from the cytoskeleton and aggregates within the soma of neurons to form the intracellular tangles (Figure 1) (Hardy and Selkoe, 2002). This process may then initiate a variety of less understood toxic insults, ultimately leading to the tremendous neuronal loss observed in AD brains. This process, which appears to be initiated by the oligomerization of Aβ, is now known as the amyloid cascade (Hardy and Selkoe, 2002).

A deadly time bomb

Surprisingly and against all predictions, Aβ was found in the early 1990s to be a physiologically normal metabolite generated in healthy persons (Hardy and Selkoe, 2002). Subtle changes in Aβ generation, or its metabolism, can occur as a result of aging, which leads to the accumulation and deposition of Aβ. Accordingly, in the rare familial AD (FAD) cases, mutations cause increased production of the two-amino-acid longer Aβ42 peptide, at the expense of the rather benign Aβ40. Aβ42 aggregates much faster, and consequently causes a much earlier onset of the disease (Hardy and Selkoe, 2002).

Generation of Aβ occurs by processing of the β-amyloid precursor protein (APP) via proteases called secretases. Three secretases are known, α-, β-, and γ-secretase. While β- and γ-secretase mediate the amyloidogenic cleavage events (Figure 1), α-secretase on the contrary prevents Aβ generation by cleaving APP in the middle of the Aβ domain (Figures 1 and 2).

β-Secretase is an aspartyl protease of the pepsin family

β-Secretase (also called BACE-1 for β-site APP-cleaving enzyme) was identified as a type 1 transmembrane protein containing aspartyl protease activity (Vassar, 2001). BACE belongs to the pepsin family of aspartyl proteases, but defines a novel subgroup of membrane-associated hydrolases. BACE-1 mediates the primary amyloidogenic cleavage of APP and generates a membrane-bound APP C-terminal fragment (APP CTFβ), which is the immediate precursor for the intramembraneous γ-secretase cleavage (Figure 1). BACE-1 is clearly the only protease with a well-defined β-secretase activity. This was unambiguously shown by the homozygous knockout of the BACE-1 gene, which does not allow any Aβ generation (Cai *et al*, 2001; Luo *et al*, 2001). A close homolog of BACE-1, BACE-2, was identified as well (Vassar, 2001); however, BACE-2 exhibits an α-secretase-like activity
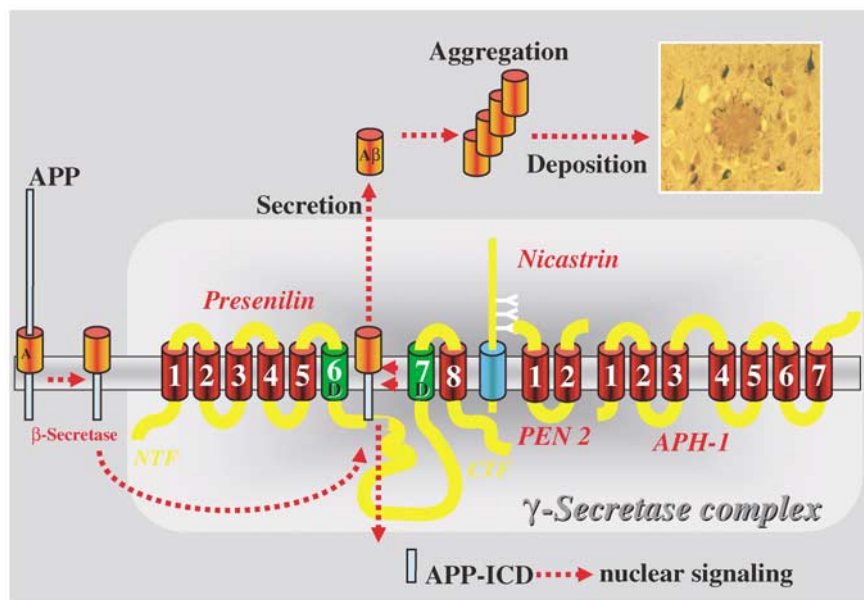
Figure 1 Generation of Aβ from APP via proteolytic processing by β- and γ-secretase (for details, see text). Aβ aggregates and finally precipitates in amyloid plaques. This event initiates the amyloid cascade resulting in additional intracellular aggregations of the tau protein, which then form tangles (the black structures surrounding the amyloid plaque).

Figure 2 Cleavage sites of α-, β-, and γ-secretase. The Aβ domain is shown in red, the TMD is underlined, and the APP-ICD is shown in blue.

(Figure 2), which cleaves APP in the middle of the Aβ domain at amino acids 19 and 20 (Farzan *et al*, 2000). Thus, BACE-2 does not contribute to the amyloidogenic processing of APP, which is consistent with the complete lack of Aβ generation in a BACE-1 knockout.

BACE-1 is generated as a *pre*proenzyme. Upon removal of the signal peptide, the prodomain is cleaved by a furin-like protease (Bennett *et al*, 2000). BACE-1 is then targeted through the secretory pathway to the plasma membrane and clusters within lipid rafts (Ehehalt *et al*, 2003). APP processing by BACE-1 also occurs preferentially within lipid rafts (Ehehalt *et al*, 2003). Consistent with the finding of BACE-1 in lipid rafts, BACE-1 is sorted apically in polarized cells (Capell *et al*, 2002), the side where rafts are thought to accumulate. At the plasma membrane, BACE-1 (like APP) can be internalized to endosomes (Huse *et al*, 2000; Walter *et al*, 2001). Thus BACE-1 and APP follow similar trafficking routes and meet within endosomes, which may be the preferential site of BACE-1 activity due to its acidic pH optimum (Vassar, 2001).

Little is known about the physiological substrates of BACE-1. As described above, BACE-1 has only limited access to basolaterally sorted APP in polarized cells, thus suggesting that APP is not the main substrate. This is supported by the finding that APP does not bear the optimal cleavage site for

BACE-1 (Liu *et al*, 2002). In fact, one of the FAD-associated mutations in APP (the so-called Swedish mutation) strongly enhances BACE-1 cleavage of APP simply by creating an 'optimized' cleavage site (Citron *et al*, 1992). Besides APP, additional candidate BACE-1 substrates were identified recently; these include P-selectin glycoprotein ligand-1 (Lichtenthaler *et al*, 2003) and the sialyl-transferase ST6Gal I (Kitazume *et al*, 2001). However, the biological function of BACE-1 remains unclear, and may be difficult to address since no phenotype is observed upon removal of BACE-1 from the mouse genome.

Most interestingly, evidence exists that BACE-1 expression is significantly enhanced in brains derived from patients with sporadic AD (Yang *et al*, 2003). The reasons for the enhanced BACE-1 expression are currently unclear. However, in this regard, it is tempting to speculate that AD-associated stressors such as oxidative stress, radicals, unfolded proteins, head trauma, and others may induce BACE-1 transcription and/or expression/activity during aging.

The γ-secretase quartet

After BACE-1 has done its job to initiate Aβ generation, γ-secretase comes into play. γ-Secretase is required for the rather unusual intramembrane cleavage event (Figures 1

and 2), a process initially thought to be biochemically impossible. However, recent evidence now suggests the convergent evolution of several polytopic proteases, which are all able to cleave proteins inside the hydrophobic environment of a lipid bilayer. These include the signal peptide peptidases and their homologs, the type 4 prepilin peptidases (although these may cleave just outside of the membrane), rhomboid, S2P, and the γ-secretase itself (Haass and Steiner, 2002; Weihofen and Martoglio, 2003). Whereas S2P, rhomboid, and type 4 prepilins work by themselves, a set of four proteins is required to build up a γ-secretase complex (De Strooper, 2003).

Presenilins

The two homologous presenilins, PS1 and PS2, are apparently of exceptional importance for the γ-secretase cleavage event. This became obvious many years ago when the genetic linkage of PSs to numerous familial AD cases was discovered (Sherrington *et al*, 1995). More than 100 autosomal dominant PS point mutations have now been identified, which all cause aggressive early-onset AD by the same mechanism. These mutations seem to influence the γ-secretase cleavage event by simply shifting it two amino acids to the C-terminus (Hardy and Selkoe, 2002). The subsequent increase of the Aβ42 to Aβ40 ratio results in the enhanced oligomerization of the highly amyloidogenic Aβ42. This already suggests that PSs must be directly involved in the γ-secretase cleavage event or even be the γ-secretase itself. The latter is supported by the pivotal observation of De Strooper *et al* (1998), who found that a PS1 knockout severely reduced Aβ generation. When the second PS gene (PS2) was eliminated as well, no Aβ generation was observed at all (Herreman *et al*, 2000; Zhang *et al*, 2000). Direct evidence for PS presenting the catalytic core of γ-secretase came from the finding that all PSs contain two functionally important and highly conserved aspartate residues within transmembrane domains (TMDs) 6 and 7 (Wolfe *et al*, 1999). This was of great interest, since biochemical evidence suggested that γ-secretase belongs to the aspartyl protease family. Do these aspartates build up the catalytic center of the γ-secretase complex? When either of the two aspartates was mutagenized, Aβ generation was indeed abolished (Wolfe *et al*, 1999). Moreover, PSs are cleaved by endoproteolysis within the large cytoplasmic loop (Figure 1) (Haass and Steiner, 2002). This cleavage results in two fragments (the N-terminal (NTF) and the C-terminal (CTF) fragment), which remain bound to each other (Capell *et al*, 1998). Upon mutagenesis of either one of the two critical aspartates, this cleavage event does not occur anymore and PS accumulated as a holoprotein (Wolfe *et al*, 1999). This phenomenon would be consistent with the holoprotein being a zymogene, which is activated by autoproteolyis (Haass and Steiner, 2002).

Another piece of evidence that PSs provide the catalytic core for the aspartyl protease activity of the γ-secretase complex is that γ-secretase inhibitors can be directly cross-linked to PS (Esler *et al*, 2000; Li *et al*, 2000). However, in contrast to BACE-1 and all other conventional aspartyl proteases, PSs lack the D(T/S)G(T/S) aspartyl protease active site; instead, they contain a GxGD motif around the critical asparate in TMD7 (Steiner *et al*, 2000; Haass and Steiner, 2002). Most interestingly, this motif is fully conserved

between presenilins, the type-4 prepilin peptidases (Steiner *et al*, 2000), and the signal peptide peptidases (Weihofen and Martoglio, 2003). This suggests convergent evolution of a novel active site for a common problem: intramembrane proteolysis.

Obviously, PSs did not evolve to produce the deleterious Aβ peptide. In fact, Aβ generation seems to be a rather unfortunate by-product of an otherwise highly important biological function of PSs. This became evident when PSs were eliminated in mice. A PS1 knockout resulted in a phenotype that closely resembled that of a Notch knockout (Selkoe and Kopan, 2003). A number of additional substrates of presenilins have also been identified besides APP and Notch (De Strooper, 2003). These include the APP homologs APLP-1 and -2, ErbB-4, E-cadherin, N-cadherin, LRP, Nectin-1-α, the Notch ligands Delta and Jagged, and CD44. In the case of Notch, the function of intramembraneous cleavage is well understood. Intramembraneous cleavage of Notch is required to liberate the Notch intracellular domain (ICD) for nuclear signaling and subsequent regulation of target gene transcription (summarized by Selkoe and Kopan, 2003). However, intramembraneous cleavage can also negatively regulate nuclear signaling. Robakis and co-workers (Marambaud *et al*, 2003) demonstrated that the ICD generated from N-cadherin functions as a potent repressor of CBP/CREB-mediated transcription. Moreover, generation of the ICD was reduced by FAD mutations. Interestingly, there is now evidence that the APP intracellular domain interacts with the nuclear adaptor protein Fe65 and with the histone acetyltransferase Tip60 to form a transcriptionally active complex (Cao and Südhof, 2001). Similar to the effects of FAD mutants on ICD generation of N-cadherin, some FAD mutations affect the generation of the APP and the Notch intracellular domain (Moehlmann *et al*, 2002). This suggests a loss of function (in signaling) in addition to the pathological gain of function related to Aβ42 generation.

Two intramembraneous cleavage events are required to liberate ICDs (Figure 2) and their corresponding Aβ or Aβ-like peptide (Sastre *et al*, 2001; Okochi *et al*, 2002). One cleavage event occurs within the middle of the TMDs (at amino acid 40 or 42). Another one occurs close to the cytoplasmic border of the TMD and is required to liberate ICD. It is possible that hydrophobic TMDs prevent efficient liberation of ICDs generated by the cleavage in the middle of the membrane, and thus require a second cleavage event to remove this 'sticky' domain. Both cleavage events appear to occur simultaneously and are fully PS dependent (Sastre *et al*, 2001).

Nct, APH-1, and PEN-2

PSs are not sufficient for γ-secretase activity, but rather require additional cofactors to form a multiprotein high-molecular-weight complex (De Strooper, 2003). Biochemical purification of this complex led to the identification of nicastrin (Nct) (Yu *et al*, 2000). The remaining components of the complex were isolated by genetic screens for enhancers of a PS-dependent Notch-deficient phenotype in *Caenorhabditis elegans*. This led to the identification of two additional components: APH-1 (anterior pharynx-defective phenotype) and PEN-2 (PS-enhancer) (Francis *et al*, 2002; Goutte *et al*, 2002; De Strooper, 2003). When all four

components were expressed together in *Saccharomyces cerevisiae*, an organism that lacks any endogenous γ-secretase activity, fully active γ-secretase was reconstituted (Edbauer *et al*, 2003).

PS expression and γ-secretase activity are regulated in a coordinated manner. Because overexpression of PS does not lead to the expression of higher levels of PS fragments (Haass and Steiner, 2002), nor to enhanced γ-secretase activity, a limiting factor regulating γ-secretase activity was postulated. Maybe there is no defined limiting factor; instead, the least abundant γ-secretase complex component, be it PS, PEN-2, APH-1, or Nct, may become limiting. If there is a disequilibrium of any of these components, excess amounts are destabilized or, in the case of Nct, fail to maturate (De Strooper, 2003).

Although the composition and the biological function of the γ-secretase complex seem to be largely solved, little is known about the function of the individual components. This is due to the difficulties in analyzing these components individually in the absence of the other partners.

Since the complete (Shirotani *et al*, 2003) luminal domain of Nct bound to the membrane (Morais *et al*, 2003) is required for γ-secretase activity within the complex, it is tempting to speculate that this globular, tightly folded ectodomain is responsible for measuring the length of the membrane-retained stub-like substrates. Such a function must be postulated for one of the γ-secretase complex components, because substrates are only recognized and proteolyzed if their ectodomain was previously truncated (Struhl and Adachi, 2000).

In the absence of PEN-2, PS is stabilized as an uncleaved holoprotein probably with the help of APH-1 and Nct (Luo *et al*, 2003; Takasugi *et al*, 2003). Thus APH-1 may be a stabilizer of the PS holoprotein, whereas PEN-2 is apparently required to initiate endoproteolysis of PS (Hu and Fortini, 2003; Luo *et al*, 2003; Takasugi *et al*, 2003). These findings may give some insights into the assembly of the γ-secretase complex. Since Nct is fairly stable in the ER, it is likely that it may be the scaffold for a second binding partner. APH-1 would then be recruited to Nct, as evidenced by the identification of a putative Nct/Aph-1 precomplex (LaVoie *et al*, 2003; Morais *et al*, 2003). Binding of PS to the APH-1/Nct complex would then result in the formation of a trimeric complex (Takasugi *et al*, 2003). Finally, PEN-2 joins the complex, thus allowing PS endoproteolysis and the eventual activation of the γ-secretase activity (Hu and Fortini, 2003; Takasugi *et al*, 2003). However, this is all still quite speculative, since it has so far proved impossible to monitor the assembly of the complex *in vivo*.

Developing drugs against AD

Having identified all the components required for γ-secretase function, it should be rather straightforward to interfere with Aβ production with the help of selective inhibitors. Several high-affinity inhibitors are indeed available. However, one concern remains. γ-Secretase is required for Notch signaling (and most likely for several other signaling pathways as well). Accordingly, deleterious side effects have been observed *in vivo* (Geling *et al*, 2002). However, as for any other novel drug, one needs to define a therapeutic window,

which allows some γ-secretase activity for signaling but reduces Aβ generation significantly enough to slow aggregation and deposition.

BACE-1 is another obvious target for Aβ-lowering drugs. In contrast to γ-secretase, BACE-1 can be fully ablated in mice without causing any deleterious phenotype. Thus, interfering with BACE-1 is unlikely to result in unwanted side effects. Moreover, the three-dimensional structure of BACE-1 has been solved (Hong *et al*, 2000), and peptidomimetic and nonpeptidomimetic inhibitors have been generated (Hong *et al*, 2000; Vassar, 2001). These need to be developed further to improve their affinity with the rather large active site cleft of BACE-1 before they can be used in human trials. However, highly sensitive inhibitors will certainly be available in the near future.

AD futurology

Besides the obvious development of secretase inhibitors, other options remain as well. In fact, in my opinion, anti-Aβ vaccination will have the highest chance to be the first 'true anti-AD' drug on the market, since first and very hopeful results from a small cohort of patients have already been published (Hock *et al*, 2003), and further trials are on the way. What these data may also tell us is that the amyloid cascade hypothesis may in fact indeed be true. Moreover, independent treatments may be important to circumvent the above-described side effects. Finally, additional options for anti-AD drugs remain, such as the nonsteroidal anti-inflammatory drugs (Weggen *et al*, 2001), and compounds aimed to prevent toxicity of Aβ and tau.

However, AD research is far from coming to an end. The secrets of the secretases are not all discovered yet. We still do not know the function of BACE or any of the γ-secretase complex components other than PS. The biggest challenge in that regard will be the discovery of the three-dimensional structure of the entire γ-secretase complex, maybe even co-crystallized with its substrate and/or inhibitors. Mechanisms of cellular toxicity are far from being understood, and finally and most embarrassingly we do not even understand the biological function of APP, the longest known player in AD pathology.

Conclusion

Thus after almost precisely 100 years, AD research came to a point where direct interference with deadly mechanisms in patients is possible and human trials with secretase inhibitors are on the way. As an important by-product, research in this field led to the elucidation of novel signaling pathways involved in cell fate decisions and shed light on completely unexpected mechanisms of intramembrane proteolysis.

Acknowledgements

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