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Aph-1, Pen-2, and Nicastrin with Presenilin Generate an Active γ-Secretase Complex

Minireview

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γ-Secretase cleaves the Amyloid Precursor Protein (APP) in its transmembrane domain, releasing the amyloid peptide Aβ. Aβ is the main constituent of the amyloid plaques in the brains of patients suffering from Alzheimer's disease. Several other type I integral membrane proteins are also cleaved by this protease. Recent work indicates that γ-Secretase is a multiprotein complex consisting of Presenilin, Nicastrin, Aph-1, and Pen-2 and that all four proteins are necessary for full proteolytic activity. Since several paralogs and alternatively spliced variants of at least Presenilin and Aph-1 have been identified as well, it is anticipated that γ-Secretase is not a homogeneous activity. γ-Secretase is an interesting but complex drug target that challenges classical thinking about proteolytic processing and intracellular signaling.

γ-Secretase is the proteolytic activity responsible for the cleavage of a series of integral membrane proteins (Table 1), most notoriously the Amyloid Precursor Protein (APP) and Notch. Generally, γ-Secretase cleaves the hydrophobic integral membrane domain of its substrates (except for N-cadherin), resulting in the release of protein fragments at the luminal (extracellular) and at the cytoplasmic side of the membrane (Annaert and De Strooper, 2002). In the case of Notch and some other substrates, the released cytoplasmic domains interact with DNA binding proteins and regulate gene transcription, linking γ-Secretase function to a series of signaling processes (Table 1). Whether the small fragments that are shed concomitantly at the luminal side also have biological functions (e.g., Aβ in case of APP or Nβ in case of Notch [Okochi et al., 2002]) remains uncertain. In any event, the Aβ fragment is pathologically important since it is the main constituent of the amyloid plaques in the brain of Alzheimer's disease patients. For this reason, inhibiting γ-Secretase is a therapeutic aim, even when doubts are raised concerning the potential side effects associated with long-term suppression of this activity (Table 1). It is still unknown, at the molecular level, whether γ-Secretase is a homogeneous or a heterogeneous activity. If the latter holds true, it is possible that different complexes preferentially cleave certain substrates. This opens the door to novel, more specific γ-Secretase inhibitors. The recent identification of the different molecular components that constitute this activity is a major step in the direction of answering this question.

The Presenilins (PS) appear to provide the active core of the protease. Two mammalian homologs, PS1 and PS2, exist. The PS (~50 kDa) span the cellular membranes several times (Table 2). Two aspartate residues (Asp257 and Asp385) located in transmembrane domains 6 and 7, respectively (Table 2), are essential for the catalytic activity of the protease. Although the working mechanism needs further scrutiny, γ-Secretase may therefore indeed be considered an aspartyl protease (Wolfe et al., 1999). PS are synthesized as precursor proteins that must become incorporated into a larger complex for stabilization. The pool that is not incorporated into these complexes is rapidly degraded by the proteasome. The stabilization of PS is accompanied by a proteolytic "maturation" cleavage performed by an unknown "presenilinase" (Thinakaran et al., 1996). The resulting amino-terminal fragment (NTF, ~30 kDa) and carboxy-terminal fragment (CTF, ~20 kDa) each contribute separately one aspartyl residue to the catalytic site. Both fragments are part of a larger complex. The exact molecular weight of this complex is an issue of debate and varies according to the techniques used. The minimal estimate is 200–250 kDa (Kimberly et al., 2003), but ~440 kDa (Edbauer et al., 2002) and even larger complexes have been described. Obviously this issue needs to be resolved, since it is a decisive piece of information for estimating the minimal number of subunits that are needed to constitute the γ-Secretase complex.

Using antibodies against the PS fragments, a second member of the complex, called Nicastrin (Nct), was purified (Yu et al., 2000). Nct is a glycosylated ~130 kDa integral membrane protein that binds relatively well to both the NTF and the CTF of PS. Nct is synthesized as a ~110 kDa precursor protein that needs PS to leave the endoplasmic reticulum and to reach the cell surface. In PS-deficient cells, the Nct precursor accumulates in the endoplasmic reticulum. Conversely, suppressing Nct expression using siRNA results in decreased steady-state levels of PS, indicating that Nct is one of the stabilizing factors of the PS fragments (Table 3; Edbauer et al., 2002). However, both proteins expressed together do not suffice to increase γ-Secretase activity in most cell lines. Therefore, other proteins are rate limiting for the assembly of the active enzyme. Geneticists, studying *C. elegans*, overcame the final hurdles. Goutte and colleagues used a screen for genes that cause an "*a*nterior *ph*arynx-defective phenotype," reflecting deficient glp-1 signaling (glp-1 and lin-12 are the two Notch receptors in *C. elegans*). They identified two such genes called Aph-1 and Aph-2. Aph-2 is the homolog of mammalian Nct, while Aph-1 is a novel ~30 kDa multimembrane spanning protein that, similar to PS, is needed for the correct subcellular transport of Aph2/Nct to the cell surface (Goutte et al., 2002). Aph-1 (Pen-1) was also identified independently in a screen for *P*resenilin *en*hancers that cause a glp-1 sterility in a *C. elegans* strain partially deficient in PS (Francis et al., 2002). This screen

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Table 1. Substrates of γ-Secretase (Mammalian)

Substrates	Priming Cleavage	Fragments and Function
APP, APLP-1, APLP-2	Bace and ADAM (constitutively)	Aβ and AICD (gene regulation?)
Notch 1, 2, 3, and 4	ADAM 17, ADAM 10 (?) (ligand-induced)	Nβ and NICD (gene regulation)
ErbB-4	Metalloproteases	B4-ICD (function?)
E-cadherin	?	ICD (disassembly of E-cadherin/catenin complex)
LRP	?	ICD (function?)
Nectin-1-α	?	ICD (function?)
Delta	ADAM?	ICD (function?)
Jagged	ADAM?	ICD (function?)
CD44	Metalloproteases	CD44β, ICD (function?)

yielded in addition the fourth γ-Secretase partner, Pen-2. Pen-2 is a small, hairpin-like membrane protein with Mr ~12 kDa (Table 2).

Aph-1 and Pen-2, like PS and Nct, are essential for normal Notch (glp-1 and lin-12) signaling in *C. elegans* (Table 3). What is, however, the evidence that they are also directly involved in the γ-Secretase activity itself? Genetics provide a partial answer. Using constructs that directly drive expression of the Notch intracellular domain, Francis et al. (2002) demonstrated that Aph-1 and Pen-2 act at or upstream of the release of the Notch intracellular domain, like Presenilin does. Downregulation of one of the two new proteins in cells in culture using siRNA leads to a decline in γ-Secretase activity (Francis et al., 2002; Takasugi et al., 2003; Lee et al., 2002), comparable to what was demonstrated before with Nct (Edbauer et al., 2002) and Presenilin (De Strooper et al., 1998) (Table 3). Thus, all four proteins are needed for cleavage of Notch and APP substrates. Conversely, overexpression of any combination of three proteins does not increase processing of APP, while overexpressing the four proteins together results concomitantly in the processing and stabilization of PS, the increased expression of fully glycosylated Nct, and a clear enhancement of γ-Secretase activity in cell-based and cell-free assays (Kimberly et al., 2003; Takasugi et al., 2003; Edbauer et al., 2003). Thus, it seems that the minimal number of components needed for the proteolytic activity of the complex have been identified, Pen-2 and Aph-1 being apparently the long-sought "limiting cellular factors" controlling PS expression (Thinakaran et al., 1996). Also, the genetic screens in *C. elegans* indicate that the core of the complex is now probably identified: only mutants of these four genes and their homologs (Table 2) but no other new ones were identified in the extensive screens by Francis et al. (2002). In mammalian species the situation is, as usual, more complicated, as several paralogs of the individual prototype proteins and a series of alternative spliced forms of Aph-1a (Gu et al., 2003) have been identified (Table 2). It should be noticed that, in addition, other proteins or lipids could be rate-limiting for the overall activity of the complex.

From the loss-of-function and overexpression experiments performed in different species (Table 3), it is obvious that the four basic components of the γ-Secretase activity influence each other's stability and maturation (Gu et al., 2003; Kimberly et al., 2003; Lee et al., 2002; Luo et al., 2003; Steiner et al., 2002; Takasugi et al., 2003; Edbauer et al., 2003). The effects can be explained

Table 2. The γ-Secretase Complex Members

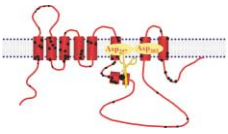
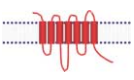
Name (Generic)		*C. elegans*	Mammalian	Functions
Presenilin		HOP-1, SEL-12 (PSEN in *Drosophila*)	PS1, PS2	Catalytic subunit and assembly of γ-secretase (glycosylation of Nct, Pen-2 expression); β-catenin phosphorylation
Nicastrin		APH-2	Nct	Assembly of γ-secretase (stabilization of PS, Pen-2)
Aph-1		APH-1 (PEN-1)	Aph1aL and Aph1aS; Aph1b (Aph1c in rodents)	Assembly of γ-secretase (stabilization of PS/Nct)
Pen-2		PEN-2	Pen-2	Assembly of γ-secretase (proteolytic processing PS)

Table 3. Loss- and Gain-of-Function Effects of the Different γ-Secretase Subunits

	Gain of Function (Overexpression in Cells)	Loss of Function (in Cells)	Loss of Function (Animals)
PS	PS full-length accumulation, no stabilization	Pen-2↓, Nct glycosylation↓, and cell surface transport Nct↓	Notch phenotype (*C. elegans, D. melanogaster, M. musculus*)
Nct	Unglycosylated Nct accumulation	PS↓; Pen-2↓	Notch phenotype (*C. elegans, D. melanogaster, M. musculus*)
Aph-1	PS full-length accumulation, stabilization Nct?	PS↓, cell surface transport Nct↓	Notch phenotype (*C. elegans*)
Pen-2	Processing of stabilized PS	Accumulation of full-length PS	Notch phenotype (*C. elegans, M. musculus*)

in different ways: each component could be essential for one step in the activation of the protease, in which case none of them needs to be a partner in the final active complex. However, the available evidence, although still somewhat shaky, suggests the alternative possibility that the four proteins are subunits of a larger, relatively stable active complex. Thus, in material precipitated from cell extracts using antibodies against one of the subunits, all other subunits are reproducibly detected. Unfortunately, all such experiments made use of Western blotting to demonstrate the presence of the associated proteins, making it hard to know how specific and quantitative the observed coprecipitations are. This assay also does not exclude the possibility that a mix of trimeric complexes is precipitated. Moreover, relatively mild detergents were used, and the studies were performed with cells that strongly overexpress the proteins. This raises concerns regarding their correct subcellular localization before solubilization of the cells and also increases the possibility of nonspecific interactions. Kimberly et al. (2003) provided an interesting alternative for the coimmunoprecipitation experiments using an affinity column that retains only the active form of γ-Secretase. Apparently all four components were retained and coeluted, in support of the hypothesis that they are all part of one enzymatic complex. However, here again, cells strongly overexpressing proteins were used as starting material. Quantitative analysis of the degree of purification of the activity and of the coenrichment of the putative γ-Secretase components was also lacking. By using this approach in combination with additional purification steps, it should be possible in principle to purify γ-Secretase to homogeneity from cells that do not overexpress proteins. This will allow researchers to really evaluate whether the different proteins are stably associated and to settle definitively whether any other factor is needed to generate the active complex. Awaiting this further work, we propose that the working hypothesis of four proteins acting together in one stable complex is reasonable, although not entirely proven.

Further work is also needed to better understand the molecular tasks of the different subunits. The loss- and gain-of-function experiments that were published over the last months (summarized in Table 3) overall show that any change in the expression level of one of the individual components affects the stability, the proteolytic maturation, the glycosylation, or the cell surface transport of the other subunits (Gu et al., 2003; Kimberly et al., 2003; Lee et al., 2002; Luo et al., 2003; Steiner et al., 2002; Takasugi et al., 2003; Edbauer et al., 2003). Apparently all four proteins closely regulate each other. Overall, Aph-1 seems to be least sensitive to changes in expression of the other proteins, and therefore could be considered as the scaffold of the complex that binds PS and Nct. Downregulation of PS affects the stability of Pen-2 and the glycosylation of Nct and, vice versa, downregulation of Nct affects PS stability. This suggests that PS and Nct need each other to leave the endoplasmic reticulum, probably in concert with Aph-1. Finally, Pen-2 downregulation results in the accumulation of full-length PS, but in a stable conformation (Takasugi et al., 2003). Thus, Pen-2 seems to be required for the "Presenilinase" cleavage of PS once PS becomes incorporated in the complex with Aph-1 and Nct. Further work is needed to know how Pen-2 controls this "Presenilinase" and to understand the kinetics of complex formation. Also, the subcellular compartments in which the different subunits bind each other remain to be investigated. It seems unlikely that the three proteins associated to PS only function in the global maturation of the PS protease. Maybe they are also important for the regulation of the activity of the complex and the recognition of the different γ-Secretase substrates.

Now that several pieces of the puzzle are coming together, it is important to tackle the question of the molecular mechanism behind the clinical mutations in PS causing Alzheimer's disease. As amply shown, the more than one hundred different missense mutations in PS1 and PS2 that cause familial Alzheimer's disease (http://molgen-www.uia.ac.be/ADMutations/) all cause a similar defect, i.e., an increase in the ratio of the $A\beta_{42}$/$A\beta_{40}$-species. The $A\beta_{42}$ peptide is, like $A\beta_{40}$, generated by γ-Secretase cleavage of APP and contains two amino acids more at its carboxyl terminus. This peptide has increased aggregation properties and is believed to trigger the pathogenic cascade leading to neurodegeneration in Alzheimer's disease. Since a loss of PS function inhibits the generation of both types of Aβ (De Strooper et al., 1998), it is tempting to consider the mutations in PS as "gain-" or "change-" of-function mutations. However, it is unclear how the many different mutations (indicated by dots in Table 2) scattered over the protein could all cause such a similar "change" of function. It is not unlikely that the PS mutations cause subtle defects in the quaternary structure of the γ-Secretase complex, which could even result in loss of function toward other substrates than APP. The fact that human PS1 is able to rescue PS deficiency in *C. elegans* and in mammalian cultured cells, while some PS mutants cannot, would argue for this possibility, at least in regard to Notch processing (Baumeister et al., 1997; Levitan et al., 1996; Song et al., 1999).

As already mentioned, γ-Secretase cleaves quite a broad range of substrates with a relaxed specificity. In fact, it looks like γ-Secretase cleaves almost by default any type I integral membrane protein whose ectodomain is shorter than a certain number of amino acid residues (Struhl and Adachi, 2000). Regulation of the process (in case of Notch) occurs at the level of a first "priming" cleavage step performed by members of the metalloprotease family ADAM (Table 1). From the novel data, a much more complicated picture is, however, emerging. If the total molecular weight of the individual subunits is taken together, a close approximation of the estimate for the minimal molecular weight of the intact complex, i.e., 200–250 kDa, is obtained (Kimberly et al., 2003). This implies a 1:1:1:1 stoichiometry. Therefore, taking into account the two mammalian PS and the two mammalian Aph-1 homologs, the existence of at least four different γ-Secretase complexes in mammalian species can be inferred. This estimate is a conservative one, since it does not take into account the alternatively spliced forms of PS and Aph-1a (for which a long and a short variant have been identified [Gu et al., 2003; Table 2]). The higher molecular weight estimates for the complex reported by other groups, would, obviously, imply an even higher degree of molecular complexity. In any event, if different complexes can be generated, the question is whether different complexes cleave different substrates, and what cellular and physiological parameters determine the assembly and disassembly of the different γ-Secretase complexes. At the biological level, some indications for such complexity do indeed exist. For instance, the gene inactivation studies of PS1 and PS2 in mice indicate that the two mammalian PS have overlapping but different functions. Mice with inactivated PS1 are lethal, displaying a partial Notch signaling deficiency, while the mice with inactivated PS2 appear largely normal. The double-deficient mouse is early embryonic lethal, displaying a severe Notch phenotype (Donoviel et al., 1999; Herreman et al., 1999). Interestingly, the many genetic crosses performed in *C. elegans* also suggest that different combinations of the proteins could have different biochemical properties. For instance, loss-of-function mutations of Pen-2 and Aph-1 in combination with partial loss-of-function mutations of Nct or the PS homologs in *C. elegans* (Table 2) cause not entirely overlapping glp-1- or lin-12-related phenotypes (Francis et al., 2002). Obviously it is also possible that this variability reflects different expression levels of the proteins in diverse tissues. Further work is clearly needed to address this important question in further detail, especially if drugs have to be designed that specifically interfere with the γ-Secretase cleavage of APP.

In conclusion, a major step forward has been made in the field by identifying the main molecular partners constituting the "minimal" core of the γ-Secretase complex. In the years to come, we will see an increasing understanding of the complexity of this enzymatic activity. To this aim, studies should focus on the complexes expressed under physiologically relevant conditions.

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