# Chapter 4

# β-Secretase, APP and Aβ in Alzheimer's Disease

Robert Vassar
*Department of Cell and Molecular Biology, The Feinberg School of Medicine, Northwestern University, Chicago Avenue, Chicago, Il, USA*

Abstract: Amyloid plaques, hallmark neuropathological lesions in Alzheimer's disease (AD) brain, are composed of the β-amyloid peptide (Aβ). A large body of evidence suggests Aβ is central to the pathophysiology of AD and is likely to start this intractable neurodegenerative disorder. Mutations in three genes (amyloid precursor protein/APP, presenilin1, presenilin2) cause early on-set familial AD by increasing synthesis of the toxic 42 amino acid species of Aβ (Aβ42). Fibrillar Aβ in amyloid plaques appears to cause neurodegeneration, although recent studies suggest soluble Aβ oligomers may also be neurotoxic. Regardless, given the strong correlation between Aβ and AD, therapeutic strategies to lower cerebral Aβ levels should prove beneficial for the treatment of AD. Aβ is derived from APP via cleavage by two proteases, β- and γ-secretase. β-secretase, recently identified as the novel aspartic protease BACE1, initiates the formation of Aβ. Consequently, BACE1 in principle is an excellent therapeutic target for strategies to reduce the production of Aβ in AD. However, the discovery of the homologue BACE2 raised the question of whether it too may be a β-secretase. To settle this issue, our group and others have used gene targeting to generate BACE1 deficient (knockout) mice. These BACE1 knockout mice have been instrumental in validating BACE1 as the authentic β-secretase *in vivo*. Here, I review the roles of BACE1, APP, and Aβ in AD and discuss the implications of therapeutic approaches that target BACE1 for the treatment of AD.

Key words: β-secretase, amyloid precursor protein (APP), β- amyloid (Aβ), BACE1

## 1.    INTRODUCTION

AD is a neurodegenerative disorder characterized by progressive dementia that inevitably leads to incapacitation and death. Synaptic loss and neuronal death occurs in AD brain regions critical for cognitive function, including cerebral cortex, entorhinal cortex, and hippocampus (reviewed in (Terry *et al.*, 1999)). The inexorable loss of neurons and synapses over the course of AD is responsible for the dementia that slowly robs AD patients of their memories, personalities, and eventually their lives. Clearly, understanding    the    pathophysiological    mechanisms    underling neurodegeneration in AD is essential for the rational design of therapies aimed at slowing or stopping disease progression.

Two characteristic neuropathological lesions define AD: 1. amyloid plaques, extracellular deposits primarily composed of the 4 kDa, 40-42 amino acid Aβ peptide (Glenner and Wong, 1984), a product of APP proteolysis, and 2. neurofibrillary tangles, intracellular aggregates of the microtubule associated protein tau (Lee *et al.*, 1991). The relationships between amyloid plaques, neurofibrillary tangles, and the pathogenic mechanisms causing AD are controversial. However, cumulative evidence from a large number of studies indicates that Aβ is critically involved at an early stage in AD pathogenesis.

A strong genetic correlation exists between early-onset familial forms of AD (FAD) and the 42 amino acid species of the Aβ peptide (Aβ42) (reviewed in Hutton *et al.*, 1998; Sisodia *et al.*, 1999; Younkin, 1998). Autosomal dominant mutations in the genes for APP, presenilin1 (PS1), and presenilin2 (PS2) all increase production of Aβ42 and cause FAD with nearly 100% penetrance. Moreover, Down's syndrome (DS) patients, who all develop early-onset AD, have an extra copy of the APP gene on chromosome 21, thus causing Aβ42 overproduction. In FAD, the Aβ42 increase occurs years before AD symptoms arise, suggesting that Aβ42 is likely to initiate AD pathophysiology. The exact structural form of Aβ42 conferring pathogenicity in AD is unclear, since both fibrillar Aβ42 found in amyloid    plaques    and    soluble    Aβ42    oligomers    appear    to    cause neurodegeneration *in vitro* and *in vivo* (see Section 9). Although the majority of AD cases appear to be sporadic, the strong association of Aβ42 with FAD argues in favor of a critical role for Aβ42 in the etiology of sporadic AD as well. Consequently, elucidating the molecular pathways responsible for the generation of Aβ, particularly Aβ42, is essential for developing rational therapeutic approaches that lower cerebral Aβ levels in AD.

The Aβ peptide is generated by the endoproteolysis of the large type I membrane protein APP (reviewed in (Selkoe, 2001; Vassar and Citron, 2000). APP is expressed ubiquitously, and Aβ is a normal product of APP

metabolism in all cells studied to date. A protease called β-secretase first cleaves APP to form the N-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Two β-secretase cleavage products are produced: a secreted ectodomain of APP named APPsβ and the C99 fragment, the membrane bound C-terminal 99 amino acids of APP. Following β-secretase cleavage, C99 is cut by a second protease called γ-secretase, which cleaves to generate the C-terminus of Aβ. Thus, the mature Aβ peptide is formed and is subsequently secreted from the cell. γ-secretase cleavage is not precise and produces a spectrum of Aβ peptides varying in length by several amino acids at the C-terminus, the majority of which end at Aβ amino acid 40. A third protease, α-secretase, cleaves APP in the middle of the Aβ domain (at Leu+17) and precludes the formation of Aβ (*see also* Chapter 5). α-Secretase cleavage produces two products: the secreted APPsα ectodomain, and the membrane bound C-terminal fragment C83, which in turn is cleaved by γ-secretase to form the non-amyloidogenic 3 kDa fragment, p3.

Interestingly, the mutations in APP that cause FAD are all located near the secretase cleavage sites and they directly affect the efficiency or position of secretase cleavage. For example, the Swedish mutation (discovered in a Swedish family), is the amino acid substitution LysMet→AsnLeu at the P2-P1 positions immediately N-terminal to the β-secretase cleavage site in APP (Mullan *et al.*, 1992a). This mutation makes APP a more efficient substrate for β-secretase and dramatically increases the rate of cleavage at the β-secretase site, thus leading to increased production of total Aβ. Several other FAD mutations have been identified near the γ-secretase site that shift the balance of γ-secretase cleavage toward greater production of Aβ42 (reviewed in Hutton *et al.*, 1998). In addition, some FAD mutations occur near the α-secretase site and appear to reduce the efficiency of α-secretase cleavage, thus providing more APP for β-secretase processing.

Until recently, the secretases were described only as APP-cleaving activities found in cells and tissues, but now molecular identities have been proposed for all. Two different proteases appear to be responsible for the α-secretase activity: TACE (TNF-α converting enzyme)(Buxbaum *et al.*, 1998) and ADAM-10 (a disintegrin and metalloprotrotease domain protein) (Lammich *et al.*, 1999). The presenilin proteins, PS1 and PS2 (Wolfe *et al.*, 1999a; Wolfe *et al.*, 1999b), appear to be components of the γ-secretase complex, along with nicastrin (Yu *et al.*, 2000), Aph1 and Pen2 (Francis *et al.*, 2002; Takasugi *et al.*, 2003). Finally, the β-secretase has been identified as the novel transmembrane aspartic protease BACE1 (for β-site APP Cleaving Enzyme 1) (Sinha *et al.*, 1999; Vassar *et al.*, 1999), also known as Asp2 (for novel aspartic protease 2) (Hussain *et al.*, 1999; Yan *et al.*, 1999) and memapsin2 (for membrane aspartic protease/pepsin 2) (Lin *et al.*, 2000).

## 2. THE FUNCTIONAL PROPERTIES OF β-SECRETASE

Following the discovery that Aβ was a product of APP endoproteolysis (Kang *et al.,* 1987; Tanzi *et al.,* 1987), a large number of studies were undertaken to define the properties of β-secretase activity in cells and tissues. This information was essential for the validation of BACE1, since the characteristics of a strong β-secretase candidate must match one-to-one with the previously established functional properties of β-secretase activity. Therefore, I summarize the major properties of β-secretase below.

β-secretase activity is present in the majority of cells and tissues of the body (Haass *et al.,* 1992), although maximal activity is found in neural tissue and neuronal cell lines (Seubert *et al.,* 1993). Interestingly, astrocytes exhibit less β-secretase activity than neurons (Zhao *et al.,* 1996). Therefore, it was predicted that the β-secretase enzyme would be widely expressed in many tissues and cell lines, but should be at higher levels in neurons of the brain.

β-secretase activity in cells efficiently cleaves membrane-bound APP substrates only: APP constructs lacking the transmembrane domain are not cleaved in transfected cells (Citron *et al.,* 1995). This implies that β-secretase is likely to be a membrane-bound protease or, alternatively, is tightly associated with a membrane protein.

β-secretase has maximal activity at acidic pH, since agents that disrupt intracellular pH inhibit β-secretase activity (Haass *et al.,* 1995a; Haass *et al.,* 1993; Knops *et al.,* 1995). Moreover, β-secretase activity is highest in the acidic subcellular compartments of the secretory pathway, including the Golgi apparatus and endosomes (Haass *et al.,* 1995b; Koo and Squazzo, 1994). These data suggest that the active site of β-secretase is located within the lumen of acidic intracellular compartments.

Site-directed mutagenesis of the amino acids surrounding the cleavage site in APP has defined the sequence preference of the β-secretase (Citron *et al.,* 1995). Substitutions of larger hydrophobic amino acids (such as Leu found in the Swedish FAD mutation) for the Met residue at P1 improve the efficiency of β-secretase cleavage. Conversely, substitution of the smaller hydrophobic amino acid Val at the same position inhibits cleavage. Many other substitutions at this site and at surrounding positions decrease cleavage, and indicate that the β-secretase is highly sequence-specific.

Radiosequencing demonstrates that Aβ isolated from amyloid plaques, as well as that produced in cell lines, predominantly begins at the Asp+1 residue of Aβ (Roher *et al.,* 1993), although minor Aβ species begin at Val-3, Ile-6, and Glu+11 (Haass *et al.,* 1992). Inhibitor studies suggest that the Val-3 and Ile-6 species are generated by a protease that is different than β-secretase (Citron *et al.,* 1996). However, the Glu+11 species is produced in

parallel with Asp+1 Aβ (Gouras *et al.*, 1998), suggesting that β-secretase is responsible for cleaving at both positions. Interestingly, the Glu+11 species is the predominant form of Aβ made in rat primary neuron cultures (Gouras *et al.*, 1998). Finally, β-secretase is insensitive to pepstatin, an inhibitor of many (but not all) aspartic proteases.

## 3. THE CLONING AND CHARACTERIZATION OF BACE1

Three different methodologies were employed to identify BACE1. We used an expression cloning strategy designed to identify genes that increase Aβ production in cells (Vassar *et al.*, 1999). Following transfection with pools of ~100 random cDNAs from an HEK 293 cell cDNA expression library, Aβ levels were measured by enzyme linked immunosorbent assay (ELISA), positive pools were broken down to single clones by sib selection, and the full-length BACE1 cDNA was isolated and sequenced. Sinha and colleagues (Sinha *et al.*, 1999) employed an affinity purification approach to isolate the β-secretase from human brain. Their affinity ligand was a peptide-based transition-state analog that strongly inhibits and binds tightly to the enzyme. Using an *in vitro* assay to follow β-secretase cleavage, the group then performed several enrichment steps before the final affinity purification step with the substrate analog inhibitor. N-terminal sequencing revealed a single amino acid sequence, which was used to isolate the full-length cDNA by conventional cloning methods. The other groups employed various genomic strategies to identify novel aspartic proteases that were evaluated for β-secretase properties (Hussain *et al.*, 1999; Lin *et al.*, 2000; Yan *et al.*, 1999). For example, based on the sequence characteristics of the β-secretase cleavage site, Yan and colleagues (Yan *et al.*, 1999) reasoned that the β-secretase might be an aspartic protease and therefore searched the C. elegans genome database to identify novel aspartic proteases. Using the C. elegans protease sequences, they searched vertebrate expressed sequence tag (EST) databases and found four novel human aspartic proteases. The group then screened the candidates for β-secretase function using antisenese technology, and determined that only one protease had bona fide β-secretase activity. Most importantly, all the groups identified exactly the same protein and concur it is β-secretase, even though the groups used very different methodological approaches. This fact provides strong support for the hypothesis that BACE1/Asp2/memapsin2 (henceforth referred to as BACE1) is the authentic β-secretase.

BACE1 exhibits all the known functional properties and characteristics of the β-secretase. First, BACE1 is clearly a protease: the 501 amino acid

sequence exhibits the hallmark features of eukaryotic aspartic proteases of the pepsin family. Two aspartic protease active site motifs of the sequence DTGS (residues 93-96) and DSGT (residues 289-292) are present in BACE1, and mutation of either aspartic acid residue inactivates the enzyme (Bennett *et al.*, 2000b; Hussain *et al.*, 1999). BACE1 has an N-terminal signal sequence (residues 1-21) and a pro-peptide domain (residues 22-45) that are removed post-translationally, so the mature enzyme begins at residue Glu46. Importantly, BACE1 has a single predicted transmembrane domain near its C-terminus (residues 455-480). Thus, BACE1 is a TypeI membrane protein with a lumenal active site, features predicted for β-secretase. The position of the BACE1 active site within the lumen of intracellular compartments provides the correct topological orientation for cleavage of APP at the β-secretase site. Similar to other aspartic proteases, BACE1 has several N-linked glycosylation sites and six lumenal cysteine residues that form three intramolecular disulfide bonds.

The expression pattern of BACE1 is consistent with that of β-secretase (Marcinkiewicz and Seidah, 2000; Vassar *et al.*, 1999; Yan *et al.*, 1999). The levels of BACE1 mRNA by Northern analysis are highest in pancreas and brain, and are significantly lower in most other tissues. Moreover, by *in situ* hybridization analysis, BACE1 is highly expressed in neurons but little, if any, is present in glial cells of the brain, as expected for β-secretase. The protein is abundant in both normal human and AD brain (Marcinkiewicz and Seidah, 2000; Vassar *et al.*, 1999). The high pancreatic mRNA expression was initially confusing, given the low levels of β-secretase activity in this tissue (Sinha *et al.*, 1999). However, recent reports indicate that a significant proportion of BACE1 mRNA in the pancreas consists of a splice variant missing the majority of exon 3 (Bodendorf *et al.*, 2001). This splice variant encodes a BACE1 isoform lacking β-secretase activity, thus providing an explanation for the low β-secretase activity found in the pancreas. The functional relevance of this pancreas-specific splice variant is unclear.

When transfected into stable APP-overexpressing cell lines, BACE1 induces a dramatic increase in β-secretase activity (Hussain *et al.*, 1999; Lin *et al.*, 2000; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999). The immediate products of β-secretase cleavage, APPsβ and C99, are increased several fold over levels found in untransfected cells. Aβ production is also elevated by BACE1 transfection in cells overexpressing wild-type APP (APPwt), but (surprisingly) not in cells overexpressing APP with the Swedish mutation (APPsw), even though C99 levels are dramatically increased. Possibly, the very high C99 levels produced by endogenous BACE1 in APPsw cells may saturate γ-secretase and prevent processing of the additional C99 made after BACE1 transfection. Interestingly, APPsα levels are reduced upon BACE1 transfection of APPwt and APPsw cells,

suggesting that α- and β-secretases compete for APP substrate in cells. Contrary to the effects of BACE1 transfection in cells, treatment of APP-overexpressing cells with BACE1 antisense oligonucleotides decreases BACE1 mRNA and inhibits β-secretase activity (Vassar *et al.*, 1999; Yan *et al.*, 1999). BACE1 antisense inhibition reduces production of APPsβ, C99, and Aβ in cells; conversely, APPsα generation is elevated.

BACE1 cleaves APP only at the known β-secretase sites of Asp+1 and Glu+11 of Aβ, as determined by radiosequencing of Aβ and APP C-terminal fragments from APP and BACE1 co-expressing cells (Vassar *et al.*, 1999). Moreover, purified recombinant BACE1 directly cleaves APP substrates at these same sites *in vitro*, demonstrating that the BACE1 molecule intrinsically exhibits protease activity (Vassar *et al.*, 1999; Yan *et al.*, 1999). The sequence specificity of purified BACE1 is the same as β-secretase: it cleaves Swedish mutant APP substrate much more efficiently than wild-type, and does not cleave a P1 Met→Val mutant substrate that is resistant to β-secretase cleavage when expressed in cells. Like β-secretase activity, BACE1 has optimal activity at ~pH 4.5, is resistant to inhibition by pepstatin, and is localized within acidic subcellular compartments of the secretory pathway, primarily the Golgi apparatus and endosomes. Taken as a whole, the properties of BACE1 correlate extremely well with the previously established functional characteristics of β-secretase in cells and tissues.

## 4.   THE HOMOLOGUE BACE2

Soon after the discovery of BACE1, searches of EST databases with the BACE1 sequence identified a homologous novel aspartic protease, BACE2 (also termed Asp1, mempapsin1, and DRAP) (Acquati *et al.*, 2000; Bennett *et al.*, 2000a; Lin *et al.*, 2000; Saunders *et al.*, 1999; Solans *et al.*, 2000; Yan *et al.*, 1999). BACE1 and BACE2 have ~64% amino acid similarity, and both have two aspartic protease active site motifs, six conserved lumenal cysteine residues, a C-terminal transmembrane domain, N-linked glycosylation sites, and other similar structural characteristics. Although BACE1 and BACE2 are most closely related to the pepsin family, they possess features that clearly set them apart from other aspartic proteases. First, BACE1 and BACE2 share only ~40-44% amino acid similarity to individual pepsin family members, while similarity within the pepsin family is significantly higher (~52-69%). Moreover, the positions of only two of the six lumenal cysteine residues of BACE1 and BACE2 are conserved with those of the pepsins. Thus, the disulfide bond structure of BACE1 and BACE2 is very different than that of the pepsin family, and may influence enzyme properties such as stability, activity, or substrate specificity (Haniu

*et al.*, 2000). Most importantly, BACE1 and BACE2 are the only aspartic proteases identified that have a C-terminal extension with a predicted transmembrane domain. Membrane attachment may facilitate intracellular localization or may serve to increase the local concentration of enzyme in the lipid bilayer for the processing of membrane-bound substrates. Taken together, the characteristics of BACE1 and BACE2 define a novel family of transmembrane aspartic proteases distinct from the pepsins and the evolutionarily more ancient retroviral aspartic proteases that include the human immunodeficiency virus (HIV) protease (Bennett *et al.*, 2000a).

The high degree of similarity between BACE1 and BACE2 initially suggested that BACE2 might also function as a β-secretase. The BACE1 gene is localized on chromosome 11q23.3 (Saunders *et al.*, 1999). So far, no mutations in the BACE1 gene have been identified that strongly associate with AD (Murphy *et al.*, 2001), although a weak association between a polymorphism in BACE1 exon5 and AD in individuals carrying an ApoE4 allele has been recently reported (Nowotny *et al.*, 2001). Intriguingly, the BACE2 gene is located in the obligate DS region on chromosome 21 (Saunders *et al.*, 1999). Thus, a third copy of the BACE2 gene (and the APP gene) is present in DS and suggests a potential role for BACE2 in the early-onset AD of DS patients. Indeed, cell transfection studies demonstrate that BACE2 cuts APP at the β-secretase site (Farzan *et al.*, 2000; Hussain *et al.*, 2000; Yan *et al.*, 2001). However, BACE2 cleaves with higher efficiency at two other positions within the Aβ domain near the α-secretase cleavage site, Phe+19 and Phe+20. Interestingly, the Flemish FAD mutation of APP (Ala→Gly at +21 of Aβ) is adjacent to the Phe+20 cleavage site and causes an increase in Aβ production that is mediated by BACE2 but not BACE1 in transfected cells (Farzan *et al.*, 2000). Thus, BACE2 may play a role in the pathogenesis of Flemish FAD. However, BACE2 acts like an alternative α-secretase on wild-type APP, so that APP processing by BACE2 typically reduces Aβ production in cells.

BACE2 mRNA is expressed in most tissues at moderate to low levels, but is nearly undetectable in whole brain by Northern analysis (Bennett *et al.*, 2000a; Marcinkiewicz and Seidah, 2000). At the cellular level, *in situ* hybridization studies reveal an intriguing distribution of BACE2 mRNA in the brain: the message is very low or undetectable in most brain regions, but appears high in neurons of a small number of discrete nuclei including ventromedial hypothalamus, mammilary body, and isolated nuclei of the brain stem (Bennett *et al.*, 2000a). The neuroanatomical pattern of BACE2 mRNA expression does not reveal a clear physiological function for BACE2, but implies a role in the processing of substrates important for these specific neuronal populations. The overall expression pattern of BACE2 mRNA in the brain contrasts markedly with that of BACE1 mRNA, which is highly

expressed in neurons of most brain regions (Vassar *et al.*, 1999). Consequently, the high levels of β-secretase activity found in the brain are inconsistent with the very low levels of cerebral BACE2 mRNA. This observation together with the α-secretase-like cleavage activity of BACE2 argues against a major role for BACE2 in Aβ generation.

## 5.    THE GENERATION AND CHARACTERIZATION OF BACE1 KNOCKOUT MICE

Soon after the discovery of BACE1, a flood of data strongly suggested BACE1 was the authentic β-secretase. However, it was still formally possible that BACE2 or an as yet unidentified enzyme might have β-secretase activity and thus contribute to Aβ generation *in vivo*. To address this issue, it was necessary to disrupt the BACE1 gene (BACE1 knockout) in the mouse in order to unequivocally prove that BACE1 was the bona fide β-secretase in the brain. BACE1 knockout mice were also required to determine whether BACE1 had a vital function *in vivo*, or if it was dispensable. These questions are of critical importance to investigators interested in the therapeutic development of BACE1 inhibitors.

Given the importance of the knockouts, several groups undertook efforts to generate BACE1 deficient mice by gene targeting. Four knockout strategies were used to inactivate the BACE1 gene: 1. deletion of exon1, thus removing the ATG start codon (Cai *et al.*, 2001); 2. insertion of a β-galactosidase reporter gene immediately downstream of the ATG start codon (Roberds *et al.*, 2001); 3. deletion of exon2, thus removing the N-terminal protease active site motif (Luo *et al.*, 2001); 4. deletion of exon4 through exon8, causing the removal of the C-terminal half of the protease domain (Roberds *et al.*, 2001).

All of the knockout strategies produced viable, fertile BACE1 deficient (BACE1$^{-/-}$) mice at the expected Mendelian frequency (Cai *et al.*, 2001; Luo *et al.*, 2001; Roberds *et al.*, 2001). Lack of expression of the BACE1 gene in the knockouts did not appear to adversely affect embryonic development, nor did it significantly affect the morphology, physiology, biochemistry, and behavior of post-natal or adult mice. Detailed analysis of the phenotype of adult BACE1$^{-/-}$ mice revealed no discernable abnormalities in tissues (morphology, weights, histology), hematology, or blood and urine chemistries, as compared to wild-type mice (Luo *et al.*, 2001; Roberds *et al.*, 2001). Since brain expresses high levels of BACE1, brain histology from the knockout mice was closely examined and no microscopic differences with wild-type brain was observed, irrespective of brain region (Luo *et al.*, 2001; Roberds *et al.*, 2001).

To more closely investigate brain function in the knockout mice, Roberds and colleagues (Roberds *et al.*, 2001) investigated gross behavioral and neuromuscular parameters of BACE1$^{-/-}$ mice and found that no demonstrable differences existed, as compared to wild-type mice. For example, BACE1$^{-/-}$ mice exhibit normal locomotor activity, gait, and exploratory behavior, and are neither hyperactive nor sedated. In addition, the knockouts have normal grip strength, righting reflex, geotaxis, eye-blink reflex, and reactions to tactile stimuli. Overall, it is clear from these studies that the absence of BACE1 is well tolerated *in vivo* and does not appear to cause untoward effects in the embryonic, post-natal, or adult mouse.

Importantly, β-secretase activity is abolished in brains and cultured neurons of BACE1$^{-/-}$ mice. Since endogenous Aβ is difficult to detect in the mouse, we mated BACE1$^{-/-}$ mice with Swedish APP-overexpressing transgenic mice (Tg2576) (Hsiao *et al.*, 1996), which produce robust levels of Aβ in the brain and develop cerebral amyloid deposits with age. BACE1$^{-/-}$•Tg2576 bigenic mice were generated, and brain extracts were analyzed for APP soluble ectodomains (APPsα, APPsβ), C-terminal fragments (C83, C99) and Aβ species (total Aβ, Aβ40, Aβ42) (Luo *et al.*, 2001). BACE1$^{-/-}$•Tg2576 mice lacked all forms of Aβ in the brain, as well as APPsβ and C99, as compared to BACE1$^{+/-}$•Tg2576 or BACE1$^{+/+}$•Tg2576 mice. In other words, all products of APP processing by β-secretase, including Aβ, were abolished in BACE1 knockout brain. This result unequivocally proves that BACE1 is the major, if not only, β-secretase responsible for Aβ generation in the brain.

The work of Cai (Cai *et al.*, 2001) and Roberds (Roberds *et al.*, 2001) also demonstrated that BACE1 is the principle β-secretase. Cai and colleagues infected cultures of BACE1$^{-/-}$ embryonic neurons with APP-expressing adenovirus and determined that Aβ and C99 are abolished in these cells by using mass spectrometry and gel electrophoresis analysis, respectively. In BACE1$^{-/-}$ cultures, the absence of Aβ species starting at Phe+19 or Phe+20 (the major sites of BACE2 cleavage) indicated that BACE2 is not significantly involved in APP cleavage in neurons (Cai *et al.*, 2001). In addition, Roberds and colleagues found no measurable Aβ by ELISA or β-secretase activity using an *in vitro* assay in extracts of whole brain or cultured neurons from BACE1$^{-/-}$ mice. Peptide-based statine inhibitors of BACE1 showed essentially the same IC50s for both purified human BACE1 and brain extracts from wild-type mice, demonstrating that mouse and human BACE1 have similar enzymatic properties, as expected (Roberds *et al.*, 2001).

Interestingly, the α-secretase cleavage products APPs α, C83, and p3 were dramatically elevated in BACE1$^{-/-}$•Tg2576 brain, demonstrating a competition between α- and β-secretases for cleavage of APP *in vivo* (Cai *et*

*al.*, 2001; Luo *et al.*, 2001). These results were similar to those obtained with BACE1 antisense inhibition experiments that show an elevation of α-secretase cleavage in BACE1 antisense treated cultured cells (Vassar *et al.*, 1999; Yan *et al.*, 1999).

By co-infecting BACE1$^{-/-}$ neurons with APP and BACE1 adenoviruses, Cai (Cai *et al.*, 2001) determined that β-secretase cleavage at Glu+11 is species-specific. Mass spectrometry analysis revealed that both the Asp+1 and Glu+11 cleavages of APP caused by BACE1 are abolished in BACE1$^{-/-}$ primary neuronal cultures. By co-expressing different combinations of human or mouse BACE1 with human or mouse APP, Cai and colleagues found that the Asp+1 site is always cleaved, regardless of whether the substrate or enzyme comes from man or mouse. In other words, the Asp+1 site shows no BACE1 species selectivity. However, the Glu+11 site is cleaved only when BACE1 enzyme and APP substrate are from the same species. These observations make sense in light of the fact that the sequence surrounding the Asp+1 site is absolutely conserved between mouse and human, while the Glu+11 sequence is divergent (Cai *et al.*, 2001). Moreover, these results suggest that APP and BACE1 have co-evolved within a species to preserve cleavage at Glu+11, although the functional significance of Glu+11 cleavage is unknown. Since Glu+11 is a major site of β-secretase cleavage in neurons, and Glu+11 Aβ appears to be more fibrillogenic and neurotoxic than Asp+1 Aβ *in vitro* (Pike *et al.*, 1995), Aβ species starting at Glu+11 may play a significant, but currently under-appreciated, role in AD pathophysiology.

Recently, we have shown BACE1$^{-/-}$•Tg2576 mice not only lack cerebral Aβ, but also fail to develop amyloid plaques with age (Luo *et al.*, 2003). Tg2576 mice begin to deposit amyloid in the brain at ~9-12 months of age. Conversely, BACE1$^{-/-}$•Tg2576 bigenic mice show no evidence of amyloid deposits even at 13 months of age. Wong and colleagues have also performed experiments similar to our own and have obtained the same result (International Alzheimer's Conference, 2002; Abstract#560). Taken together, these results demonstrate BACE1 is required for amyloid formation. Since BACE1$^{-/-}$•Tg2576 mice have elevated α-secretase cleavage, as judged by increased APPsα and C83, their p3 levels are also likely to be increased. Although it remains to be confirmed that BACE1$^{-/-}$•Tg2576 mice have increased p3 levels, the apparent lack of amyloid deposits in these mice implies that p3 is in fact non-amyloidogenic, as expected. Wong and colleagues are currently investigating whether partial inhibition of BACE1, as occurs in heterozygous BACE1$^{+/-}$ mice, significantly delays the on-set of amyloid formation in BACE1$^{+/-}$•APP mice. If so, it implies that only partial (~50%) therapeutic inhibition of BACE1 may be sufficient to delay AD pathogenesis in human patients.

As a whole, the results of the BACE1 knockout experiments conclusively demonstrate that BACE1 is the major, if not only, β-secretase *in vivo*. Therefore, BACE2 and other proteases can be excluded from serious consideration as β-secretase candidates (with the possible exception of a role for BACE2 in Flemish FAD; (Farzan *et al.*, 2000)). The normal appearing phenotype of BACE1$^{-/-}$ mice indicates that BACE1 is dispensable for normal development and physiological functions *in vivo*. In contrast, the ablation of the PS1 gene, which is required for γ-secretase function, causes embryonic lethality. This suggests the possibility that, unlike γ-secretase inhibitors, BACE1 inhibitors may not be associated with mechanism-based toxicity in human beings. Finally, the lack of Aβ generation in the brains of BACE1 deficient mice indicates that therapeutic inhibition of BACE1 should reduce Aβ levels and amyloid development, an outcome widely believed to be beneficial for the treatment of AD.

## 6.    BACE1 OVEREXPRESSING TRANSGENIC MICE

Recently, transgenic mice that overexpress BACE1 in the brain have been generated (Bodendorf *et al.*, 2002). The rationale for these experiments was 1.) to determine if elevated BACE1 expression would increase the steady-state levels of Aβ in the brain, and 2.) to test whether increased steady-state levels of Aβ caused by BACE1 overexpression could accelerate amyloid deposition. The transgenic mice of Bodendorf and colleagues overexpress human BACE1 driven by the neuron-specific mouse Thy1 promoter. Both single BACE1 transgenics and mice bigenic for both BACE1 and APP transgenes show a significant increase in the steady state levels of all APP cleavage products made by β-secretase (APPsβ, C99, C89, Aβ40 and Aβ42), but increases are relatively modest (~1.5-2 fold) probably due to high levels of clearance in the rodent brain. Levels of the APP metabolites C99, APPsβ, and Aβ all show a similar increase in BACE1 transgenic brain, indicating a direct relationship between amyloidogenic processing of APP and an increase in Aβ. This suggests that β-secretase cleavage of APP by BACE1, rather than γ-secretase cleavage, is the rate-limiting step in the production of Aβ *in vivo*.

In cultured cells, BACE1 not only cleaves full-length APP, but also cuts C99 at Glu+11 to produce C89 (Farzan *et al.*, 2000). To explore whether this occurs *in vivo*, Bodendorf (Bodendorf *et al.*, 2002) compared the steady-state levels of C89 and C99 in transgenic mice expressing matched levels of either wild-type APP (APPwt) or APP with the Swedish mutation (APPsw). As expected, since APPsw is a better substrate for BACE1 than APPwt, the APPsw mice expressed about 4 fold more C99 than the APPwt mice.

Interestingly, the levels of C89 were the same in both APPsw and APPwt mouse brains. Since C89 levels were not increased in APPsw mice, the authors concluded that, in contrast to the results observed in cell culture, *in vivo* C89 is not made by BACE1 cleavage of C99, but rather C89 is generated directly from full-length APP. Finally, the study by Bodendorf and colleagues found no evidence for the species-specificity of the Glu+11 cleavage, again in contrast to cell culture experiments (Cai *et al.*, 2001). Clearly, the resolution of the discrepancies surrounding Glu+11 generation and species-specificity must await further investigation.

Thus far, it has not yet been reported whether BACE1 overexpression accelerates amyloid deposition in APP transgenic mice, analogous to the effects of mutant PS1 transgenes. These experiments will provide insight into the potential role of BACE1 overexpression in humans as a pathophysiological initiator or accelerant during AD. In the future, it will be useful to introduce a human BACE1 transgene into BACE1 knockout mice in order to test the specificity and efficacy of BACE1 inhibitors *in vivo*, thus preventing any confounding effects from endogenous mouse BACE1. In addition, testing BACE1 inhibitors in BACE1$^{-/-}$ mice will also be informative for investigating possible non-specific side effects that are not directly due to inhibition of BACE1.

## 7. BACE1 X-RAY STRUCTURE

Structural information about the interaction of substrate with the active site of BACE1 would greatly facilitate the rational design of small molecule BACE1 inhibitors. Toward this end, Sauder et al. (Sauder *et al.*, 2000) used molecular modeling to simulate the BACE1 active site bound with wild-type or mutant APP substrates. Since the basic structure of most aspartic protease active sites is well conserved, the X-ray structure of pepsin was used to model BACE1. X-ray structural information of a peptide inhibitor bound to rhizopuspepsin was also incorporated to model the interaction with APP. The molecular modeling identified several residues in BACE1 that potentially contribute to substrate specificity. In particular, Arg296 forms a salt-bridge with the P1' Asp+1 residue of the β-secretase cleavage site, thus explaining the unusual preference of BACE1 among aspartic proteases for substrates that are negatively charged at this position. In addition, several hydrophobic residues in BACE1 form a pocket for the hydrophobic P1 residue. The model also showed that the Swedish FAD mutation, LysMet→AsnLeu at P2-P1, interacts more favorably with Agr296 and the hydrophobic pocket of BACE1 than does wild-type substrate, providing an explanation for the enhanced cleavage of this mutation. Conversely, the

substitution of Met→Val at P1 blocks the catalytic Asp93 residue, explaining the lack of cleavage of this mutation by BACE1.

Shortly after the molecular modeling study, the X-ray structure of the BACE1 protease domain co-crystalized with a transition-state inhibitor was determined to 1.9 Å resolution (Hong *et al.*, 2000). As expected, the BACE1 catalytic domain is similar in structure to pepsin and other aspartic proteases, despite the relatively low sequence similarity. Interestingly, the BACE1 active site is more open and less hydrophobic than that of other aspartic proteases. Four hydrogen bonds from the catalytic aspartic acid residues (Asp93 and Asp289) and ten additional hydrogen bonds from various residues in the active site are made with the inhibitor, most of which are conserved in other aspartic proteases. The X-ray structure indicates that Arg296 and the hydrophobic pocket of the active site play an important role in substrate binding, confirming the results of the molecular modeling study. In addition, the bound inhibitor has an unusual kinked conformation from P2' to P4'. The BACE1 X-ray structure suggests that small molecules targeting Arg296 and the hydrophobic pocket residues should inhibit β-secretase cleavage. Moreover, mimicking the unique P2'-P4' conformation of the bound inhibitor may increase the selectivity of inhibitors for BACE1 over BACE2 and the other aspartic proteases.

## 8.    BACE1 INHIBITOR DEVELOPMENT

The challenges facing BACE1 inhibitor development are significant, but not insurmountable. Two transition-state analog inhibitors of BACE1 modeled on the β-secretase cleavage site of the Swedish mutation have been reported with relatively low IC50 concentrations. The first inhibitor, P10-P4'StatVal, contains Asn at P2 (like the Swedish mutation), a statine group at P1, and Val at P1' and has an IC50 of ~30nM (Sinha *et al.*, 1999). The second, called OM99-2, has an IC50 of ~1.6nM and is P4-P4' with AsnLeu at P2-P1, Ala at P1', and a hydroxyethylene isostere between P1 and P1' (Ghosh *et al.*, 2000). The former inhibitor was used as the affinity ligand for the purification of BACE1 protein from human brain, and the later inhibitor was co-crystallized with BACE1 for the X-ray structure determination. Enzyme inhibitors with therapeutic potential are preferably smaller than 700 daltons and have low nanomolar IC50 concentrations or better, so these large peptide-based inhibitors are not viable drug candidates. However, they are starting points for rational drug design efforts and are useful reagents for studying the enzymatic properties of BACE1. For example, one group has recently used P10-P4'StatVal to study the kinetics of BACE1 inhibition (Marcinkeviciene *et al.*, 2001).  They concluded that inhibition involves a

two-step process: a fast initial association between inhibitor and enzyme, followed by a slower "tightening up" of the complex due to conformational changes in the flap region and displacement of the catalytic water in the active site of BACE1. Such studies enhance our mechanistic understanding of BACE1 inhibition and provide the foundation for further advances in BACE1 inhibitor development.

Several other factors must be taken into consideration for the development of a viable BACE1 drug candidate: 1. in addition to small size, high potency, and low toxicity, the candidate should exhibit favorable pharmacokinetic properties; 2. the inhibitor must possess sufficient lipophilicity to a) efficiently cross the blood-brain barrier and achieve high concentrations in the brain, and b) to traverse two lipid bilayers and reach BACE1 localized in the TGN/endosomal lumen (although the periodic cycling of BACE1 to the cell surface may facilitate enzyme-inhibitor binding); 4. since it is not yet known whether BACE2 is dispensable *in vivo*, it may be necessary to design BACE1-selective drugs that exhibit minimal cross-inhibition of BACE2 and other aspartic proteases. Regarding this latter point, the high degree of homology between BACE1 and BACE2 suggests that the active sites of the two proteases are quite similar, thus making the design of BACE1-selective drugs potentially difficult.

## 9.    SOLUBLE Aβ OLIGOMERS AS PATHOGENIC AGENTS IN AD

The amyloid plaque is a deposit in the parenchyma of the brain composed mainly of insoluble fibrils of the Aβ peptide (reviewed in Selkoe, 2001). The more fibrillogenic Aβ42 is the predominant species of Aβ in amyloid plaques, and is increased in production in autosomal dominant forms of FAD (see Section 1). Given these associations, and the fact that Aβ fibrils appear neurotoxic *in vitro*, it was postulated that amyloid plaques are pathogenic and cause neurodegeneration in AD, as stated in the original amyloid cascade hypothesis (reviewed in Hardy and Selkoe, 2002). However, direct evidence that amyloid plaques are pathogenic *in vivo* has been difficult to obtain. Transgenic mice that overexpress APP with FAD mutations have elevated cerebral Aβ levels and develop amyloid deposits with age (reviewed in Hsiao Ashe, 2001). APP transgenics have many of the neuropathological characteristics of AD, including neurodegenerative changes, and they exhibit memory deficits. On the other hand, the mice do not develop neurofibrillary tangles, nor do they have significant neuronal loss, both important features of AD. Moreover, plaque number poorly correlates with severity of dementia in AD (Terry *et al.*, 1999). Although the

total amount of Aβ in the brain (Aβ load) directly correlates with dementia (Cummings and Cotman, 1995; Parvathy *et al.*, 2001), the fact that plaque number does not correlate, together with the incomplete recapitulation of AD pathology in APP transgenic mice, calls into question the role of amyloid plaques in AD pathogenesis.

Recently, research has begun to focus on soluble, non-fibrillar oligomeric forms of Aβ as potential toxic agents in AD. The first indication that soluble forms of Aβ may play a role in AD came from Roher and colleagues who determined that the amount of water-soluble Aβ in AD brain tissue was significantly greater than in control tissue (Kuo *et al.*, 1996). Interestingly, evidence suggests amyloid deposits in APP transgenic mice are dynamic and can increase in size or be cleared over time (Bacskai *et al.*, 2001). These results imply that Aβ can make the transition from soluble (oligomer) to insoluble (plaque) form in a reversible fashion. Given the high concentrations of soluble Aβ in AD brain, and that it may diffuse large distances through the parenchyma, soluble Aβ may have great neurotoxic potential.

Next, studies investigated whether soluble Aβ was toxic to neurons, causing neuronal dysfunction and neurodegenerative changes (reviewed in (Klein *et al.*, 2001)). Aβ derived diffusible ligands (ADDLs) (Lambert *et al.*, 1998) and protofibrils (Hartley *et al.*, 1999) are non-fibrillar Aβ assemblies that are soluble in physiological solutions. Although the precise structures of ADDLs and protofibrils are still under investigation, gel electrophoresis and atomic force microscopy indicates they are small oligomers probably composed of less than ~10 Aβ molecules in ADDLs and perhaps a few dozen Aβ molecules in protofibrils (*see also* Chapter 1). Preparations of these Aβ assemblies are potent neurotoxins and kill neurons at nanomolar concentrations *in vitro* (Hartley *et al.*, 1999; Lambert *et al.*, 1998). Interestingly, ADDLs and protofibrils disrupt neuronal physiology including long-term potentiation, a cellular correlate of memory and learning, in hippocampal slices. Thus, soluble Aβ oligomers appear toxic to neurons and interfere with physiological mechanisms of memory that are likely to be important in AD.

Evidence that soluble Aβ oligomers may be toxic to neurons *in vivo* and could impair memory has begun to emerge recently. APP transgenic mice develop memory deficits prior to the formation of amyloid deposits, implying that increased cerebral levels of soluble Aβ disrupt memory function (Hsiao Ashe, 2001). Moreover, studies with these APP transgenic mice have demonstrated that anti-Aβ antibodies, either generated endogenously by Aβ vaccination (Schenk *et al.*, 1999) or administered exogenously by peripheral injection (Bard *et al.*, 2000), reduce amyloid deposition and Aβ levels in the brain. Aβ-immunized APP transgenic mice

also show improved performance in memory tests as compared to non-immunized transgenics (Janus *et al.*, 2000; Morgan *et al.*, 2000). Passive immunization of APP transgenics with anti-Aβ monoclonal antibodies demonstrated rapid memory improvement (Dodart *et al.*, 2002; Kotilinek *et al.*, 2002), in one study occurring as early as 24 hours after a single peripheral injection of antibody (Dodart *et al.*, 2002). Since amyloid burden was unaffected by passive immunization in these studies, the authors concluded that some form of soluble Aβ (rather than the amyloid deposit) was likely to cause memory deficits in the APP transgenic mice and in AD. Although the underlying mechanisms of memory rescue in these cases are unknown, presumably antibody-mediated sequestration and clearance of soluble Aβ in the brain is responsible.

Finally, we have recently conducted studies to determine whether BACE1 deficiency, and the consequent ablation of Aβ, is sufficient to rescue memory deficits in Tg2576 APP transgenic mice (Ohno *et al.*, 2004). Since Tg2576 mice develop memory impairments at an early age before the onset of amyloid deposition (Hsiao Ashe, 2001), we chose to study young pre-deposit mice to analyze the contribution of soluble Aβ to memory dysfunction. Our work demonstrated that memory deficits and cholinergic dysfunction in the hippocampus did not develop in BACE1$^{-/-}$•Tg2576 bigenic mice that lacked Aβ, while florid deficits were apparent in Aβ-overproducing Tg2576 monogenics. Because the Aβ in Tg2576 at the time of testing was non-fibrillar and soluble, we concluded that soluble Aβ assemblies rather than amyloid plaques are responsible for at least some aspects of AD-related memory deficits. Moreover, our work is further validation of BACE1 as a prime therapeutic target for AD.

To put these studies into perspective, it was recently discovered that soluble Aβ potentially has a normal function as a negative regulator of excitatory synaptic transmission (Kamenetz *et al.*, 2003). Upon electrical stimulation of glutaminergic pathways in hippocampal slices, presynaptic terminals release Aβ, which then inhibits postsynaptic neurons from further depolarization. Such a mechanism may be important for "putting on the brakes" on excitatory transmission in order to protect against over-stimulation under certain conditions. Most importantly, these results convincingly suggest a normal function for soluble Aβ and indicate that cerebral Aβ levels may need to be maintained within a narrow margin, or else memory function may become disrupted.

Taken together, the evidence suggests that soluble Aβ oligomers are an important pathogenic agent in AD. However, significant work remains before we can confidently conclude that soluble Aβ is at the root of AD etiology. In fact, amyloid plaques are also likely to contribute to pathogenicity in AD, since clear signs of inflammation and

neurodegeneration such as dystrophic neurites are increased in the immediate vicinity of amyloid deposits in both AD and APP transgenic mice (Selkoe, 2001). In the end, it is likely that both soluble Aβ oligomers and amyloid plaques are involved in the pathophysiology and progression of AD.

## 10.    CONCLUSIONS

A large body of evidence has demonstrated that BACE1 is the authentic β-secretase. Yet, these are still early days in the study of BACE1 (and BACE2) and its potential as a drug target in AD. The lack of Aβ production and amyloid deposition in BACE1 deficient mice clearly validates BACE1 as the β-secretase and indicates that BACE1 inhibitors should reduce cerebral Aβ levels, but questions remain. What percentage of BACE1 inhibition is required to significantly delay amyloid deposition? In addition, does the other β-secretase cleavage product, Glu+11 Aβ, have any role in amyloid deposition? These questions have important implications for both AD pathogenesis and therapeutic development and thus require further investigation.

Despite progress in characterizing the molecular and cellular properties of BACE1 and BACE2, little is known about the substrates (other than APP) and the biological functions of these two proteases. Recent results suggest that a Golgi-resident sialyltransferase, ST6Gal I (Kitazume *et al.*, 2001), and P-selectin glycoprotein ligand-1 (Lichtenthaler *et al.*, 2003) are BACE1 substrates, however the functional significance of these data is unknown and further work in this area is required. Although the apparently normal phenotype of BACE1 knockout mice is uninformative regarding BACE1 function *in vivo*, it is possible that subtle effects of BACE1 deficiency may be revealed with challenge. Phenotypes uncovered by different challenges or stresses could provide a clue to the biological function of BACE1 and would guide predictions regarding potential side effects of BACE1 inhibitors in humans under certain conditions.

Regarding BACE2 function, the intriguing pattern of BACE2 expression in the brain suggests an important role for BACE2 in specific neuronal subpopulations. The generation of BACE2 knockout mice will be instrumental for addressing this question. BACE1 and BACE2 are highly homologous and have expression patterns that partially overlap, implying that these enzymes may be functionally redundant in some tissues. The analysis of $BACE1^{-/-} \cdot BACE2^{-/-}$ compound knockout mice could provide insight into this issue. Clearly, identification of other substrates for BACE1 and BACE2 will vastly increase our understanding of the functional role of this novel family of transmembrane aspartic proteases.

To date, no mutations in the BACE1 gene have been identified that definitively associate with AD. Still, it is possible that mutations in the BACE1 gene could increase the risk of AD by elevating either BACE1 gene expression or enzyme activity. Such BACE1 mutations would be expected to increase the production of Aβ, thus potentially contributing to AD pathogenesis. Increasing the level of Aβ by 50% is enough to cause early on-set AD in DS, and it is possible that even much smaller Aβ increases may have profound effects over time to cause some forms of late-onset AD. In this light, it is interesting to note that BACE1 levels appear to be increased by ~2.7 fold or more in the brains of at least some late-onset AD cases, as compared to age-matched controls, and that C99 levels were ~2 fold higher (Holsinger *et al.*, 2002). A recently identified polymorphism in exon5 of the BACE1 gene has been found to weakly associate with AD (Nowotny *et al.*, 2001) and one may speculate that such mutations may increase BACE1 and Aβ levels, although currently there is no evidence to support this hypothesis. In any case, further analysis of BACE1 protein levels and BACE1 gene sequences from AD patients is warranted and may eventually reveal mutations that increase the risk of AD.

Finally, as the key enzyme that initiates Aβ formation *in vivo*, BACE1 is a prime drug target for inhibiting the production of Aβ. Regardless of whether soluble or fibrillar Aβ is the pathogenic agent in AD, therapeutic inhibition of BACE1 is expected to lower cerebral Aβ levels, which should prove beneficial for AD. Although the creation of pharmaceutically viable BACE1 inhibitors will be challenging, it is likely that BACE1 drugs will be developed with time. Drugs that inhibit other therapeutically important aspartic proteases such as renin and the HIV protease have been successfully developed, and these drugs provide paradigms for the rational design of BACE1 inhibitors for the treatment of AD.

## ACKNOWLEDGEMENTS

# REFERENCES

Acquati, F., Accarino, M., Nucci, C., Fumagalli, P., Jovine, L., Ottolenghi, S., and Taramelli, R., 2000, The gene encoding DRAP (BACE2), a glycosylated transmembrane protein of the aspartic protease family, maps to the Down'scritical region. *FEBS Lett.* **468**: 59-64.

Bacskai, B.J., Kajdasz, S.T., Christie, R.H., Carter, C., Games, D., Seubert, P., Schenk, D., and Hyman, B.T., 2001, Imaging of amyloid-beta deposits in brains of living mice permits direct observation of clearance of plaques with immunotherapy. *Nat Med.*, **7**: 369-372.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D., and Yednock, T., 2000, Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat. Med.* **6**: 916-919.

Bennett, B.D., Babu-Khan, S., Loeloff, R., Louis, J.-C., Curran, E., Citron, M., and Vassar, R., 2000a, Expression analysis of BACE2 in brain and peripheral tissues. *J. Biol. Chem.* **275**: 20647-20651.

Bennett, B.D., Denis, P., Haniu, M., Teplow, D.B., Kahn, S., Louis, J.-C., Citron, M., and Vassar, R., 2000b, A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's β-secretase. *J. Biol. Chem.* **275**: 37712-37717.

Bodendorf, U., Danner, S., Fischer, F., Stefani, M., Sturchler-Pierrat, C., Wiederhold, K.H., Staufenbiel, M., and Paganetti, P., 2002, Expression of human β-secretase in the mouse brain increases the steady-state level of β-amyloid. *J. Neurochem.*, **80**: 799-806.

Bodendorf, U., Fischer, F., Bodian, D., Multhaup, G., and Paganetti, P., 2001, A splice variant of β-secretase deficient in the amyloidogenic processing of the amyloid precursor protein. *J. Biol. Chem.* **276**: 12019-12023.

Buxbaum, J.D., Liu, K.N., Luo, Y., Slack, J.L., Stocking, K.L., Peschon, J.J., Johnson, R.S., Castner, B.J., Cerretti, D.P., and Black, R.A., 1998, Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. *J. Biol. Chem.* **273**: 27765-27767.

Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D.R., Price, D.L., and Wong, P.C., 2001, BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nature Neurosci.* **4**: 233-234..

Citron, M., Diehl, T.S., Capell, A., Haass, C., Teplow, D.B., and Selkoe, D.J., 1996, Inhibition of amyloid ß-protein production in neural cells by the serine protease inhibitor AEBSF. *Neuron* **17**: 171-179.

Citron, M., Teplow, D.B. and Selkoe, D.J., 1995, Generation of amyloid β-protein from its precursor is sequence specific. *Neuron* **14**: 661-670.

Cummings, B.J. and Cotman, C.W., 1995, Image analysis of beta-amyloid load in Alzheimer's disease and relation to dementia severity. *Lancet* **346**: 1524-1528.

Dodart, J.C., Bales, K.R., Gannon, K.S., Greene, S.J., DeMattos, R.B., Mathis, C., DeLong, C.A., Wu, S., Wu, X., Holtzman, D.M., and Paul, S.M., 2002, Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. *Nat. Neurosci.* **5**: 452-457.

Farzan, M., Schnitzler, C.E., Vasilieva, N., Leung, D., and Choe, H., 2000, BACE2, a β-secretase homolog, cleaves at the β site and within the amyloid-β region of the amyloid-β precursor protein. *Proc. Natl. Acad. Sci. USA* **97**: 9712-9717.

Francis, R., McGrath, G., Zhang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C., Parks, A.L., Xu, W., Li, J., Gurney, M., Myers, R.L., Himes, C.S., Hiebsch, R., Ruble, C., Nye, J.S., and Curtis, D., 2002, aph-1 and pen-2 are required for

Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. *Dev. Cell* **3**: 85-97.

Ghosh, A.K., Shin, D., Downs, D., Koelsch, G., Lin, X., Ermolieff, J., and Tang, J., 2000, Design of potent inhibitors for human brain memapsin 2 (β-secretase). *J. Am. Chem. Soc.* **122**: 3522-3523.

Glenner, G.G., and Wong, C.W., 1984, Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**: 885-890.

Gouras, G.K., Xu, H., Jovanovic, J.N., Buxbaum, J.D., Wang, R., Greengard, P., Relkin, N.R., and S., G., 1998, Generation and regulation of β-amyloid peptide variants by neurons. *J. Neurochem.* **71**: 1920-1925.

Haass, C., Capell, A., Citron, M., Teplow, D.B., and Selkoe, D.J., 1995a, The vacuolar H+ ATPase inhibitor bafilomycin A1 differentially affects proteolytic processing of mutant and wild-type β-amyloid precursor protein. *J. Biol. Chem.* **270**: 6186-6192.

Haass, C., Hung, A.Y., Schlossmacher, M.G., Teplow, D.B., and Selkoe, D.J., 1993, β-amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J. Biol. Chem.* **268**: 3021-3024.

Haass, C., Lemere, C.A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D.J., 1995b, The Swedish mutation causes early-onset Alzheimer's disease by β-secretase cleavage within the secretory pathway. *Nature Med.* **1**: 1291-1296.

Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J., 1992, Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature* **359**: 322-325.

Haniu, M., Denis, P., Young, Y., Mendiaz, E.A., Fuller, J., Hui, J.O., Bennett, B.D., Kahn, S., Ross, S., Burgess, T., Katta, V., Rogers, G., Vassar, R, and Citron, M., 2000, Characterization of Alzheimer's β-secretase protein BACE. *J. Biol. Chem.* **275**: 21099-21106.

Hardy, J., and Selkoe, D.J., 2002, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**: 353-356.

Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vasquez, S., Vassilev, P.M., Teplow, D.B., and Selkoe, D.J., 1999, Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* **19**: 8876-8884.

Holsinger, R.M.D., McLean, C.A., Beyreuther, K., Masters, C.L., and Evin, G., 2002, Increased expression of the amyloid precursor β-secretase in Alzheimer's disease. *Ann. Neurol.* **51**: 783-786.

Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A.K., Zhang, X.C., and Tang, J., 2000, Structure of the protease domain of memapsin 2 (β-secretase) complexed with inhibitor. *Science* **290**: 150-153.

Hsiao Ashe, K., 2001, Learning and Memory in Transgenic Mice Modeling Alzheimer's Disease. *Learn. Mem.* **8**: 301-308.

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G., 1996, Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. *Science* **274**: 99-103.

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M., Smith, T.S., Simmons, D.L., Walsh, F.S., Dingwall, C., and Christie, G., 1999, Identification of a novel aspartic protease (Asp 2) as β-Secretase. *Mol. Cell. Neurosci.* **14**: 419-427.

Hussain, I., Powell, D.J., Howlett, D.R., Chapman, G.A., Gilmour, L., Murdock, P.R., Tew, D.G., Meek, T.D., Chapman, C., Schneider, K., Ratcliffe, S.J., Tattersall, D., Testa, T.T., Southan, C., Ryan, D.M., Simmons, D.L., Walsh, F.S., Dingwall, C., and Christie, G., 2000, ASP1 (BACE2) cleaves the amyloid precursor protein at the β-secretase site. *Mol. Cell. Neurosci.* **16**: 609-619.

Hutton, M., Perez-Tur, J., and Hardy, J., 1998, Genetics of Alzheimer's disease. *Essays Biochem.* 33: 117-131.

Janus, C., Pearson, J., McLaurin, J., Mathews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., Mount, H.T., Nixon, R.A., Mercken, M., Bergeron, C., Fraser, P.E., St George-Hyslop, P., and Westaway, D., 2000, Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* **408**: 979-982.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S. and Malinow, R., 2003, APP processing and synaptic function. *Neuron* **37**: 925-937.,

Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B., 1987, The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**: 733-736.

Kitazume, S., Tachida, Y., Oka, R., Shirotani, K., Saido, T.C., and Hashimoto, Y., 2001, Alzheimer's β-secretase, β-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase. *Proc. Natl. Acad. Sci. USA* **98**: 13554-13559.

Klein, W.L., Krafft, G.A., and Finch, C.E., 2001, Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci.* **24**: 219-224.

Knops, J., Suomensaari, S., Lee, M., McConlogue, L., Seubert, P., and Sinha, S., 1995, Cell-type and amyloid precursor protein-type specific inhibition of Aβ release by bafilomycin A1, a selective inhibitor of vacuolar ATPases. *J. Biol. Chem.* **270**: 2419-2422.

Koo, E.H., and Squazzo, S., 1994, Evidence that production and release of amyloid β-protein involves the endocytic pathway. *J. Biol. Chem.* **269**: 17386-17389.

Kotilinek, L.A., Bacskai, B., Westerman, M., Kawarabayashi, T., Younkin, L., Hyman, B.T., Younkin, S., and Ashe, K.H., 2002, Reversible memory loss in a mouse transgenic model of Alzheimer's disease. *J. Neurosci.* **22**: 6331-6335.

Kuo, Y.M., Emmerling, M.R., Vigo-Pelfrey, C., Kasunic, T.C., Kirkpatrick, J.B., Murdoch, G.H., Ball, M.J., and Roher, A.E., 1996, Water-soluble Aβ (N-40, N-42) oligomers in normal and Alzheimer disease brains. *J. Biol. Chem.* **271**: 4077-4081.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., and Klein, W.L., 1998, Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. U S A* **95**: 6448-6453.

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., and Fahrenholz, F., 1999, Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc. Natl. Acad. Sci. U S A* **96**: 3922-3927.

Lee, V.M.-Y., Balin, B.J., Otvos, L. and Trojanowski, J.Q., 1991, A major subunit of paired helical filaments and derivatized forms of normal tau. *Science* **251**: 675-678.

Lichtenthaler, S.F., Dominguez, D.I., Westmeyer, G.G., Reiss, K., Haass, C., Saftig, P., De Strooper, B. and Seed, B., 2003, The cell adhesion protein P-selectin glycoprotein ligand-1 is a substrate for the aspartyl protease BACE1. *J. Biol. Chem.* **278**: 48713-48717.

Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J., 2000, Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc. Natl. Acad. Sci. USA* **97**: 1456-1460.

Luo, Y., Bolon, B., Damore, M.A., Fitzpatrick, D., Liu, H., Zhang, J., Yan, Q., Vassar, R., and Citron, M., 2003, BACE1 (β-secretase) knockout mice do not acquire compensatory gene expression changes or develop neural lesions over time. *Neurobiol. Dis.* **4**: 81-88.

Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J.-C., Yan, Q., Richards, W.G., Citron, M., and Vassar, R., 2001, Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nature Neurosci.* **4**: 231-232.

Marcinkeviciene, J., Luo, Y., Graciani, N.R., Combs, A.P., and Copeland, R.A., 2001, Mechanism of inhibition of β-site amyloid precursor protein-cleaving enzyme (BACE) by a statine-based peptide. *J. Biol. Chem.* **276**: 23790-23794.

Marcinkiewicz, M., and Seidah, N.G., 2000, Coordinated expression of β-amyloid precursor protein and the putative β-secretase BACE and α-secretase ADAM10 in mouse and human brain. *J. Neurochem.* **75**: 2133-2143.

Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M., and Arendash, G.W., 2000, Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* **408**: 982-985.

Mullan, M., Crawford, F., Houlden, H., Axelman, K., Lilius, L., Winblad, B.,and Lannfelt, L., 1992a, A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β-amyloid. *Nat. Genet.* **1**: 345-347.

Murphy, T., Yip, A., Brayne, C., Easton, D., Evans, J.G., Xuereb, J., Cairns, N., Esiri, M.M., and Rubinsztein, D.C., 2001, The BACE gene: genomic structure and candidate gene study in late-onset Alzheimer's disease. *Neuroreport* **12**: 631-634.

Nowotny, P., Kwon, J.M., Chakraverty, S., Nowotny, V., Morris, J.C., and Goate, A.M., 2001, Association studies using novel polymorphisms in BACE1 and BACE2. *Neuroreport* **12**: 1799-1802.

Ohno, M., Sametsky, E.A., Younkin, L.H., Oakley, H., Younkin, S.G., Citron, M., Vassar, R., and Disterhoft, J.F., 2004, BACE1 Deficiency Rescues Memory Deficits and Cholinergic Dysfunction in a Mouse Model of Alzheimer's Disease. *Neuron* **41**: 27-33.

Parvathy, S., Davies, P., Haroutunian, V., Purohit, D.P., Davis, K.L., Mohs, R.C., Park, H., Moran, T.M., Chan, J.Y., and Buxbaum, J.D., 2001, Correlation between Abetax-40-, Abetax-42-, and Abetax-43-containing amyloid plaques and cognitive decline. *Arch. Neurol.* **58**: 2025-2032.

Pike, C.J., Overman, M.J., and Cotman, C.W., 1995, Amino-terminal deletions enhance aggregation of β-amyloid peptides in vitro. *J. Biol. Chem.* **270**: 23895-23898.

Roberds, S.L., Anderson, J., Basi, G., Bienkowski, M.J., Branstetter, D.G., Chen, K.S., Freedman, S.B., Frigon, N.L., Games, D., Hu, K., Johnson-Wood, K., Kappenman, K.E., Kawabe, T.T., Kola, I., Kuehn, R., Lee, M., Liu, W., Motter, R., Nichols, N.F., Power, M., Robertson, D.W., Schenk, D., Schoor, M., Shopp, G.M., Shuck, M.E., Sinha, S., Svensson, K.A., Tatsuno, G., Tintrup, H., Wijsman, J., Wright, S., and McConlogue, L., 2001, BACE knockout mice are healthy despite lacking the primary β-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum. Mol. Genet.* **10**: 1317-1324.

Roher, A.E., Lowenson, J.D., Clarke, S., Wolkow, C., Wang, R., Cotter, R.J., Reardon, I., Zurcher-Neely, H.A., Heinrikson, R.L., Ball, M.J., and Greenberg, B.D., 1993, Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.* **268**: 3072-3083.

Sauder, J.M., Arthur, J.W., and Dunbrack, R.L., Jr., 2000, Modeling of substrate specificity of the Alzheimer's disease amyloid precursor protein β-secretase. *J. Mol. Biol.* **300**: 241-248

Saunders, A.J., Kim, T.-W., Tanzi, R.E., Fan, W., Bennett, B.D., Babu-Khan, S., Luo, Y., Louis, J.-C., McCaleb, M., Citron, M., Vassar, R., and Richards, W.G., 1999, *BACE* maps

to chromosome 11 and a BACE homolog, *BACE2*, reside in the obligate Down'ssyndrome region of chromosome 21. *Science* **286**: 1255a.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D., and Seubert, P., 1999, Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**: 173-177.

Selkoe, D.J., 2001, Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* **81**: 741-766.

Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomqist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., Lieberburg, I., and Schenk, D.B., 1993, Secretion of β-amyloid precursor protein cleaved at the amino-terminus of the β-amyloid peptide. *Nature* **361**: 260-263.

Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S.M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V., 1999, Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature* **402**: 537-540.

Sisodia, S.S., Kim, S.H., and Thinakaran, G., 1999, Function and dysfunction of the presenilins. *Am. J. Hum. Genet.* **65**: 7-12.

Solans, A., Estivill, X., and de La Luna, S., 2000, A new aspartyl protease on 21q22.3, BACE2, is highly similar to Alzheimer's amyloid precursor protein β-secretase. *Cytogenet. Cell Genet.* **89**: 177-184.

Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the gamma-secretase complex. *Nature* **422**: 438-441.

Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A.B., St. George-Hyslop, P.H., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M., and Neve, R.L., 1987, Amyloid β-protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science* **235**: 880-884.

Terry, R.D., Masliah, E., and Hansen, L.A., 1999, The neuropathology of Alzheimer disease and the structural basis of its cognitive alterations. In *Alzheimer Disease* (R.D. Terry, R. Katzman, K.L. Bick, and S.S. Sisodia, eds.), Lippincott Williams and Wilkins, Philadelphia, pp. 187-206.

Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.-C., Collins, F., Treanor, J., Rogers, G., and Citron, M., 1999, β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**: 735-741.

Vassar, R., and Citron, M., 2000, Aβ-generating enzymes: recent advances in β- and γ-secretase research. *Neuron* **27**: 419-422.

Wolfe, M.S., Angeles, J.D.L., Miller, D.D., Xia, W., and Selkoe, D.J., 1999a, Are presenilins intramembrane-cleaving proteases? Implications for the molecular mechanism of Alzheimer's disease. *Biochemistry* **38**: 11223-11230.

Wolfe, M.S., Xia, W., Ostaszewski, B.L., Diehl, T.S., Kimberly, W.T., and Selkoe, D.J., 1999b, Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* **398**: 513-517.

Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashler, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E., Carter, D.B., Tomaselli, A.G., Parodi, L.A.,

Heinrikson, R.L., and Gurney, M.E., 1999, Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **402**: 533-537.

Yan, R., Munzner, J.B., Shuck, M.E., and Bienkowski, M.J., 2001, BACE2 functions as an alternative α-secretase in cells. *J. Biol. Chem.* **276**: 34019-34027.

Younkin, S.G., 1998, The role of Abeta 42 in Alzheimer's disease. *J. Physiol. (Paris)* **92**: 289-292.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.S., Holmes, E., Milman, P., Liang, Y., Zhang, D.M., Xu, D.H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L.S., Sorbi, S., Bruni, A., Fraser, P., and St George-Hyslop, P., 2000, Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* **407**: 48-54.

Zhao, J., Paganini, L., Mucke, L., Gordon, M., Refolo, L., Carman, M., Sinha, S., Oltersdorf, T., Lieberburg, I., and McConlogue, L., 1996, β-Secretase processing of the β-amyloid precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. *J. Biol. Chem.* **271**: 31407-31411.