A non-amyloidogenic function of BACE-2 in the secretory pathway

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Abstract

β-Site amyloid precursor protein cleavage enzyme (BACE)-1 and BACE-2 are members of a novel family of membrane-bound aspartyl proteases. While BACE-1 is known to cleave β-amyloid precursor protein (βAPP) at the β-secretase site and to be required for the generation of amyloid β-peptide (Aβ), the role of its homologue BACE-2 in amyloidogenesis is less clear. We now demonstrate that BACE-1 and BACE-2 have distinct specificities in cleavage of βAPP in cultured cells. Radiosequencing of the membrane-bound C-terminal cleavage product revealed that BACE-2 cleaves βAPP in the middle of the Aβ domain between phenylalanines 19 and 20, resulting in increased secretion of APPs-α- and p3-like products and reduced production of Aβ species. This cleavage can occur in the Golgi and later secretory compartments. We also demonstrate that BACE-1-mediated cleavage of βAPP at Asp1 of the Aβ domain can occur as early as in the endoplasmic reticulum, while cleavage at Glu11 occurs in later compartments. These data indicate that the distinct specificities of BACE-1 and BACE-2 in their cleavage of βAPP differentially affect the generation of Aβ.

Keywords: Alzheimer's disease, β-amyloid precursor protein, β-secretase, β-site amyloid precursor protein cleaving enzyme.

Alzheimer's disease (AD) is characterized by the invariant accumulation of senile plaques and neurofibrillary tangles in certain areas of the brains of AD patients (Selkoe 1999). The major constituent of senile plaques is the amyloid β-peptide (Aβ), which derives by endoproteolytic processing of the β-amyloid precursor protein (βAPP; Steiner and Haass, 2000).

Aβ is generated by sequential cleavages of βAPP by β- and γ-secretase. β-Secretase cleaves βAPP at the N terminus of the Aβ-domain, resulting in the generation of soluble APPs-β and a membrane-associated C-terminal fragment (CTF) bearing the complete Aβ-domain (CTF-β). Subsequent cleavage of this fragment by γ-secretase, which critically requires presenilins, results in the release and secretion of Aβ (Steiner and Haass, 2000). In an alternative pathway, βAPP is cleaved by α-secretase within the Aβ domain, therefore precluding the formation of Aβ (Steiner and Haass, 2000). α-Secretase cleavage results in the secretion of APPs-α and the generation of CTF-α, which can also be processed further

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Abbreviations used: APP, amyloid precursor protein; Aβ, amyloid β-peptide; AD, Alzheimer's disease; BACE, β-site APP cleaving enzyme; BFA, brefeldin A; endo, H, endoglycosidase H; HEK, human embryonic kidney; PNGase F, peptide-*N*-glycosidase F; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

by γ-secretase to yield p3 (Haass *et al.* 1993; Steiner and Haass, 2000).

The membrane-bound aspartyl protease beta-site APP-cleaving enzyme (BACE)-1 with β-secretase activity was identified in human embryonic kidney (HEK) 293 cells and brain tissue (Hussain *et al.* 1999; Sinha *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Lin *et al.* 2000). BACE-1 and its homologue BACE-2 are type I membrane proteins sharing significant homology with other members of the aspartyl protease family (Fan *et al.* 1999; Hussain *et al.* 1999; Saunders *et al.* 1999; Sinha *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Acquati *et al.* 2000; Lin *et al.* 2000). While BACE-1 is highly expressed in neurons, BACE-2 expression is low in the central nervous system, but higher in peripheral tissues including pancreas, placenta and stomach (Bennett *et al.* 2000a; Vassar and Citron, 2000). BACE-1 appears to be the major enzyme in amyloidogenesis in brain, because BACE-$1^{-/-}$ mice have greatly reduced brain Aβ levels (Cai *et al.* 2001; Luo *et al.* 2001; Roberds *et al.* 2001). Moreover, antisense inhibition of BACE-1, but not of BACE-2, reduces the generation of Aβ in cultured cells (Vassar *et al.* 1999; Yan *et al.* 1999).

Consistent with the highly similar structural features of BACE-1 and BACE-2, both enzymes undergo similar post-translational modifications by complex *N*-glycosylation and proteolytic removal of their prodomains (Vassar and Citron, 2000; Walter *et al.* 2001a). However, while propeptide cleavage of BACE-1 is mediated by proteases of the furin family or by furin itself (Bennett *et al.* 2000b; Capell *et al.* 2000; Huse *et al.* 2000; Creemers *et al.* 2001), the propeptide of BACE-2 appears to be removed autocatalytically (Hussain *et al.* 2001; Yan *et al.* 2001a).

The majority of BACE-1 is localized within Golgi and endosomal compartments, where it co-localizes with βAPP (Hussain *et al.* 1999; Vassar *et al.* 1999; Capell *et al.* 2000; Huse *et al.* 2000; Lin *et al.* 2000; Walter *et al.* 2001b). The subcellular localization is consistent with the acidic pH optimum of the enzyme and the known subcellular sites of βAPP processing by β-secretase (Vassar and Citron, 2000; Walter *et al.* 2001a). The intracellular trafficking of BACE-1 is regulated by a di-leucine motif within its cytoplasmic domain and by phosphorylation/dephosphorylation of an adjacent serine residue (Huse *et al.* 2000; Walter *et al.* 2001b). In addition, the transmembrane domain of BACE-1 plays a role for its retention in the trans-Golgi network (Yan *et al.* 2001b).

While the catalytic activity of BACE-1 in cultured cells is well understood (Hussain *et al.* 1999; Sinha *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Lin *et al.* 2000), the role of BACE-2 in amyloidogenesis is less clear. Studies *in vitro* (Farzan *et al.* 2000; Hussain *et al.* 2000; Lin *et al.* 2000) and with transiently transfected cells (Yan *et al.* 2001a) indicate that BACE-2 cleaves βAPP much less efficiently at the known β-secretase sites as compared with BACE-1.

Materials and methods

Cell culture, cDNAs and transfection

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM; Gibco Life Sciences, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Gibco Life Sciences). The cell lines stably overexpressing βAPP_{695} (Haass *et al.* 1992) and BACE-1 (Capell *et al.* 2000; Walter *et al.* 2001b) have been described previously. BACE-2 cDNA was provided by Dr R. Tamarelli (Acquati *et al.* 2000) and was subcloned into the *Apa*I/*Hin*dIII sites of pcDNA3.1 hygro (–) expression vector (Invitrogen, Groningen, the Netherlands). Transfection of cells with BACE-2 cDNAs was carried out using Fugene reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instructions. Single-cell clones were generated by selection in 100 μg/mL hygromycine (Invitrogen).

Antibodies, metabolic labeling, immunoprecipitation and immunoblotting

The polyclonal antibodies 7524 and 7525 were obtained from rabbits, which were inoculated with synthetic peptide representing amino acids 492–518 of BACE-2 coupled to keyhole limpet hemocyanin. Antibody 7520, directed against the C-terminus of BACE-1 (Cappell *et al.* 2000; Walter *et al.* 2001) and antibody 3926 against Aβ (Wild-Bode *et al.* 1997), has been described previously. Antibodies 1736 and 192wt were provided by Drs D. J. Selkoe (Haass *et al.* 1992) and D. Schenk (Seubert *et al.* 1993), respectively, and 6E10 was obtained from Senetek Inc (Napa, CA, USA).

To radiolabel cellular proteins, cells were incubated at 37°C in methionine-free, serum-free MEM (Gibco Life Sciences) for 60 min. Cells were then incubated with fresh medium supplemented with [^{35}S]methionine/[^{35}S]cysteine (Promix; Amersham Pharmacia Biotech, Freiburg, Germany) and kept at 37°C for the time periods indicated in the respective experiment. The secretion of βAPP variants in pulse–chase experiments was calculated by setting the amount of cellular radiolabeled βAPP after the pulse as 100%. In some experiments, brefeldin A (BFA) or monensin was added during the preincubation and the labeling period. As a control, cells were incubated with the carrier (ethanol) alone.

For immunoprecipitations cells were lysed in STEN buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA) supplemented with 1% nonidet P-40 (NP-40)/1% Triton X-100/2% bovine serum albumin (BSA) on ice for 10 min. Lysates were clarified by centrifugation for 20 min at 14 000 *g* and immunoprecipitated for 3 h at 4°C. After separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Imobilon, Millipore Corporation, Eschborn, Germany) and analyzed by autoradiography or phosphoimaging. Alternatively, proteins were detected by immunoblotting using an enhanced chemiluminescence technique (Amersham Pharmacia Biotech).

Radiosequencing

Cells were labeled with [^{3}H]valine or [^{3}H]phenylalanine for 6 h and βAPP CTFs were isolated by immunoprecipitation with antibody 6687, separated by SDS–PAGE and transferred to PVDF membrane. Radiolabeled proteins were detected by autoradiography, and

subjected to radiosequencing as described previously (Haass *et al.* 1992).

Deglycosylation experiments

BACE-1 and BACE-2 were immunoprecipitated from cell lysates as described above and precipitates were incubated in the presence of endoglycosidase H (endo H; Roche Molecular Biochemicals) or PNGase F (Roche Molecular Biochemicals) for 14 h at 37°C in the appropriate buffer. Reactions were stopped by the addition of SDS sample buffer and reaction mixtures were separated by SDS–PAGE.

Cell surface biotinylation and uptake assay

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated on ice with PBS containing 0.5 mg/mL EZ-link^{TM} Sulfo-NHS-SS-Biotin (Pierce, Bonn, Germany) for 30 min. Cells were then washed three times with ice-cold PBS supplemented with 20 mM glycine for 5 min each and finally washed twice with PBS. To allow re-internalization, cells were then incubated with DMEM (10% fetal calf serum) at 37°C for the time periods indicated. Subsequently, cells were washed three times with glutathione buffer (50 mM glutathione; 90 mM NaCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 0.2% BSA, pH 8.6) for 15 min each. After two final washes with PBS, cells were lysed in STEN buffer containing 1% NP-40/1% Triton X-100/1% BSA.

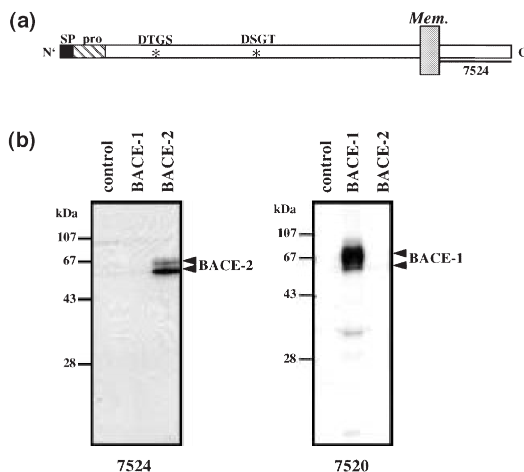


Fig. 1 Stable expression of BACE-1 and BACE-2 in HEK 293 cells. (a) Schematic of BACE-2 showing the epitope of antibody 7524. The aspartyl protease active site motifs (DTGS and DSGT) are indicated by asterisks. The signal peptide (SP) is indicated by a black box, and the propeptide (pro) is indicated by a hatched box. (b) Membranes of untransfected HEK 293 cells or cells stably overexpressing BACE-1 or BACE-2 were probed with antibodies 7524 (left panel) or 7520 (right panel) by western immunoblotting. Note that antibodies 7520 and 7524 specifically detect BACE-1 and BACE-2, respectively.

Results

Cellular expression and post-translational modifications of BACE-2

We generated HEK 293 cells which stably express human BACE-2. BACE-2 was detected in membrane preparations by western blotting with the polyclonal antibody 7524 directed to the cytoplasmic domain of BACE-2 (Fig. 1a). Two bands of ~62 kDa and ~65 kDa were specifically detected in cells transfected with BACE-2 (Fig. 1b, left panel). No reactivity was observed in untransfected cells or in cells overexpressing BACE-1. Similar results were obtained with antibody 7525 directed to the same epitope of BACE-2 as antibody 7524 (data not shown). In contrast, antibody 7520 directed to the C-terminus of BACE-1 (Capell *et al.* 2000; Walter *et al.* 2001b), selectively detected a ~65 kDa and a ~70 kDa protein in membrane preparations of BACE-1-transfected cells (Fig. 1b, right panel), representing immature and mature forms of the enzyme (Capell *et al.* 2000; Walter *et al.* 2001b). These data demonstrate that antibodies 7524 and 7520 selectively recognize BACE-2 and BACE-1, respectively.

To prove whether the 62 kDa and 65 kDa variants of BACE-2 result from post-translational modifications we performed pulse–chase experiments. Cells were pulse-labeled with [^{35}S]methionine, and BACE-2 was immunoprecipitated at the chase time periods as indicated. After pulse labeling a band of 62 kDa was detected (Fig. 2a, upper panel). During the chase, levels of the 65 kDa species increased with time, becoming the predominant species after 2 h. This species almost completely disappeared after 4 h of chase (Fig. 2a, upper panel). In contrast, BACE-1 appeared to be more stable, with an estimated half-life of more than 8 h [Fig. 2a, lower panel; (Capell *et al.* 2000; Huse *et al.* 2000; Walter *et al.* 2001b)].

The molecular weight shift from 62 kDa to 65 kDa indicates that BACE-2 matures by post-translational modification, as previously described for BACE-1 (Capell *et al.* 2000; Haniu *et al.* 2000; Huse *et al.* 2000; Benjannet *et al.* 2001; Walter *et al.* 2001b). To prove whether BACE-2 is modified by *N*-glycosylation, cells were pulse-labeled with [^{35}S]methionine and chased for 1 h in the presence or absence of tunicamycin, which inhibits *N*-glycosylation of proteins in the endoplasmic reticulum. Immediately after the pulse the 62 kDa immature species was detected, which matures to higher molecular weight species during the 1 h chase (Fig. 2b). In the presence of tunicamycin BACE-2 migrated at the expected molecular mass of ~50 kDa, demonstrating that *N*-glycosylation of BACE-2 was inhibited.

To further prove whether BACE-2 undergoes complex *N*-glycosylation during its maturation, cells were labeled with [^{35}S]methionine and then incubated in the presence or absence of PNGase F or endo H. While PNGase F removes all types of N-linked sugars, endo H selectively cleaves mannose-rich N-linked sugars (Capell *et al.* 2000; Benjannet *et al.* 2001; Walter *et al.* 2001b). Treatment of BACE-2 with

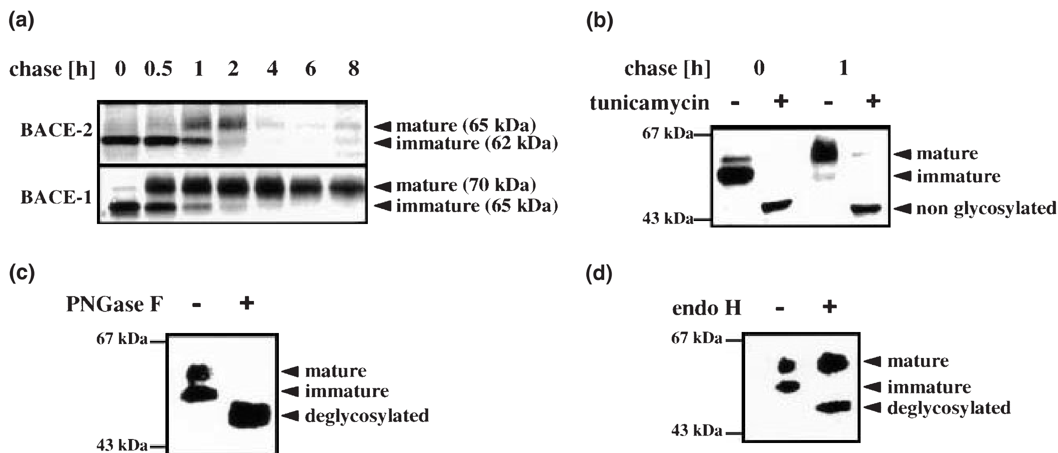


Fig. 2 BACE-2 matures by complex *N*-glycosylation. HEK 293 cells expressing BACE-1 or BACE-2 were pulse labeled with [^{35}S]methionine for 10 min and chased for the time-points indicated. (a) BACE-1 and BACE-2 were isolated by immunoprecipitation with antibodies 7520 and 7525, respectively, and detected by autoradiography. Mature and immature forms are indicated by arrowheads. (b) Cells were labeled in the presence or absence of 10 μg/mL tunicamycin. After the time periods indicated BACE-2 was immunoprecipitated and analyzed by autoradiography. (c and d) Immunoprecipitated BACE-2 was incubated in the presence of PNGase F (c) or endo H (d). Samples were separated by SDS–PAGE and radiolabeled BACE-2 was detected by autoradiography.

PNGase F resulted in a molecular weight shift of both the 62 kDa and the 65 kDa species of BACE-2 to approximately 50 kDa (Fig. 2c). In contrast, endo H selectively deglycosylated the 62 kDa species of BACE-2, while the 65 kDa species was endo H resistant (Fig. 2d), indicating that BACE-2 undergoes complex *N*-glycosylation during its maturation. This finding also demonstrates that BACE-2 is transported from the endoplasmic reticulum to the Golgi, where complex *N*-glycosylation occurs.

To investigate whether BACE-2 is further transported to the cell surface, we carried out biotinylation assays. Plasma membrane proteins of BACE-2-expressing cells were biotinylated and then isolated by precipitation with streptavidin-conjugated agarose beads. In parallel, BACE-2 was isolated by immunoprecipitation with antibody 7525 and precipitates were separated by SDS–PAGE. Biotinylated BACE-2 was detected by western immunoblotting as a single 65-kDa band (Fig. 3a). In contrast, immunoprecipitation with antibody 7525 revealed the presence of both immature (62 kDa) and mature (65 kDa) species of BACE-2 (Fig. 3a). The selective biotinylation of the 65 kDa species indicates that mature BACE-2 is transported to the cell surface. To test whether BACE-2 is reinternalized from the plasma membrane, cells were kept at 37°C for up to 60 min after surface biotinylation. After the indicated time periods, biotin bound to surface proteins was removed by incubation with glutathione buffer on ice. Under these conditions biotin was selectively removed from proteins localized at the cell surface, while internalized proteins were protected against removal of biotin. These experiments revealed that BACE-2 is reinternalized very inefficiently, since little if any biotinylated BACE-2 was detected upon treatment with glutathione buffer at all time periods tested (Fig. 3b, upper panel). Consistent with previous results (Huse *et al.* 2000; Walter *et al.* 2001b) and in contrast to BACE-2, BACE-1 was readily endocyto-

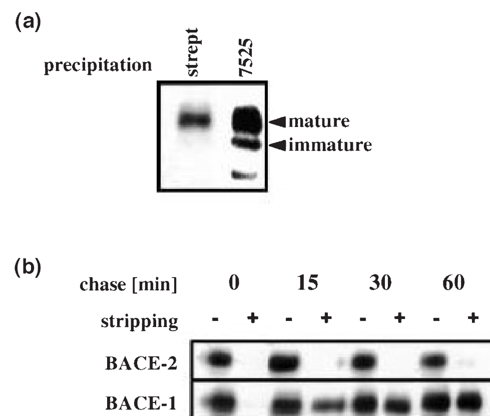
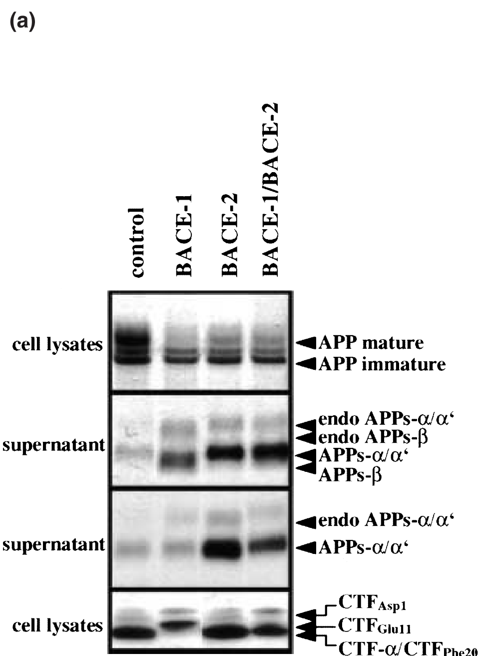


Fig. 3 Detection of BACE-2 at the cell surface. (a) Cell surface proteins were labeled with biotin and isolated by precipitation with streptavidin-conjugated sepharose beads. In parallel, BACE-2 was immunoprecipitated with antibody 7525. Precipitates were separated by SDS–PAGE and BACE-2 was detected by western immunoblotting. Note that selectively the mature form of BACE-2 is biotinylated, demonstrating selective labeling of cell surface proteins. (b) Internalization of cell-surface-located BACE-2 and BACE-1 was analyzed by incubation of cells at 37°C after biotinylation. At the time-points indicated cells were treated with glutathione buffer (+) or left untreated (–). Biotinylated proteins were precipitated with streptavidin-conjugated Sepharose beads and BACE-2 and BACE-1 were detected by western immunoblotting with antibodies 7524 and 7520, respectively. The increased amounts of biotinylated BACE-1 both with and without stripping at 30 and 60 min is due to experimental variability.

sed under these conditions, as indicated by the resistance of biotin-label of BACE-1 against removal with glutathione (Fig. 3b, lower panel).

BACE-2 cleaves βAPP within the Aβ domain

To characterize the catalytic specificity of BACE-2 in comparison to that of BACE-1 we analyzed proteolytic processing of βAPP. In cells that stably overexpress BACE-1, BACE-2 or both enzymes, the levels of mature βAPP were strongly reduced as compared to control cells (Fig. 4a, first panel). Accordingly, conditioned media of BACE-1- or BACE-2-transfected cells contained increased levels of secreted βAPP (APPs), indicating secretory processing of βAPP by BACE-1 and BACE-2 (Fig. 4a, second panel). Notably, APPs in media from BACE-2-expressing cells co-migrated with APPs-α from control cells, while APPs from BACE-1-expressing cells migrated slightly faster (Fig. 4a, second panel). This is consistent with predominant cleavage of βAPP by BACE-1 at the β-site resulting in slightly smaller APPs-β as compared to APPs-α (Citron *et al.* 1992; Haass *et al.* 1995). We also precipitated conditioned media with antibody 1736 that recognizes selectively APPs cleaved at, or close to, the α-secretase site (Haass *et al.* 1992). In the media of BACE-2-expressing cells significantly increased levels of APPs recognized by antibody 1736 were detected, demonstrating that BACE-2 elevates the secretion of APPs cleaved at (APPs-α), or close to (APPs-α′) the α-secretase site (Fig. 4a, third panel). Quantification revealed that BACE-2-expressing cells secreted approximately sevenfold more APPs-α/α′ than BACE-1-expressing cells (Fig. 4b). These data indicate that BACE-2 cleaves βAPP at or close to the cleavage site of α-secretase and therefore has distinct substrate specificity than its homologue BACE-1 in cultured cells. We next analyzed the βAPP CTFs and predominantly detected CTF-α in control cells (Fig. 4a, fourth panel). Only low amounts of CTF_{Asp1} were detected in these cells, consistent with predominant α-secretory processing in HEK 293 cells (Haass *et al.* 1992). In line with increased processing of βAPP by BACE-2 at or close to the α-site, the level of a βAPP fragment co-migrating with CTF-α



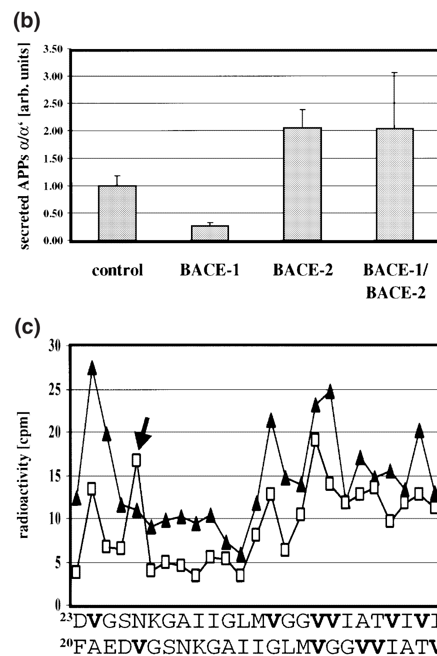


Fig. 4 BACE-2 cleaves βAPP within the Aβ domain. (a) HEK 293 cells stably expressing BACE-1, BACE-2 or both were labeled with [^{35}S]methionine, and intracellular βAPP and its C-terminal fragments were precipitated with antibody 6687 from cell lysates (upper and lower panel). Secreted forms of βAPP were precipitated from conditioned media with antibody 5313 detecting both APPs-α and APPs-β (second panel) or with antibody 1736, which selectively detects APPs-α/α′ (third panel). Secreted species derived from endogenous (endo) APP are indicated by arrow heads. The identity of the additional CTF species migrating slightly below CTF_{Asp1} in control cells and BACE-2-transfected cells (lower panel) is unknown. Since HEK 293 cells secrete N-terminal truncated forms of Aβ (Haass *et al.* 1992), this species likely represents an N-terminally truncated CTF. (b) Quantification of APPs-α/α′ normalized to βAPP expression in stably transfected cells was carried out as described in the Methods section. Values represent means ± SD of three independent experiments. APPs-α secretion of cells expressing only βAPP was set as 1. (c) Radiosequencing of [^{3}H]valine-labeled CTF-α from untransfected cells (▲) and BACE-2-transfected cells (□). The values are given in cpm per sequencing cycle. Amino acid sequences matching to the radiosequences are given below in single letter code with valine residues in bold. The peak in cycle 5 of CTFs from BACE-2-expressing cells (arrow) is consistent with a CTF starting at Phe20.

increased in BACE-2-expressing cells (Fig. 4a, fourth panel). To determine the cleavage site of BACE-2 within βAPP, CTFs from cells metabolically labeled with [^{3}H]valine were radiosequenced. In control cells, prominent peaks in cycles 2, 14, 17 and 18 were detected, indicating that βAPP CTFs predominantly start at Asp23 while the quantity of CTFs starting at Leu17 appears to be low (Fig. 4c). CTFs starting at Asp23 have been described previously (Zhong *et al.* 1994) and might originate from subsequent processing of CTF_{Leu17} to CTF_{Asp23}. Importantly, sequencing of βAPP CTFs from BACE-2-transfected cells revealed a distinct profile of radioactivity. Here, a peak in cycle 5 demonstrated that BACE-2 cleaved βAPP between Phe19 and Phe20 to generate CTF_{Phe20} (Fig. 4c). The additional peaks in cycles 2, 14 and 17 in the BACE-2 sample show that CTFs beginning both at Asp23 and Phe20 are present (Fig. 4c). Cleavage of βAPP by BACE-2 at the Phe19–Phe20 bond in cultured cells is consistent with *in vitro* experiments demonstrating that BACE-2 can cleave synthetic peptides at this site (Farzan *et al.* 2000). Cells overexpressing BACE-1 contain predominantly two slower migrating CTF species (Fig. 4a, lower panel), consistent with cleavage of βAPP by BACE-1 at Asp1 (CTF_{Asp1}) and Glu11 (CTF_{Glu11}) of the Aβ domain (Vassar *et al.* 1999; Farzan *et al.* 2000; Creemers *et al.* 2001). Interestingly, cells expressing BACE-1 together with BACE-2 revealed low amounts of CTF-β_{Asp1} and predominantly CTF-α/CTF_{Phe20}, while CTF_{Glu11}, which is the major species in BACE-1 transfected cells, was almost absent (Fig. 4a, lower panel). Taken together, these data indicate that the combined activities of BACE-1 and BACE-2 determine the generation of βAPP CTFs and Aβ species and suggest a non-amyloidogenic activity of BACE-2.

We therefore investigated the effect of BACE-2 expression on the generation of Aβ- and p3-species. Control cells secrete more p3 than $A\beta_{1-40/42}$ (Fig. 5a), due to predominant non-amyloidogenic processing of βAPP in HEK 293 cells (Haass *et al.* 1992). Consistent with previous reports (Hussain *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Capell *et al.* 2000; Creemers *et al.* 2001), BACE-1 expression leads to increased production of $A\beta_{1-40/42}$ and even more $A\beta_{11-40/42}$. As expected, the increased amyloidogenic activity of BACE-1 strongly reduced p3 production (Fig. 5a). Cells expressing BACE-2 did not secrete significant amounts of $A\beta_{11-40/42}$, but produced predominantly p3 species and low amounts of $A\beta_{1-40/42}$. Based on the identified cleavage specificity of BACE-2 between Phe19 and Phe20, we assume that the p3 species secreted from BACE-2-expressing cells contained p3 starting at Phe20 ($p3_{Phe20}$). Interestingly, cells expressing both BACE-1 and BACE-2 secreted strongly decreased levels of $A\beta_{11-40/42}$ compared to BACE-1-expressing cells. Due to the activity of BACE-2, double-transfected cells secreted significantly higher amounts of p3/$p3_{Phe20}$ as compared to cells expressing BACE-1 alone. In addition, double-transfected cells also generated significant amounts of $A\beta_{1-40/42}$, most likely reflecting the activity of BACE-1. Quantification of these βAPP derivatives confirmed that BACE-2 selectively reduced $A\beta_{11-40/42}$ levels, while $A\beta_{1-40/42}$ generation was not significantly affected (Fig. 5b,c).

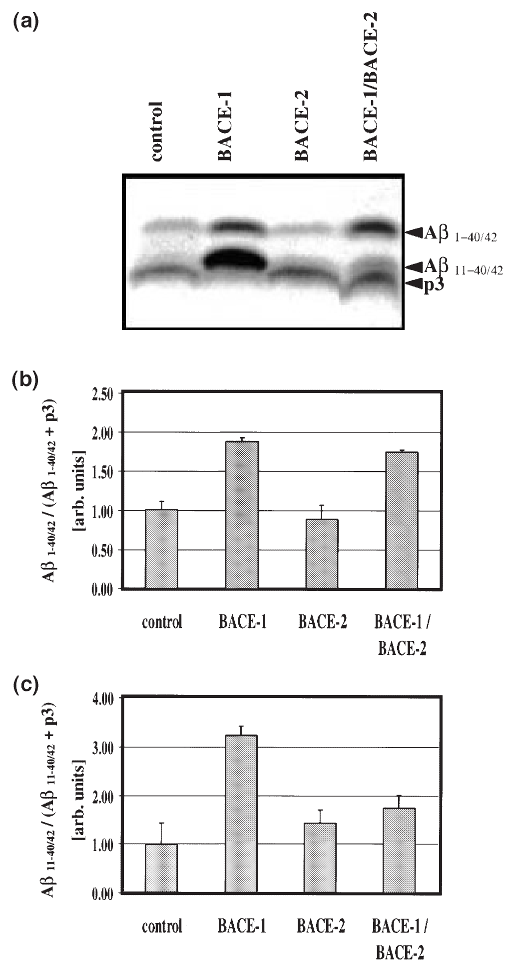


Fig. 5 BACE-2 selectively reduces the generation of Aβ starting at Glu11. (a) Cells were labeled for 4 h with [^{35}S]methionine and conditioned media were precipitated with antibody 3926 recognizing the Aβ domain of βAPP. Precipitates were separated on 10–20% Tris–tricine gels and radiolabeled Aβ and p3 species were detected by autoradiography. (b,c) Quantification of secreted $A\beta_{1-40/42}$ and $A\beta_{11-40/42}$. Values represent means ± SD of three independent experiments. Values of cells expressing βAPP only were set as 1.

Distinct activities of BACE-1 and BACE-2 in the secretory pathway

The distinct effects of BACE-2 on Aβ and CTF species starting either at Asp1 or at Glu11 suggest that these BACE-1-mediated cleavages occur sequentially. To address this question, cells were labeled with [^{35}S]methionine in the presence or absence of inhibitors of protein forward transport. We used BFA to inhibit protein transport from the endoplasmic reticulum to Golgi compartments or monensin to inhibit transport from the Golgi to later secretory

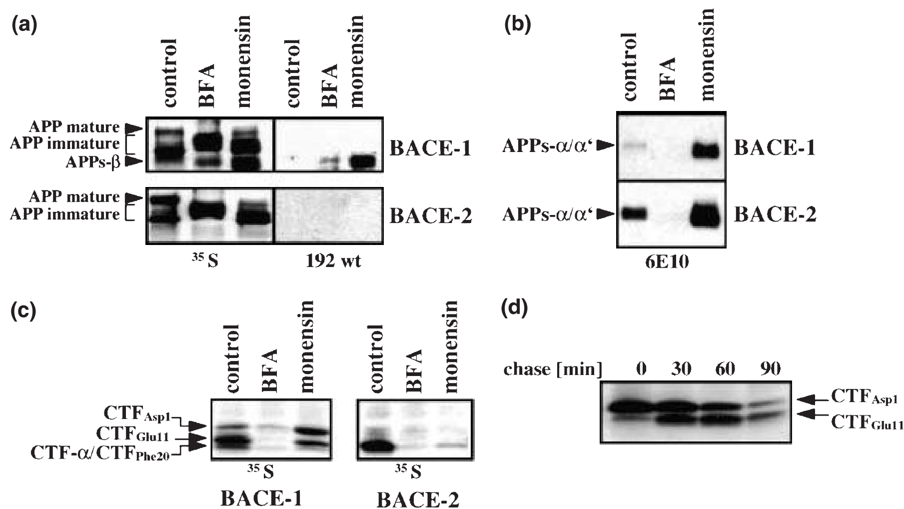


Fig. 6 Cleavage of βAPP by BACE-1 and BACE-2 in distinct subcellular compartments. Cells expressing BACE-1 or BACE-2 were labeled with [^{35}S]methionine in the presence or absence of BFA or monensin. (a) Intracellular βAPP was precipitated from cell lysates with antibody 5313 and detected by autoradiography (left panels). The same membrane was probed with antibody 192wt to detect intracellular APPs-β (right panels). (b) Cell lysates were precipitated with antibody 1736 and detected with antibody 6E10. (c) βAPP CTFs were precipitated with antibody 6687 from cell lysates and detected by autoradiography. (d) Cells expressing βAPP K670N/M671L ('Swedish mutation') and BACE-1 were pulse-labeled with [^{35}S]methionine and chased for the time periods indicated. βAPP CTFs were isolated by immunoprecipitation with antibody 6687 and detected by autoradiography.

compartments (Haass *et al.* 1993; Wild-Bode *et al.* 1997). Holo-βAPP and its proteolytic processing products were isolated from cell lysates and conditioned media. Immunoprecipitation of intracellular βAPP with antibody 5313 directed against the βAPP ectodomain revealed two major variants of βAPP in untreated cells, representing immature and mature βAPP in both BACE-1 and BACE-2 expressing cells (Fig. 6a, left panels). BFA and monensin treatment led to the detection of βAPP variants migrating between mature and immature βAPP due to aberrant glycosylation (Haass *et al.* 1993; Wild-Bode *et al.* 1997). BFA and monensin also blocked secretion of APPs in all cell lines analyzed (data not shown), indicating inhibition of protein forward transport. In BACE-1-transfected cells, BFA treatment induced the accumulation of an additional intracellular βAPP derivative. This species was detected by the neoepitope-specific antibody 192wt that selectively recognizes APPs-β but not holo-βAPP [Seubert *et al.* 1993; Fig. 6(a), upper right panel]. Moreover, antibody 6E10 directed against amino acids 1–16 of the Aβ domain did not recognize this species (data not shown), indicating that βAPP is selectively cleaved at the β-site within the endoplasmic reticulum. Monensin treatment led to an increased accumulation of intracellular APPs-β recognized by antibody 192wt (Fig. 6a). These results indicate that BACE-1 can cleave βAPP at the β-site in early secretory compartments, namely in the endoplasmic reticulum and Golgi. Intracellular APPs-β was not observed in BACE-2-expressing cells (Fig. 6a, lower panels), consistent with the distinct specificity of BACE-2 in cleavage of βAPP between Phe19 and Phe20. We therefore analyzed the levels of intracellular APPs-α/α′ in BACE-1- or BACE-2-expressing cells. Only low amounts of intracellular APPs-α/α′ were detected in untreated cells expressing BACE-1 (Fig. 6b, upper panel), because of predominant cleavage of βAPP at the β-site and residual α-secretase cleavage at or close to the cell surface followed by rapid secretion of APPs-α/α′ (Sisodia *et al.* 1990). Significantly higher levels of intracellular APPs-α/α′ were detected in untreated cells expressing BACE-2 (Fig. 6b, lower panel). Cell treatment with BFA strongly inhibited production of intracellular APPs-α/α′, also indicating that cleavage of βAPP by BACE-2 predominantly occurred in post-endoplasmic reticulum compartments. Indeed, the detection of high amounts of APPs-α/α′ in cells treated with monensin demonstrated that BACE-2 can efficiently cleave βAPP in the Golgi compartment. To prove this, we next analyzed the levels of βAPP CTFs. In BACE-1-expressing cells, CTF_{Asp1} and predominantly CTF_{Glu11} were detected (Fig. 6c, left panel). BFA treatment strongly increased the ratio of CTF_{Asp1}/CTF_{Glu11}. While the generation of CTF_{Glu11} was strongly reduced, significant amounts of CTF_{Asp1} were produced. Again, these data indicate that BACE-1-mediated cleavage of βAPP at Asp1 can occur in the endoplasmic reticulum, while cleavage at Glu11 is very inefficient in this compartment. By using βAPP bearing the K670N/M671L double mutation ('Swedish mutation'), we also observed cleavage at Asp1 within the endoplasmic reticulum even without overexpression of BACE-1, therefore indicating endogenous β-secretase activity in this compartment (data not shown). Interestingly, treatment of BACE-1-expressing cells with monensin allowed generation of

CTF_{Glu11}, indicating that this cleavage can occur in the Golgi (Fig. 6c, left panel). In BACE-2-transfected cells, BFA strongly inhibited the cleavage of βAPP as indicated by the significant reduction of CTF-α/CTF_{Phe20} (Fig. 6c, right panel). However, treatment with monensin allows generation of βAPP CTF-α/CTF_{Phe20} to some extent, indicating that BACE-2 can cleave βAPP within the Golgi apparatus. Taken together these results demonstrate that cleavage of βAPP by BACE-2 between Phe19 and Phe 20 and by BACE-1 between Tyr10 and Glu11 can occur within the same subcellular compartments, namely the Golgi and later secretory compartments, while cleavage of the Met–Asp bond at the beginning the Aβ domain mediated by BACE-1 can also occur earlier within the endoplasmic reticulum. Pulse–chase analysis of βAPP containing the K670N/M671L 'Swedish' double mutation revealed that CTF_{Asp1} was generated earlier as CTF_{Glu11}, also indicating sequential cleavages of βAPP by BACE-1 first at Asp1 and then at Glu11 (Fig. 6d).

Discussion

This study demonstrates that BACE-1 and BACE-2 undergo similar post-translational modifications and subcellular trafficking, but have distinct specificity in cleavage of βAPP. Like BACE-1, BACE-2 matures by complex *N*-glycosylation during its transport through the secretory pathway. However, pulse–chase experiments revealed that BACE-2 is less stable than BACE-1. Cell surface biotinylation demonstrates that both BACE-1 and BACE-2 are transported to the plasma membrane. Unlike BACE-1, which is readily re-internalized into endosomes, BACE-2 can hardly be detected in these compartments. This may suggest that BACE-2 is degraded immediately after its uptake in endosomes (Fig. 2). BACE-2 might also be secreted into the media to some extent, a mechanism shown previously for minor portions of BACE-1 (Benjannet *et al.* 2001). The detection of membrane-bound C-terminal fragments of 15–20 kDa might indeed indicate internal cleavage of BACE-2 and subsequent shedding of the enzyme (data not shown).

By analyzing βAPP processing we demonstrate that BACE-2 strongly increases the secretion of an APPs-α-like variant which is recognized by the neoepitope-specific antibody 1736. In line with this finding, radiosequencing reveals the accumulation of an intracellular βAPP CTF-α-like species starting at Phe20 in BACE-2-transfected cells, demonstrating that BACE-2 cleaves βAPP between Phe19 and Phe20 within the Aβ domain, close to the α-secretase site. Our data are fully consistent with the findings that BACE-2 cleaves synthetic peptides *in vitro* more efficiently at this site than at the Met–Asp bond at the N terminus of the Aβ domain (Farzan *et al.* 2000), and that cells transiently transfected with BACE-2 secrete increased levels of an APPs-α-like species (Yan *et al.* 2001a). Notably, fibroblasts from Down's syndrome patients have been shown to produce significantly more βAPP CTF starting at Phe20 (Zhong *et al.* 1994). This might indicate a higher BACE-2 activity due to triplication of the BACE-2 gene, which is localized in the Down critical region of chromosome 21 (Fan et al. 1999; Saunders *et al.* 1999; Acquati *et al.* 2000). However, our data do not exclude the possibility that BACE-2 cleaves βAPP at the β-secretase site(s) to some extent. Indeed, it was shown that the FAD-associated K670N/M671L double mutation ('Swedish mutation') of βAPP is efficiently cleaved by BACE-2 at the β-secretase site (Farzan *et al.* 2000; Hussain *et al.* 2000) therefore indicating that BACE-2 might also function as β-secretase and might be involved in increased Aβ production in Swedish mutant cases.

In contrast to BACE-2-mediated cleavage at Phe20, our data indicate that BACE-1 generates two CTFs starting at Asp1 and Glu11 that can subsequently be cleaved by γ-secretase to produce the respective Aβ species (Fig. 5). The reason for the distinct cleavage specificity of BACE-1 and BACE-2 is unclear. Both enzymes are highly similar in their primary structure (Hussain *et al.* 1999; Sinha *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Lin *et al.* 2000). However, subtle structural differences might determine the cleavage specificity of both enzymes. In addition, differences in the post-translational modifications, like propeptide cleavage or distinct *N*-glycosylation might also contribute to the distinct substrate specificity. Comparison of the crystal structure of BACE-2, which remains to be determined, with that of BACE-1 (Hong *et al.* 2000) might answer this question.

We demonstrate that the distinct cleavages of βAPP by BACE-1 at Asp1 and Glu11 can occur in distinct subcellular compartments. By treatment with BFA we found that BACE-1 can cleave βAPP at Asp1 as early as in the endoplasmic reticulum. It will be interesting to determine whether this cleavage is involved in the production of intracellular Aβ that can be detected in early secretory compartments (Cook *et al.* 1997; Hartmann *et al.* 1997; Wild-Bode *et al.* 1997). In contrast, the Glu11 cut of βAPP, also mediated by BACE-1, is almost completely inhibited by BFA, indicating selective Glu11 cleavage in post-endoplasmic reticulum compartments. It has been shown that the pro-domain of BACE-1 is cleaved by furin-like proteases or furin itself (Bennett *et al.* 2000b; Capell *et al.* 2000; Huse *et al.* 2000; Benjannet *et al.* 2001; Creemers *et al.* 2001), which are active predominantly in post-endoplasmic reticulum compartments. Therefore, propeptide removal might contribute to the distinct substrate specificities of BACE-1 in distinct subcellular compartments. In addition, differences in the *N*-glycosylation pattern of BACE-1 in the endoplasmic reticulum and the Golgi might alter its cleavage specificity.

Interestingly, co-expression of BACE-1 and BACE-2 almost completely inhibits the production of CTF_{Glu11} but has little effect on the levels of CTF_{Asp1}. Together with the data derived from pharmacological cell treatment, the

following picture of sequential βAPP cleavage by BACE-1 and BACE-2 emerges. βAPP can be first cleaved by BACE-1 at Asp1 as early as in the endoplasmic reticulum. BACE-2 appears not to cleave βAPP efficiently in this compartment, because neither APPs-α′ nor the corresponding CTF starting at Phe20 accumulates upon BFA treatment. Rather, BACE-2 can cleave βAPP in post-endoplasmic reticulum compartments, like the Golgi and later secretory compartments during transport of βAPP to the cell surface as indicated by an increase of intracellular APPs-α′ in BACE-2-transfected cells treated with monensin (Fig. 6). Within these compartments BACE-1 and BACE-2, together with α-secretase, determine the relative amounts of βAPP CTFs and Aβ species. Indeed, cell treatment with monensin reveals that cleavage of βAPP by BACE-1 at Glu11 and by BACE-2 at Phe20 can both occur in the Golgi compartment. The strong reduction of both intracellular βAPP CTF_{Glu11} and secreted $A\beta_{11-40/42}$ of cells expressing BACE-1 and BACE-2 might be due to a higher affinity of BACE-2 for its cleavage site in βAPP, than that of BACE-1 for cleavage at Glu11. The CTF_{Glu11} generated by BACE-1 might also be processed further by BACE-2 before it is finally cleaved by γ-secretase.

This study might help to understand why brain neurons secrete the highest levels of both $A\beta_{1-40/42}$ and $A\beta_{11-40/42}$ (Gouras *et al.* 1998; Cai *et al.* 2001). Because BACE-1 is expressed in neurons at much higher levels than BACE-2 (Bennett *et al.* 2000a; Hussain *et al.* 2000), there might be less competition between both proteases, favoring therefore the generation of these Aβ species. In cells expressing higher levels of BACE-2, the enzyme might, in addition to α-secretase, efficiently compete with BACE-1, favoring the generation of APPs-α- and p3-like species, and thereby inhibit the production of Aβ. These data suggest that in the secretory pathway, amyloidogenic or non-amyloidogenic processing of βAPP involves the distinct enzymatic activities of BACE-1 and BACE-2. Specific facilitation of BACE-2 activity or inhibition of BACE-1 activity would be therefore attractive therapeutic strategies in the treatment of AD.

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Note added in proof

While this manuscript was under consideration, similar results were described by the groups of Drs V. M. Lee and R. W. Doms demonstrating that cleavage of βAPP at Aspl and Glu11 can occur in distict subcellular compartments (Huse *et al.* 2002; Liu *et al.* 2002).

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