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Presenilins and nicastrin are components of the γ -secretase complex involved in APP and Notch processing

Introduction – Background of the project

Alzheimer's disease (AD) is a neurological disorder that is clinically characterized by the progressive loss of memory, disorientation, disturbances in behaviour, and ultimately full dementia. With the increasing number of elderly in the world, AD represents more and more a major health problem. Worldwide, about 20 million people suffer from Alzheimer's disease. Therefore, it is important to understand the pathogenic mechanisms involved in AD, in order to develop effective treatments that ameliorate or even prevent this illness. The disease is characterized by excessive protein deposits called neurofibrillary tangles and amyloid plaques. The amyloid plaques are composed of extracellular deposits of amyloid β peptide ($A\beta_{40}$ and the more pathogenic $A\beta_{42}$). Neurofibrillary tangles are intraneuronal cytoplasmic lesions mainly composed of tau. Two types of AD exist: familial AD (FAD), which is characterized by autosomal dominant inheritance, and sporadic AD, where no clear inheritance pattern is seen. The clinical and pathological features of both types of AD are very similar, therefore knowledge of the familial form of AD is important and essential to unravel the mechanisms involved in both sporadic and genetic AD. Three genes have been identified to be associated with familial AD: APP, PS1 and PS2, located respectively on chromosome 21, 14 and 1.

In order to solve the AD puzzle, it is important to identify the trigger of the disease process. $A\beta$ is a 4 kDa peptide generated from the 110-130 kDa amyloid precursor protein (APP). Mutations in this APP gene and the two other genes involved in FAD, PS1 and PS2, change the APP processing such that more $A\beta$ peptide, and specifically more of the pathogenic $A\beta_{42}$ is produced. The amyloid hypothesis considers the $A\beta$ peptide as the initiator of a pathological cascade that leads to amyloid plaques, neurofibrillary tangle formation, neuronal dysfunction, possibly inflammatory responses, and finally dementia in the patient (Hardy *et al.*, 1997; Selkoe *et al.*, 1999). Downregulation of $A\beta$ in brain, by inhibiting its production, or by promoting its degradation is considered as a promising therapeutic target. A lot of attention therefore goes to the proteases that are responsible for the generation of the $A\beta$ peptide. β -secretase cleaves APP at the aminoterminal of the $A\beta$ sequence and is the first prerequisite for the generation of $A\beta$ peptides. So far, two β -secretases have been identified: BACE1 and BACE2. In contrast to β -secretase, γ -secretase has not been identified unequivocally. However, several lines of evidence implicate presenilin (PS) in this proteolytic event. PS are hydrophobic transmembrane proteins, which span the cellular membrane 8 times, according to the most propagated model. Shortly after biosynthesis, the PSs are cleaved within the loop domain between transmembrane domain 6 and 7 by an unknown protease, presenilinase, resulting in the generation of a 30 kDa aminoterminal and a 20 kDa carboxyterminal fragment. These fragments are stably incorporated in high molecular weight complexes (HMW), which are believed to be the biological active form of PS. A major clue for the role of presenilins in the γ -secretase processing of APP was the discovery that FAD missense mutations in PSs selectively elevate the production of the pathogenic $A\beta_{42}$. Direct evidence for the role of PS in γ -secretase processing was provided by our group in 1998, just before the start of this project. To study

the function of the presenilins and their possible effect on APP processing, mice were generated with inactivated PS1 gene. Inactivation of PS1 resulted in a significant reduction of A β production, together with an accumulation of APP C-terminal fragments. PS1 deficiency results in a severe phenotype characterized by late embryonic lethality, disturbed somitogenesis, mid-line closure deficiencies and malformations of the central nervous system, most significantly underdevelopment of the subventricular zone and a neuronal migration disorder mimicking human lissencephaly type II (Wong *et al.*, 1997; Shen *et al.*, 1997; Hartmann *et al.*, 1999). Based on earlier studies in *C. elegans* and *Drosophila* demonstrating a genetic link between Notch signalling and PS-function, some authors described this PS1 knockout phenotype as a partial Notch1 knockout phenotype. It was however not known how PS1 and Notch might interact. The Notch receptors are large transmembrane receptor proteins that interact with numerous signal transduction pathways. They are essential for many cell differentiation events, including the delineation of boundaries between tissues and inductive interactions between neighbouring tissues during development. They play an important role in neurogenesis and somitogenesis. Mouse embryos lacking Notch1 gene activity display a severe lethal phenotype at embryonic day 10 (E10) characterized by excessive neuronal differentiation and delayed and disorganized somitogenesis.

Project

As discussed above, inactivation of PS1 results in downregulation of the γ -secretase processing of APP, leading to a significant reduction of A β production. However, some residual A β production can still be detected. Furthermore, the PS1 knockout phenotype is reminiscent of the Notch1 knockout, but the similarity is only partial. These observations led us to the following questions. Is the residual γ -secretase activity due to PS2? PS1 and PS2 protein show a high level of amino acid identity (67%), which suggests that they have related, if not overlapping, molecular activities. Can PS2 compensate to some extent for the loss of PS1? Is the PS1 knockout phenotype indeed related to the Notch1 knockout phenotype? In order to solve these questions, PS2 knockout mice were generated. We analysed the PS2 knockout phenotype and the effect of PS2 deficiency on γ -secretase processing of APP. The PS2 gene was inactivated by homologous recombination (Lutgarde Serneels). Exon 5 was replaced by a hygromycine cassette under the control of the PGK promoter. In contrast to PS1^{-/-} mice, which display a truncated skeleton and forebrain hemorrhaging, PS2^{-/-} mice are viable and fertile and develop only mild pulmonary fibrosis and hemorrhage with age. Apparently, PS1 can compensate for the loss of PS2 during development, whereas the reverse is not true. We next investigated the processing of APP in PS2^{-/-} neurons. We speculated that the residual γ -secretase activity observed in PS1^{-/-} neurons was due to the presence of PS2. Quite surprisingly and unexpectedly, deficiency of PS2 does not significantly alter the processing of APP. No significant accumulation of carboxyterminal APP α - and β -stubs, or reduction of A β peptide could be observed in the absence of PS2. This result implies that PS2 mutations causing AD and increasing the generation of A β ₄₂, must act on APP via a gain-of-function mechanism.

The generation of the PS2^{-/-} mice did not answer the question whether PS2 is a functional homologue of PS1 or not. In a further step, the complete deletion of both PS2 and PS1 genes was therefore pursued. PS1^{+/-} mice were crossed with PS2^{-/-} mice to obtain PS1^{+/-}PS2^{+/-} double heterozygotes. These mice were then crossed again with PS2^{-/-} mice to generate PS1^{+/-}PS2^{-/-} mice. Even with only one active PS gene left, these mice remain viable and fertile. From 8 months on, however, these animals display skin tumors and suffer from an autoimmune disease. These mice were then intercrossed to obtain the PS double deficient mice. We failed to detect liveborn double homozygous offspring. At E9.5 (embryonic day), however, we could recover homozygous PS1^{-/-}PS2^{-/-} embryos in a nearly Mendelian distribution. PS1^{-/-}PS2^{-/-} embryos displayed a very severe phenotype characterized by delayed vasculogenesis of the yolk sac. Although an initial vascular plexus and red blood cells had formed, organization into a discrete network of vitelline vessels was always lacking. The embryo itself was devoid of blood circulation, and appeared posteriorly truncated. Some embryos showed heart looping defects, and occasionally an enlarged pericardial sac was observed. Somitogenesis was disturbed and fusion of the headfolds was delayed. The neural tube often had a kinked appearance. This phenotype is clearly different from the PS2^{-/-} embryos, which appear normal, and the PS1^{-/-} embryos, which are only marginally retarded at E9.5. These data imply that PS1 and PS2 have indeed partially overlapping, but not identical functions.

The similarity of the PS double knockout and the Notch1 knockout mice, however, is striking. Both display an early lethal phenotype, characterized by a severe deficit in posterior development. We wondered therefore whether the loss of both PS genes results in a complete inhibition of Notch signalling. To analyse this hypothesis, we investigated the effect of PS1/2 deficiency on target genes of the Notch1 signalling pathway via *in situ* hybridisation. The Notch signalling pathway is initiated by binding of the Notch receptor to a member of the DSL ligand family (Delta, Serrate, Lag-2), expressed by a surrounding cell. Ligand binding triggers the proteolytic processing of the Notch protein, releasing the Notch intracellular domain (NICD). NICD translocates then to the nucleus, where it activates Hes genes which function as negative regulators of lineage specific gene expression. The activity of these genes also inhibits the ligand expression in the receiving cell. The final result of this pathway is the inhibition of differentiation in the receiving cell. We expected to observe differences in the expression of the Notch ligand Dll-1 and the Notch downstream signalling molecule Hes-5 in E9.5 PS double deficient embryos compared to control littermates. Indeed, the expression patterns of Dll-1 and Hes-5 are disturbed in PS1/PS2 double deficient embryos. These data establish the absolute requirement of both PS1 and PS2 in Notch signalling (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999) , and provide *in vivo* evidence for the hypothesis that PS2 has a similar molecular function as PS1.

In cells derived from single PS1- or PS2 deficient mice, residual γ -secretase activity can be observed. The question is now whether cells that are devoid of PS1 and PS2 maintain this γ -secretase activity or not. Unfortunately, PS-null mice die early in embryogenesis (E9.5), making it impossible to generate sufficient cells to perform the required biochemical experiments. We therefore generated pluripotent embryonic stem cell lines from PS-null blastocysts obtained by mating PS1^{+/-}PS2^{-/-} mice (in collaboration with Thromb-X, NV (Belgium)). In contrast to the residual γ -secretase activity in PS1

knockout cells, and the absence of effects on γ -secretase activity in PS2 knockout cells, γ -secretase activity dropped to an undetectable level in these mutant cell lines. No A β secretion could be observed. We next analysed the effect of PS deficiency on Notch processing. Our *in vivo* data already demonstrated the involvement of both PSs in Notch signalling. Furthermore, Notch signal transduction requires the intramembrane processing of Notch, releasing the NICD. This proteolytic event is reminiscent of the intramembrane cleavage of APP, and therefore we wondered whether γ /PS could be involved in this specific step. Indeed, proteolysis of Notch was completely inhibited in absence of both presenilins. These observations extend to the molecular level our previous conclusion that the phenotype of double deficient PS1^{-/-}-PS2^{-/-} embryos is very similar to that of Notch1-deficient embryos.

In conclusion, we showed that inactivation of PS2 results in no apparent phenotype. Furthermore, PS2 deficiency has no significant effect on γ -secretase processing of APP. However, inactivation of both PS1 and PS2 results in a very severe phenotype, implying that PS1 and PS2 have partially overlapping, but not identical functions. Deficiency of both presenilins results in complete inhibition of γ -secretase processing of APP and Notch, providing biochemical evidence for the Notch-like phenotype. So, PS1 and PS2 are indeed functional homologues, both involved in γ -secretase cleavage of APP and Notch. These results prove that the presenilins are indispensable components of the γ -secretase complex.

The question whether presenilin itself is γ -secretase has been an issue of debate for several years. As discussed above, our studies clearly demonstrated the absolute requirement of PSs in the γ -secretase cleavage of APP and Notch. However, these results do not prove that the PSs themselves have catalytic activities. We cannot exclude the possibility that the PS indirectly affects the cleavage through effects on protein transport, folding or stability. Recently, the question whether PS is or is not γ -secretase turns out to be more complex. Several findings indicate that PS is part of a complex and γ -secretase activity requires the interaction between PS and other proteins. Apart from PS1, three other components of the γ -secretase complex have been identified: Nicastrin (NCT), APH-1a/b and PEN-2.

In the last part of this project, we focussed on nicastrin. Nicastrin, a type I transmembrane glycoprotein, was the second member of the γ -secretase complex identified by immuno-affinity purification. We wondered then what was the relation of NCT and PS in the complex. We tried to address this question by analysing the effect of PS deficiency on NCT. The major posttranslational modification of NCT is glycosylation. We investigated the functional relevance of this important modification. NCT is synthesized in fibroblasts and neurons as an endoglycosidase H sensitive glycosylated precursor protein (immature nicastrin) and then modified by complex glycosylation in the Golgi apparatus and by sialylation in the trans-Golgi network (mature nicastrin). These modifications are not observed with exogenously overexpressed nicastrin. Under normal cell culture conditions, only mature nicastrin is expressed at the cell surface, and binds to the presenilin heterodimers. Mature nicastrin has a half-life of more than 24 hours. In the absence of presenilin 1 and 2, nicastrin remains entirely endoglycosidase H sensitive, is retained in the endoplasmic reticulum and is slowly degraded. Single presenilin 1 or presenilin 2 deficiency affects glycosylation of nicastrin to a lesser extent than

the combined presenilin deficiencies. Apparently, there is a PS-dose dependent effect on NCT glycosylation. These effects reflect very closely the effects of PS deficiency on γ -secretase activity, suggesting a correlation between either the transport of nicastrin out of the endoplasmic reticulum or the concomitant complex glycosylation of nicastrin, and γ -secretase activity. However, when complex glycosylation of nicastrin was inhibited using mannosidase I inhibitors, γ -secretase cleavage of APP or Notch was not inhibited while the immature nicastrin still associates with presenilin and appears at the cell surface. Complex glycosylation of nicastrin is therefore not needed for γ -secretase activity. Because the trafficking of nicastrin to the Golgi apparatus is dependent on presenilins, our data point to a central role of presenilin in nicastrin maturation/localization.

In conclusion these data provide basic information in regard to the cellular distribution of nicastrin, its posttranslational modifications and their role in the generation of the PS/nicastrin complex and the effect of PS deficiency on nicastrin biosynthesis and trafficking.

Major conclusions – Implications for Alzheimer's Disease

Although we cannot conclude that the PSs have catalytic activities, we provided direct evidence that the PSs are indispensable components of the γ -secretase complex.

The γ -secretase cleavage of Notch and APP occurs within the predicted transmembrane, in a highly hydrophobic environment. This concept is provocative, since water is required to hydrolyse peptide bonds. Apparently, γ -secretase activity is a particular example of a completely new signalling concept, called Regulated Intramembrane Proteolysis (RIP). Recently, several transmembrane proteins have been identified which can be cleaved by intramembrane-cleaving proteases (I-CLIPs), releasing protein fragments at both sides of the membrane (Wolfe et al., 1999). This mechanism is conserved from bacteria up to higher vertebrates and is involved in processes as cellular differentiation, lipid metabolism, and the unfolded protein response (Brown et al., 2000).

So, γ -secretase cleavage of APP is an example of a very general biological phenomenon, and therefore further understanding of its molecular biology is of major importance. PS-dependent γ -secretase activity appears to be involved in the intramembrane processing of the APP homologues APLPs, (Naruse et al., 1998), the four Notch receptors (De Strooper et al., 1999; Mizutani et al., 2001; Saxena et al., 2001), the ErbB-4 receptor (Ni et al., 2001), CD44 (Lammich et al., 2002), LRP (May et al., 2002), the Notch ligands Delta and Jagged (Ikeuchi and Sisodia, 2003), members of the syndecan family (Schulz and David, unpublished), and E-cadherin (Marambaud et al., 2002). Probably, this list is far from complete. It is obvious that this finding complicates the use of γ -secretase inhibitors in the treatment of AD. For instance, inhibition of Notch signalling by γ -secretase inhibitors could interfere in the hematopoietic/immunological system causing major side effects.

In this regard it is very important to clarify whether these different substrates can be cleaved by different γ -secretase complexes. If one can distinguish between the γ -secretase processing of different

substrates, one could focus on A β inhibition, without affecting the processing of other substrates. The minimal core of the complex, PS, NCT, APH-1 and PEN-2, was recently identified. The fact that two PS homologues, two APH-1 homologues (APH-1a en b), and two APH-1a splicing variants exist (Francis et al., 2002; Goutte et al., 2002), suggests that several γ -secretase complexes indeed exist. Further investigation is required to confirm this, and to examine whether these complexes show any substrate and/or tissue specificity. The answer to these questions is very important for the development of specific γ -secretase inhibitors in AD treatment. This will be the subject of my postdoctoral research project.

Finally, we would like to emphasize the advantages/possibilities of using double PS1 and PS2 deficient embryonic stem cells. These novel cell lines can be used for *in vitro* screening of modulators of γ -secretase activity. In addition, the double PS deficient ES cells can be used as a cellular background to express presenilin clinical mutations. Such a cell line can then be used to screen for drugs that specifically inhibit the production of the pathogenic A β ₄₂ peptide. Furthermore, such cell lines provide the perfect background to perform structure-function analysis on PS. Because almost all available structure-function studies have been performed in cells that endogenously express PS1 or at least PS2, the interpretation of the obtained results depends on the so-called replacement phenomenon. It is believed that the overexpressed PS competes with the endogenous PS for incorporation into the high molecular weight complexes. Obviously, it is virtually impossible to exclude the possibility that some endogenous wild-type PS1 or PS2 remains available and still contributes to the overall effects observed in the transfected cells. This explains probably some of the contradictory findings in the field. In this regard, the use of PS-null cells should be encouraged. Another important advantage of multipotent embryonic stem cells is the ability to differentiate these cells into different specialized cell lines such as neurons, myocytes, macrophages, adipocytes, blood islands etc. In this way, the effect of PS-deficiency in differentiation processes can be studied.

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