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Identification of a β -Secretase Activity, Which Truncates Amyloid β -Peptide after Its Presenilin-dependent Generation*

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The β -amyloid precursor protein (β APP) is proteolytically processed by two secretase activities to produce the pathogenic amyloid β -peptide ($A\beta$). N-terminal cleavage is mediated by β -secretase (BACE) whereas C-terminal intramembrane cleavage is exerted by the presenilin (PS) γ -secretase complex. The $A\beta$ -generating γ -secretase cleavage principally occurs after amino acid 40 or 42 and results in secretion of $A\beta$ -(1–40) or $A\beta$ -(1–42). Upon overexpression of BACE in cultured cells we unexpectedly noticed a reduction of secreted $A\beta$ -(1–40/42). However, mass spectrometry revealed a truncated $A\beta$ species, which terminates at amino acid 34 ($A\beta$ -(1–34)) suggesting an alternative γ -secretase cut. Indeed, expression of a loss-of-function variant of PS1 inhibited not only the production of $A\beta$ -(1–40) and $A\beta$ -(1–42) but also that of $A\beta$ -(1–34). However, expression levels of BACE correlate with the amount of $A\beta$ -(1–34), and $A\beta$ -(1–34) is produced at the expense of $A\beta$ -(1–40) and $A\beta$ -(1–42). Since this suggested that BACE is involved in a C-terminal truncation of $A\beta$, we incubated purified BACE with $A\beta$ -(1–40) *in vitro*. Under these conditions $A\beta$ -(1–34) was generated. Moreover, when conditioned media containing $A\beta$ -(1–40) and $A\beta$ -(1–42) were incubated with cells expressing a loss-of-function PS1 variant together with BACE, $A\beta$ -(1–34) was efficiently produced *in vivo*. These data demonstrate that an apparently γ -secretase-dependent $A\beta$ derivative is produced after the generation of the non-truncated $A\beta$ via an additional and unexpected activity of BACE.

Because of the increasing mean life expectancy, there is considerable interest in the understanding of the molecular and biochemical mechanisms of age-related diseases. By far the

most frequent age-related neurological disorder is Alzheimer's disease (AD).¹ During the aging process the patients accumulate insoluble amyloid β -peptide ($A\beta$), which is deposited in senile plaques and microvessels in the brain. $A\beta$ is generated by endoproteolytic processing of the β -amyloid precursor protein (β APP), involving β - and γ -secretase (1).

β -secretase (also called BACE; β -site APP-cleaving enzyme) was identified as a membrane-associated aspartyl protease (2–6). BACE mediates the primary amyloidogenic cleavage of β APP and generates a membrane-bound β APP C-terminal fragment (APP CTF β), which is the immediate precursor for the intramembrane γ -secretase cleavage (1). BACE also generates N-terminally truncated $A\beta$ species starting with amino acid 11 of the $A\beta$ domain (2, 7–9). A close homologue (BACE-2) (6, 10–12) can also mediate the typical β -secretase cut although with much lower efficiency (13). BACE-2 rather exhibits an α -secretase-like activity, which cleaves in the middle of the $A\beta$ domain at amino acid 19 and 20 (9, 13, 14). Apparently BACE-2 does not contribute to the amyloidogenic processing of $A\beta$, since the deletion of BACE fully abrogates $A\beta$ generation (15–17).

γ -Secretase activity is associated with a protein complex, composed of presenilins (PS1 or PS2), Nicastrin (Nct), PEN-2, APH-1a, and APH-1b (18–25). The expression of these complex components is coordinately regulated, and γ -secretase activity is only detected in the presence of all subunits (21, 23–25). Removing a single subunit results in the destabilization or reduced maturation of the remaining components (23–26). The catalytic activity is most likely contributed by the PSs (1, 27). PSs are polytopic transmembrane proteins, which together with the signal peptide peptidases and the type-4 prepilin peptidases may belong to a novel family of aspartyl proteases of the GXGD type (for review see Ref. 1). The cleavage of BACE-generated CTF β by γ -secretase results in the secretion of $A\beta$ into biological fluids (1). This cleavage principally occurs after amino acid 40 and 42, the latter being enhanced by numerous familial AD-associated mutations in the PS genes and β APP itself (28). Beside the predominant cleavage after amino acid 40 and 42 slightly shorter peptides have been observed as well, suggesting that the γ -secretase has loose sequence specificity (29). This includes peptides terminating after amino acid 34,

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¹ The abbreviations used are: AD, Alzheimer's disease; $A\beta$, amyloid β -peptide; β APP, β -amyloid precursor protein; PS, presenilin; BACE, β -site APP-cleaving enzyme; sBACE, soluble BACE; wt, wild type; HEK, human embryonic kidney; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

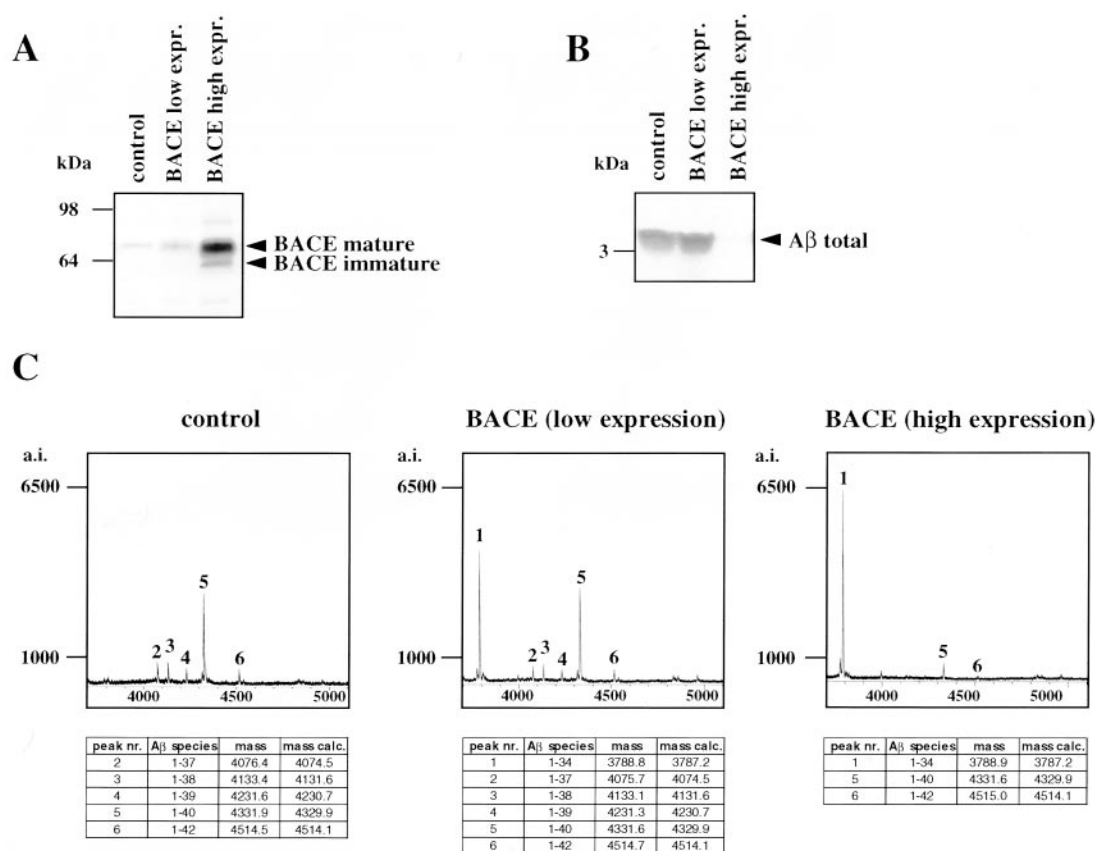


FIG. 1. Production of A β (1-34) correlates with BACE expression. A, HEK 293 cells stably overexpressing β APP₆₉₅ sw (control; lane 1) or β APP₆₉₅ sw and BACE (lanes 2 and 3) were labeled with [³⁵S]methionine. BACE was immunoprecipitated from cell lysates with antibody 7520. B, conditioned media were analyzed for A β accumulation by immunoprecipitation with antibody 3926. C, MALDI-TOF MS of A β peptides immunoprecipitated from conditioned media of the three cell lines with antibody 3926. Arbitrary intensities are given on the y-axis (a.i.). The tables below the spectra indicate the peak masses obtained by mass spectrometry (mass) and the respective calculated masses (mass calc.). Note that the relative levels of A β (1-34) correlate with increasing amounts of BACE expression while other A β species are reduced.

37, 38, and 39 (29). In addition or in parallel to these cleavages, γ -secretase also cleaves within the transmembrane domain shortly before the cytoplasmic border after amino acid 49 to liberate the β APP intracellular domain (AICD) (30-33), which may be involved in nuclear signaling (34, 35). The biological function of γ -secretase is related to the very similar intramembraneous processing of Notch. Indeed, a depletion of PS1 leads to a very severe Notch phenotype (summarized in Ref. 36).

BACE and γ -secretase are obvious targets for therapeutic strategies aimed to inhibit A β generation. Unfortunately γ -secretase inhibitors not only block A β generation but also interfere with Notch signaling (37-39). Therefore treatment of patients with such inhibitors remains problematic. On the other side it has been shown that the gene encoding BACE can be removed without any deleterious effects (15-17). Therefore inhibition of BACE with small chemical compounds seems to be a safer approach for long term treatment. However, a detailed understanding of the cleavage specificity and the substrate specificity of BACE is required for the generation of selective drugs. Surprisingly, overexpression of BACE in cell culture models leads to reduced A β secretion (2). In order to investigate this paradox we analyzed A β peptides secreted from cells stably expressing various levels of BACE and made the surprising observation that BACE can also cleave 34 amino acids C-terminal from its primary cleavage site, thus mimicking a PS-like cleavage specificity.

MATERIALS AND METHODS

Cell Culture, cDNAs, and Transfection—HEK293 were cultured as described (9). The cell lines stably overexpressing wild type β APP₆₉₅

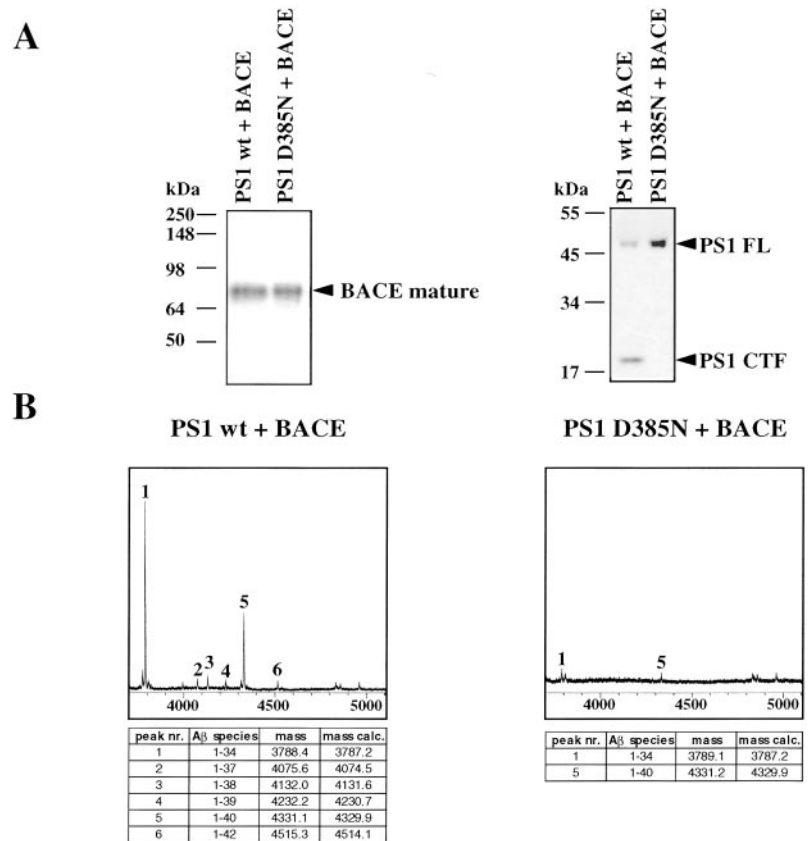
(40) or β APP₆₉₅ containing the Swedish double mutation (β APP_{sw}) (41) and cell lines co-expressing either PS1 wt or PS1 D385N have been described (42). To transfect BACE into these cell lines, the BACE cDNA was cloned into the *EcoRI/XhoI* sites of pcDNA3.1 hygro (+) expression vector (Invitrogen). Transfection was carried out using FuGENE 6 reagent (Roche Molecular Biochemicals). Pooled stable cell clones were selected in 150 μ g/ml hygromycin (Invitrogen). The cell lines expressing BACE-2 have been described previously (9).

Antibodies, Metabolic Labeling, Immunoprecipitation, and Immunoblotting—Antibodies 7520 (43, 44) and 7524 (9) directed against the respective C termini of BACE or BACE-2 and antibody 3926 (45) against the A β domain of β APP, as well as the antibodies 6687, against the C terminus of β APP, and 5313, against the N terminus of β APP (46), have been described previously. The monoclonal antibody 6E10 directed against amino acids 1-17 of the A β domain was obtained from Senetek Inc. For immunodetection of PS1 the polyclonal and monoclonal antibodies against the large hydrophilic loop of PS1 (3027 and BI.3D7) were used (47, 48). Metabolic labeling, immunoprecipitations, and Western blotting were carried out as described previously (9).

BACE Activity Assay—The fluorometric BACE activity assay was carried out as described previously (46). To selectively inhibit BACE activity GL189 was used as described previously (46). Soluble BACE (sBACE) was isolated and incubated with synthetic A β as follows: HEK 293 cells expressing sBACE were incubated with OptiMem 1 containing Glutamax (Invitrogen) for 24 h. 400 ml of the conditioned medium were purified using a mono Q-Sepharose column (Amersham Biosciences). 20 μ l of the fraction derived from cells expressing sBACE or from control fractions not containing sBACE were incubated with 50 μ g of synthetic A β (1-40) for MALDI-TOF MS and with 6 μ g of synthetic A β (1-40) for gel analysis at 37 °C for the indicated time points. The pH was adjusted to 4.5 with acetic acid. Samples were dried in a Speed Vac and resuspended in acetic acid. Subsequently samples were purified by using a Zip-Tip column and were then subjected to MALDI-TOF MS.

Mass Spectrometry/MALDI-TOF—Cells were grown on 10-cm

FIG. 2. Production of A β -(1–34) is PS1-dependent. *A, left panel*, BACE expression in cells co-expressing PS1 wt or PS1 D385N. Membrane fractions were probed with antibody 7520. Immature BACE can only be detected upon longer exposure (data not shown). *Right panel*, PS1 derivatives were identified by a combined immunoprecipitation/immunoblotting protocol using antibody 3027 and BI.3D7 (47). Note full replacement of endogenous PS1 by the uncleaved non-functional PS1 D385N mutant. *B*, MALDI-TOF MS of A β peptides immunocaptured from conditioned media of cell lines shown in *A* using antibody 3926. The tables below the spectra indicate the peak masses measured (*mass*) and the respective masses calculated (*mass calc.*). Note that in the presence of a non-functional PS1 mutant (PS1 D385N) and BACE almost no A β species is generated, whereas in the presence of PS1 wt and BACE A β -(1–34) is the predominant species.



dishes and incubated with 4 ml of Dulbecco's modified Eagle's medium high glucose (DMEM; PAA Laboratories) supplemented with 10% fetal calf serum (PAA Laboratories) and penicillin/streptomycin for 24 h. Subsequently the samples were prepared for mass spectrometry as described previously (49–51). Samples were analyzed on MALDI-target plates by matrix-assisted laser desorption ionization (Brucker Reflex III).

RESULTS

In order to analyze the A β species secreted by BACE-expressing cells we collected conditioned media from HEK 293 cells stably transfected with Swedish mutant β APP₆₉₅ (β APPsw) and BACE. Cells expressing either endogenous levels of BACE or moderate or high levels of transfected BACE were investigated (Fig. 1A). To prove the catalytic activity of BACE in these cell lines we performed *in vitro* activity assays using solubilized membranes of the respective cell lines (46). As expected we found substantially increased β -secretase activity in the cell line expressing high levels of BACE as compared with non-transfected cells or cells expressing low levels of BACE (data not shown). These cell lines were labeled with [³⁵S]methionine and conditioned media were immunoprecipitated with the anti-A β antibody 3926. Surprisingly, increasing BACE expression negatively correlated with A β production (Fig. 1B). This is consistent with previous findings by Vassar *et al.* (2) who observed reduced A β production despite increased BACE activity in cells transfected with β APPsw (2). This paradoxical finding raised the possibility that A β species produced under these conditions could either not be metabolically labeled or not detected by conventional gel electrophoresis. We thus used an independent method and attempted to identify secreted A β species by a combined immunoprecipitation/MALDI-TOF MS method. As expected cells expressing endogenous BACE secreted predominantly A β -(1–40) (Fig. 1C). In addition we also obtained small amounts of A β -(1–42) and A β -(1–37/38/39) (Fig. 1C). Upon expression of moderate levels of BACE we

observed an additional A β species (A β -(1–34); Fig. 1C). In order to analyze if the production of this truncated species is related to BACE expression levels, we next investigated A β species secreted from cells expressing higher levels of BACE (Fig. 1A). This revealed robust amounts of A β -(1–34), which was accompanied by reduced levels of A β -(1–40), A β -(1–42), and A β -(1–37/38/39) (Fig. 1C). Similar results were obtained using cell lines co-expressing wtAPP and BACE (data not shown). The detection of robust levels of A β -(1–34) upon expression of BACE explains the lack of its detection upon metabolic labeling, since the single radioactively labeled Met residue at position 35 of the A β peptide has been removed by the additional cleavage.

Although the above described results suggest that BACE is directly involved in the enhanced production of A β -(1–34), previous observations indicated that C-terminally truncated A β species including A β -(1–34) are generated by the γ -secretase complex in a PS-dependent manner (52, 53). In order to analyze if a PS-dependent γ -secretase activity is required for A β -(1–34) generation, we co-expressed BACE with either PS1 wt or the non-functional PS1 D385N mutant (Fig. 2A). As shown previously (42), PS1 wt undergoes endoproteolysis whereas no endoproteolysis was obtained in cells expressing PS1 D385N (Fig. 2A, right panel; Ref. 27). The non-functional PS1 D385N fully replaced biologically active endogenous PS (Fig. 2A, right panel). Whereas robust levels of A β -(1–34) and A β -(1–40) (and all other minor A β species) were produced from cells co-expressing PS1 wt and BACE, A β -(1–34) generation as well as generation of all other A β species was almost completely inhibited in the presence of the non-functional PS1 D385N (Fig. 2B, right panel). This clearly demonstrates that a PS-dependent γ -secretase activity is involved directly or indirectly in the production of A β -(1–34). However, the results described in Fig. 1, demonstrated that upon BACE

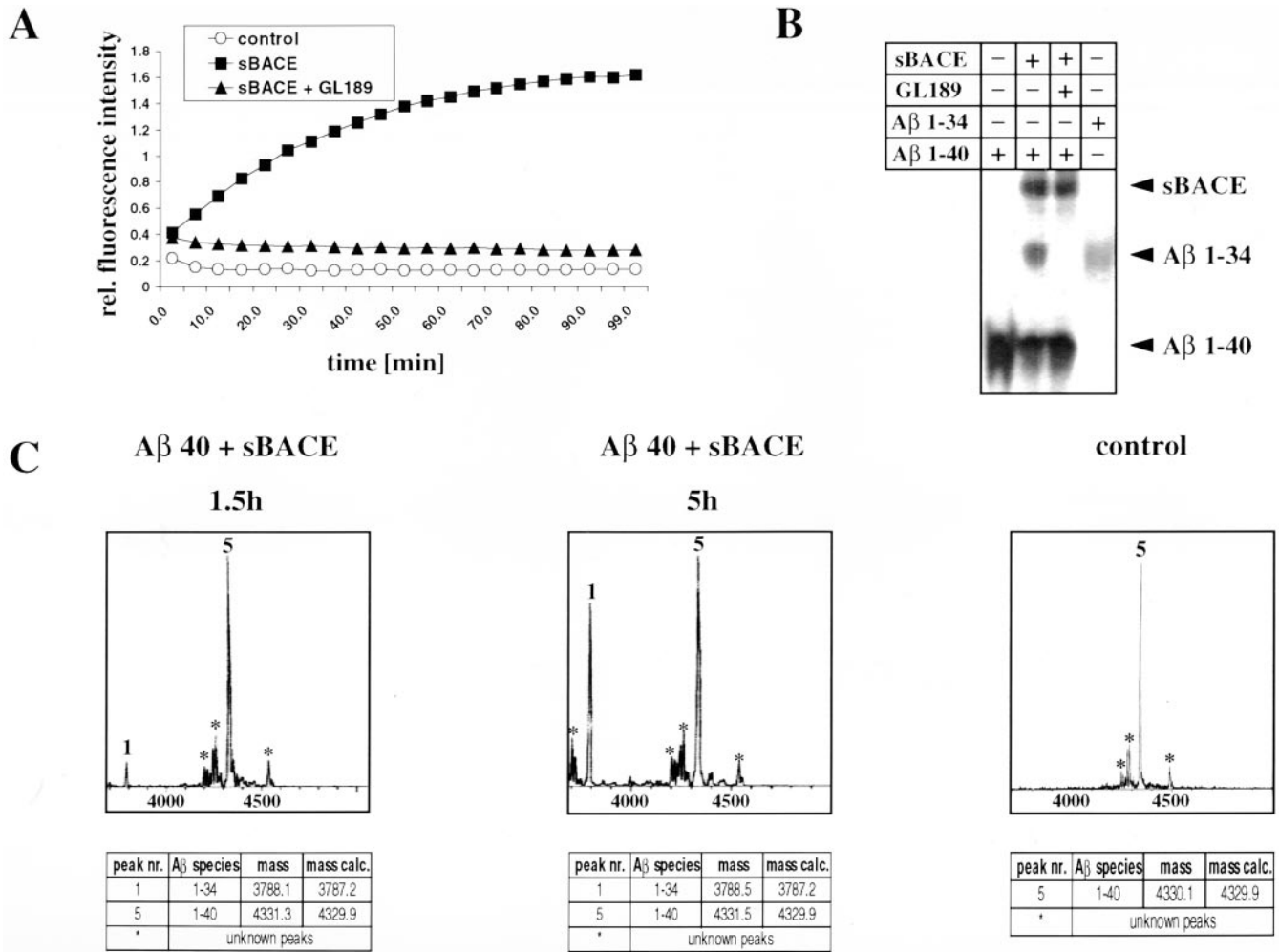


FIG. 3. BACE cleaves synthetic Aβ-(1-40) after amino acid 34 *in vitro*. A, conditioned media from cells expressing sBACE were analyzed *in vitro* for β-secretase activity. Note that medium containing sBACE (filled squares) shows significant activity upon incubation with the fluorogenic substrate, while the control medium without sBACE (open circles) and sBACE in the presence of the specific BACE inhibitor GL189 (filled triangles) do not display activity. B, sBACE was incubated with 6 μg of synthetic Aβ-(1-40) overnight at 37 °C in the presence or absence of the inhibitor GL189. As a control the synthetic peptides Aβ-(1-40) and Aβ-(1-34) were co-migrated. The proteins were visualized by Coomassie Blue staining. Note the aberrant migration of Aβ-(1-34). Significant *in vitro* production of an Aβ species co-migrating with synthetic Aβ-(1-34) was obtained. The production of this peptide was fully inhibited by the specific BACE inhibitor GL189. C, analysis of *in vitro* produced Aβ-(1-34) by MALDI-TOF MS. 50 μg of synthetic Aβ-(1-40) were incubated at 37 °C for 1, 5, or 8 h with purified sBACE or a control fraction not containing sBACE. The tables below the spectra indicate the peak masses measured (mass) and the respective masses calculated (mass calc.). Note that BACE is capable of producing Aβ-(1-34) under *in vitro* conditions.

expression Aβ-(1-34) generation occurs to the expense of the production of all other Aβ variants and thus suggests a direct involvement of BACE in the cleavage of Aβ at position 34. This apparent paradox may indicate that γ-secretase activity is required first to produce secreted Aβ species, which are then trimmed at their C termini by a so far unknown BACE activity.

In order to prove this hypothesis, synthetic Aβ-(1-40) was incubated with purified BACE isolated from conditioned media of cells secreting a soluble version of BACE lacking the transmembrane domain and the C terminus (43). To prove the catalytic activity of secreted BACE we carried out *in vitro* assays (46). Soluble BACE was fully active in the *in vitro* assay, whereas no activity was obtained in control media (Fig. 3A). This activity was fully blocked by the BACE-specific inhibitor GL189 (Fig. 3A). Upon incubation of synthetic Aβ-(1-40) with soluble BACE an additional peptide, which co-migrated with synthetic Aβ-(1-34) was detected on a gel system capable to separate low molecular weight peptides (Fig. 3B; Ref. 54). *In vitro* generation of Aβ-(1-34) was completely inhibited upon addition of the specific BACE in-

hibitor GL189 (Fig. 3B). Using MALDI-TOF MS we confirmed a time-dependent generation of Aβ-(1-34) during incubation of soluble BACE with Aβ-(1-40) (Fig. 3C). In contrast incubation of synthetic Aβ-(1-40) with conditioned media, not containing soluble BACE does not reveal truncated Aβ species (Fig. 3C). This finding demonstrates that BACE has the ability to cleave Aβ after amino acid 34 and, together with the results shown in Fig. 1, excludes artificial trimming by exopeptidases.

Because *in vivo*, only very minor amounts of BACE are secreted (data not shown and Ref. 55) we next investigated if membrane-bound BACE can convert secreted Aβ-(1-40/42) to Aβ-(1-34) in living cells. To do so we collected conditioned media (Fig. 4A) from cells expressing βAPPsw and endogenous BACE. These media were then added either to cells expressing PS1 D385N alone or to cells co-expressing PS1 D385N and BACE. Because of the lack of γ-secretase activity in the latter cell line almost no *de novo* synthesis of any Aβ species occurs (Ref. 42; compare also Fig. 2B). Therefore any truncation of Aβ should occur independent of γ-secretase activity. In conditioned media incubated with cells expressing PS1 D385N no

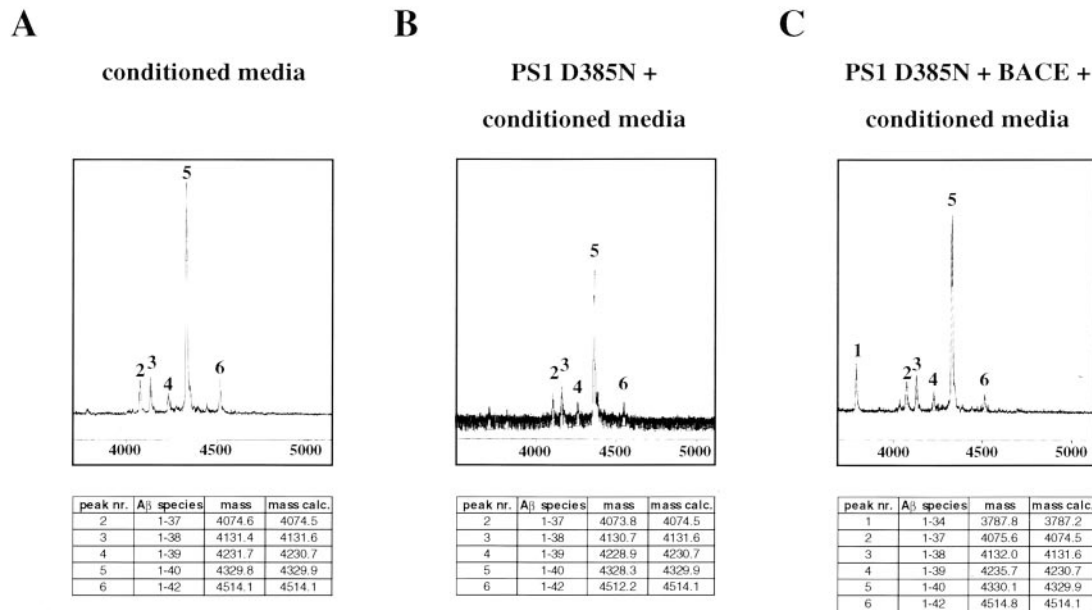


FIG. 4. Membrane-bound BACE generates A β (1–34) in living cells. A, HEK 293 cells stably expressing β APP₆₉₅ sw were incubated with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 48 h. One-third of the conditioned media of HEK 293 cells was immunoprecipitated with antibody 3926. Immunoprecipitated A β was analyzed by MALDI-TOF MS. The remaining conditioned media (see A) was added to cells expressing either β APP₆₉₅ sw and PS1 D385N alone (B) or β APP₆₉₅ sw, PS1 D385N, and BACE (C). A β species were immunoprecipitated from the media after an incubation period of 24 h using antibody 3926. Subsequently samples were subjected to MALDI-TOF MS. The tables below the spectra indicate the peak masses measured (*mass*) and the respective masses calculated (*mass calc.*). Note that although no A β species are secreted in cells expressing PS1 D385N (compare Fig. 2), A β (1–34) is generated in conditioned media when BACE is overexpressed.

detectable conversion of A β (1–40) to A β (1–34) was observed (Fig. 4B). However, upon addition of the conditioned media to cells expressing both PS1 D385N and BACE, A β (1–34) was readily produced (Fig. 4C). Taken together these results demonstrate that BACE can proteolytically modify A β species, which were originally produced by a γ -secretase-dependent pathway.

While BACE is the protease with the major β -secretase activity (15–17), the homologous BACE-2 can also proteolytically process β APP to some extent (9, 13, 14, 56). To investigate if the BACE homologue BACE-2 may also be able to convert A β (1–40/42) into A β (1–34) we co-expressed wild type β APP together with either moderate or high levels of BACE-2 (Fig. 5A). Low level expression of BACE-2 allowed the recovery of very small amounts of A β (1–34), which were not detectable in cells expressing no exogenous BACE-2 (Fig. 5B). However, high level expression of BACE-2 allowed the generation of increased amounts of A β (1–34) thus demonstrating a BACE-2-dependent generation of A β (1–34). These data demonstrate that both BACE and BACE-2 have the unexpected ability to generate C-terminally truncated A β species.

DISCUSSION

BACE plays a central role in the pathogenesis of AD and appears to be the sole β -secretase, since its knock-out in mice fully abolishes A β generation (15–17). In addition and in contrast to the loss of γ -secretase activity the knock out of BACE has no obvious phenotype (15–17). Thus BACE became a primary target for the development of therapeutic strategies. However, very little is known about the biological function of BACE. In addition we do not yet know precisely where the major proteolytic activity of BACE is localized within the cell. BACE is co-translated into the endoplasmic reticulum (ER) as a pro-enzyme (43, 57). During its trafficking through the secretory pathway the pro-domain is removed, and complex glycosylation occurs (43, 57–61). Upon reaching the plasma membrane BACE is reinternalized and targeted to endosomes (44, 62). From endosomes BACE is retrieved in a phosphorylation-

dependent manner and transported back to the *trans*-Golgi network (44). Although BACE has an acidic pH optimum it is apparently active in early compartments such as the ER, since small amounts of A β can accumulate in pre-Golgi compartments (45). Work on the Swedish β APP mutation also demonstrated β -secretase activity within the *trans*-Golgi network (64). In contrast wild type β APP is apparently processed by BACE within early endosomes after reinternalization from the plasma membrane (65). So far no proteolytic activity of BACE was demonstrated on the cell surface. Here we show that A β can be truncated by BACE at its C terminus after its generation by γ -secretase (Fig. 6). Thus it appears likely that secreted A β is further processed by BACE at or close to the plasma membrane although we cannot exclude uptake of A β prior to its processing by BACE.

Our data demonstrate a novel cleavage site at position 34 of the A β domain. This was unexpected since full-length β APP is cleaved by BACE in a highly sequence-specific manner (66). Moreover, previous work demonstrated that *in vivo* a membrane bound substrate is required for recognition by BACE (66). Obviously, soluble A β escapes these requirements and is cleaved by BACE after amino acid 34 of the A β domain. This suggests that the initial cleavage of BACE at the Met-Asp bond of the A β domain occurs in a different structural context than the secondary cut at position 34. This latter cleavage occurs only after A β (1–40/42) generation, whereas the first cleavage requires the membrane bound precursor with a specific recognition sequence at the Met-Asp bond. Furthermore, BACE-2, which differs in its cleavage specificity of β APP (Fig. 6) by predominantly generating an α -secretase-like cleavage after amino acid 19 of the A β domain (9, 13, 14) is also able to generate A β (1–34) from A β (1–40/42). Both enzymes apparently share similar sequence requirements for recognition and cleavage of soluble A β but different preferences for cleavage of full-length β APP. This may also have implications for the search for physiological substrates of BACE. So far only a sialyltransferase (ST6 Gal 1) has been identified as a putative

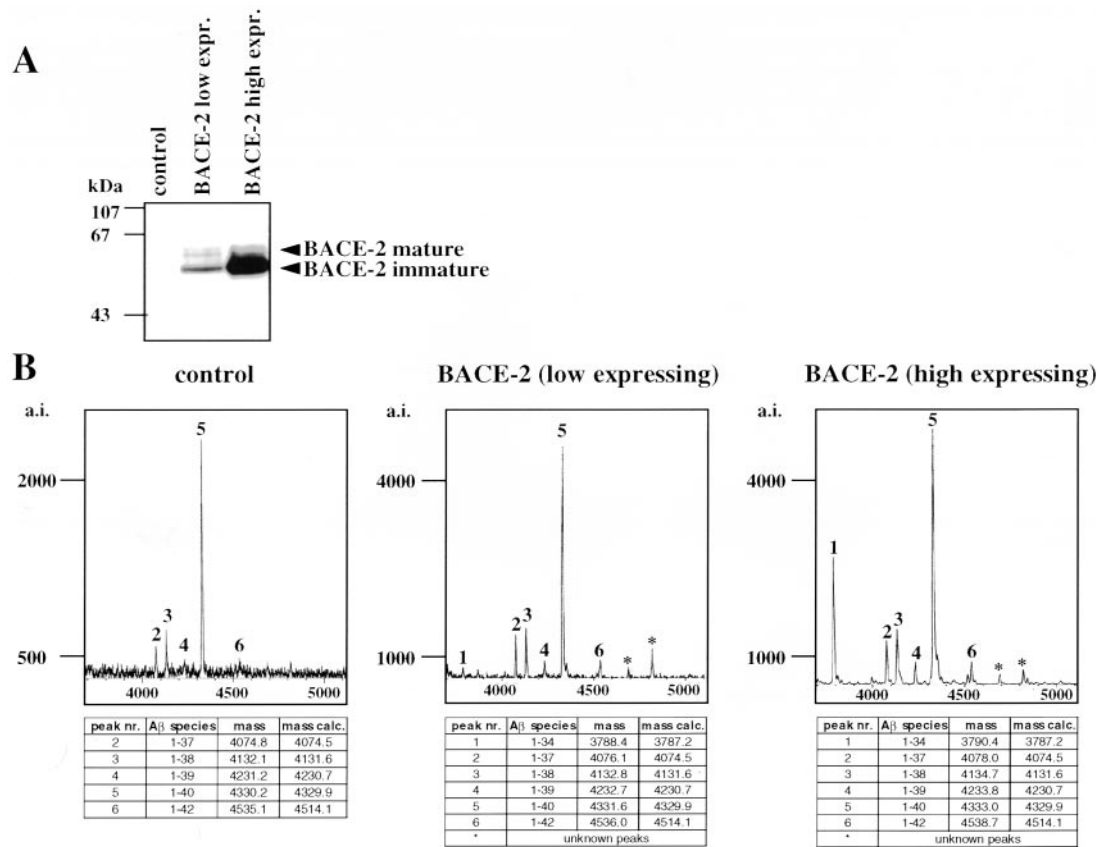
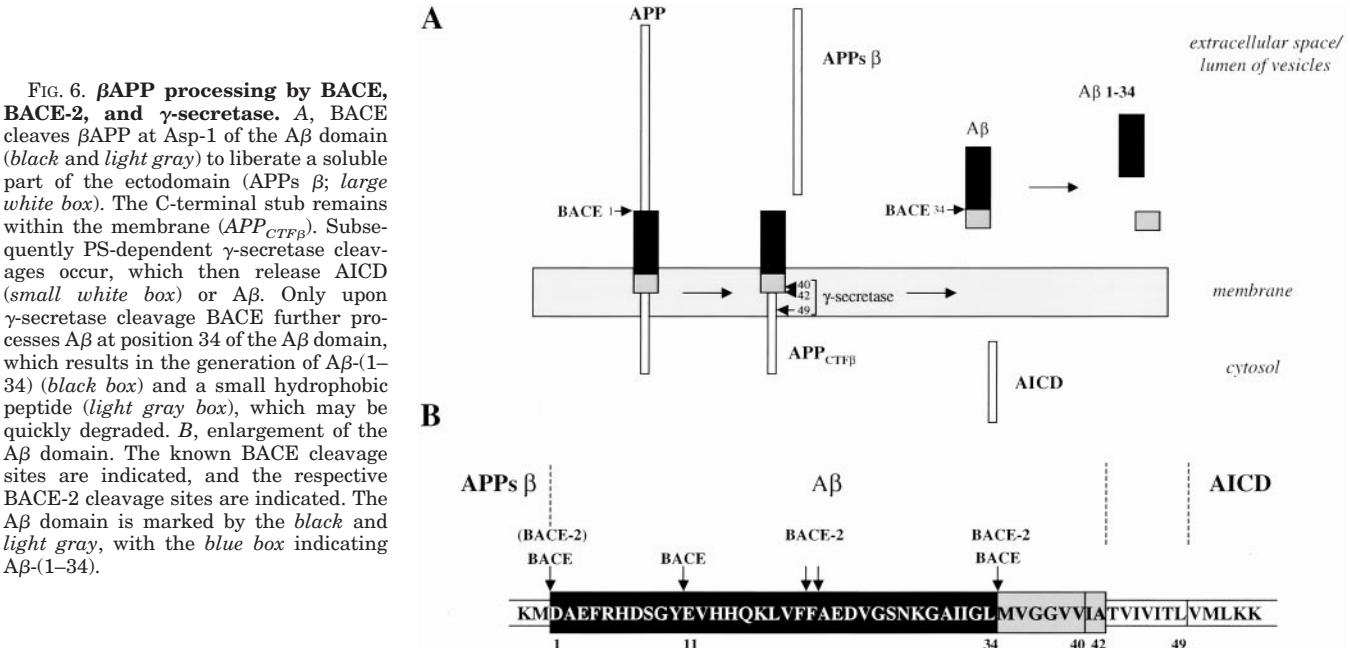


FIG. 5. **Aβ(1-34) generation correlates with BACE-2 expression.** A, membranes of HEK 293 cells stably overexpressing either βAPP₆₉₅ wt (control; lane 1) or additionally BACE-2 (lanes 2 and 3) were probed with antibody 7524. Note the increasing amounts of BACE-2 expression in the three cell lines. Longer exposure reveals endogenous BACE-2 (data not shown). B, MALDI-TOF MS of Aβ peptides immunocaptured directly from conditioned media of the cell lines shown in A, using antibody 3926. Arbitrary intensities are given on the y-axis (a.i.). The tables below the spectra indicate the peak masses measured (mass) and the respective masses calculated (mass calc.). Note that Aβ(1-34) levels correlate with BACE-2 expression.



BACE substrate beside βAPP (67). Our results suggest that substrate-mimicking peptides have to be considered as natural BACE substrates *in vivo*. Aβ(1-34) has been shown to exist *in vivo* (29). The novel cleavage activity of BACE and BACE-2 removes the most hy-

drophobic sequences of the Aβ domain. This may inhibit aggregation and thus facilitate proteolytic clearance by the insulin-degrading enzyme (68) or neprilysin (63, 69). Thus BACE may play an unexpected role in Aβ clearance. Finally, our data also demonstrate that an apparently PS-

and γ -secretase-dependent cut is in fact mediated by BACE. Since the cleavage after amino acid 34 is fully dependent on the previous PS/ γ -secretase cleavage, the involvement of proteases in the generation of C-terminally truncated A β peptides may have been misinterpreted (52, 53). Moreover, our results also solve the apparent paradox that increased BACE expression results in reduced A β production (2), since the truncated A β -(1–34) species cannot be detected by autoradiography due to the loss of the Met residue at position 35 of the A β domain. Furthermore, under the electrophoretic conditions used (54), the peptide aberrantly migrates at a higher molecular weight as expected, again confusing the analysis of A β produced in BACE-expressing cells. Finally our findings may also suggest that substrate-mimetic peptides could represent a novel therapeutic approach *in vivo*.

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Identification of a β -Secretase Activity, Which Truncates Amyloid β -Peptide after Its Presenilin-dependent Generation

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