BACE2 Functions as an Alternative \(\alpha\)-Secretase in Cells*

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BACE1 and BACE2 define a new subfamily of membrane-anchored aspartyl proteases. Both endoproteases share similar structural organization including a prodomain, a catalytic domain formed via DTG and DSG active site motifs, a single transmembrane domain, and a short C-terminal tail. BACE1 has been identified as the Alzheimer’s\(\beta\)-secretase, whereas BACE2 was mapped to the Down’s critical region of human chromosome 21. Herein we show that purified BACE2 can be autoactivated in vitro. Purified BACE2 cleaves human amyloid precursor protein (APP) sequences at the \(\beta\)-secretase site, and near the \(\alpha\)-secretase site, mainly at A\(\beta\)-Phe\(_{20}\) \| Ala\(_{21}\) and also at A\(\beta\)-Phe\(_{19}\) \| Phe\(_{20}\). Alternatively, in cells BACE2 has a limited effect on the \(\beta\)-secretase site but efficiently cleaves the sequences near the \(\alpha\)-secretase site. The in vitro specificity of APP processing by BACE2 is distinct from that observed in cells. BACE2 localizes in the endoplasmic reticulum, Golgi, trans-Golgi network, endosomes, and plasma membrane, and its cellular localization patterns depend on the presence of its transmembrane domain. BACE2 chimeras that increase localization of BACE2 in the trans-Golgi network do not change its APP processing patterns. Thus, BACE2 can be distinguished from BACE1 on the basis of autoprocessing of the prosegment, APP processing specificity, and subcellular localization patterns.

The amyloid peptide (A\(\beta\)), one of the major components of amyloid plaques (1), has been considered one of the causal factors for the pathogenesis of Alzheimer’s disease (reviewed in Refs. 2–4). A\(\beta\) is excised from a large membrane-bound amyloid precursor protein (APP) by two endopeptidases, \(\beta\)-secretase and \(\gamma\)-secretase. \(\beta\)-secretase and \(\gamma\)-secretase cleave APP sequentially to generate the N and C termini of the A\(\beta\) peptide, respectively. The C terminus of the A\(\beta\) peptide is processed heterogeneously by the \(\gamma\)-secretase and produces various lengths of A\(\beta\) peptides ranging from 39 to 43 amino acids. The longer ones tend to aggregate more readily and are more amyloidogenic. Another endopeptidase, \(\alpha\)-secretase, cleaves between residues 16 and 17 of the A\(\beta\) peptide and disrupts formation of these amyloidogenic peptides (5).

BACE1, which was identified recently as the \(\beta\)-secretase (6–10), is a membrane-bound aspartyl protease. Immunofluorescent studies of BACE1-transfected cells indicate various secretory locations for BACE1 including early Golgi (6–7), later Golgi (11), endosomes (10, 12–13) and plasma membrane (12). Endogenous BACE1 is located predominantly in the later Golgi and TGN.\(^2\) The TGN is a major site for cellular APP processing by BACE1.\(^2\) BACE1 has four N-linked glycosylation sites that play a role in the protease activity in vitro (15). Like many aspartyl proteases, BACE1 is also synthesized as a pro-BACE1, and the prodomain is removed by furin or furin-like proteases (12, 16). Although the prodomain is required for the proper folding of BACE1, the presence of the prodomain does not substantially suppress its protease activity (17).

BACE2 was identified as a homolog of BACE1 through expressed sequence tags data base searching (8, 10, 18–19) and genomic cloning (20). Both proteins have similar structural organization and share 51% identity at the amino acid level. BACE2 transcripts are expressed in the central nervous system and many peripheral tissues; however, its expression level in neurons is substantially lower than BACE1 (18–19). BACE2 maps to 21q22.3 (8, 20–22), the Down’s syndrome critical region, providing a logical link between this gene product and APP processing. In vitro enzymatic assays with peptide substrates demonstrate that BACE2 cleaves \(\beta\)-secretase substrates, similar to BACE1, processing both the wild-type and Swedish mutant APP (19, 23). In addition, BACE2 also cleaves between Phe\(_{19}\)-Phe\(_{20}\) and Phe\(_{20}\)-Ala\(_{21}\) within the A\(\beta\) peptide (23). A Flemish mutant APP was preferentially cleaved at the \(\beta\)-secretase site in the cell, presumably because of disruption of the recognition site at Phe\(_{20}\)-Ala\(_{21}\) (23).

Our previous antisense experiments showed that the reduction of BACE2 mRNA did not substantially affect the levels of secreted A\(\beta\) (8). We also noticed no increased production of \(\beta\)-secretase cleavage products after cells were transfected with BACE2 plasmid DNA. In this study, we systematically examined the role of BACE2 in APP processing using both purified BACE2 enzyme as well as expression of BACE2 and BACE1 chimeras in cells. In addition, we also studied whether the subcellular localization of BACE2 will affect its APP processing pattern.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies—Antibody 6E10, which recognizes residues 1–16 of A\(\beta\), was purchased from Synetec, PLC (St. Louis, MO). The antibody 22C11 recognizing the N-terminal region of APP and anti-GFP antibody were purchased from Roche Molecular Biochemicals. Antibodies against syntaxin 6 and EEA1 were purchased from Transduction Laboratory (Lexington, KY). The anti-p56 was purchased from Sigma. The antibody against \(\beta\)-COP was purchased from Affinity Bioreagents (Golden, CO). Antibody B280 recognizing the sequence DDSLEPFFDSLKVQTHV (191–207 of human BACE1) was produced by Covance (Denver, PA). Antibody C8, which recognizes the C-terminal 15 amino acids of APP, was provided by Dr. Dennis Selkoe (Harvard Medical School, Boston, MA). The immunofluorescent Alexa-488 or Texas red-labeled secondary antibodies were purchased from Molecular Probes (Eugene, OR). All expression constructs were engineered by

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The abbreviations used are: A\(\beta\), amyloid peptide; APP, amyloid precursor protein; TGN, trans-Golgi network; GFP, green fluorescent protein; HPLC, high pressure liquid chromatography.

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polarized chain reaction amplification of human BACE1 or BACE2, and the sequence of each plasmid was confirmed by DNA sequencing. All the GFP-tagged recombinant constructs were generated by cloning regions of BACE1 or BACE2 into the GFP expression vector pEGFP-N3 (CLONTECH). For the expression of plasmids in mammalian cells, BACE1 and BACE2 coding regions were cloned into the expression vector pcDNA3.1/hygro (Invitrogen, Carlsbad, CA).

Expression of Soluble Human BACE2—The coding sequence of human BACE2 was engineered for expression using polymerase chain reaction. The 5′-sense primer (5′-CGCTTTAAGCTTGGCACCACATGG-GCGACTGGCUGCgggccc-3′) introduced a consensus Kozak translation initiation sequence and a HindIII restriction site to facilitate cloning. The 3′-antisense primer (5′-CGCTTTAAGCTTGGCACCACATGGATGggtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg...
and Ala and a minor cleavage between Aβ-Phe and Phe. This substrate recognition is in the vicinity of the known APP α-secretase cleavage site between Lys and Leu in the Aβ sequence and may result in α-secretase-like activity with APP (see below). Treatment of the APP β-secretase substrates with BACE2 resulted in specific hydrolysis at the β-secretase site, and the rate of cleavage was enhanced 20-fold in the substrate containing the Swedish mutation. Finally, an APP γ-secretase peptide substrate was not cleaved by BACE2 under reaction conditions where cleavage of the β-secretase, γ-secretase, and prosegment substrates was readily detected.

Comparison of the relative rates of hydrolysis of these various peptides showed that the substrate based on the BACE2 prosegment was cleaved the fastest, followed by the β-secretase Swedish substrate, the α-secretase substrate, and finally the β-secretase wild-type sequence (Table I).

**TABLE I**

Comparison of the relative rates of hydrolysis of selected peptide substrates by soluble BACE2

| Recognition site | Substrate/Cleavage site | Relative rate |
|------------------|-------------------------|---------------|
| BACE2 processing site | ERHADGLAL | ALEPA | 8.0 |
| APP α-secretase | VHHQKLVF | MajAEDVSN | 0.25 |
| APP β-secretase | SEVNL | DAEFR | 0.05 |
| APP β-secretase (Swedish) | SEVNL | DAEFR | 1.0 |
| APP γ-secretase | RGGVVIATIVGER | 0 |

**Knock-down of BACE2 mRNA Has Minimal Effects on the Level of Aβ Peptides**—125.3 cells, expressing high levels of Swedish mutant APP, treated with BACE1 antisense oligomers produce substantially reduced levels of secreted Aβ, consistent with its function as the cellular β-secretase (6, 8). Conversely, treatment of 125.3 cells with specific antisense oligomers against BACE2, although resulting in a marked reduction of BACE2 transcripts (Fig. 2A), did not show reduced levels of secreted Aβ (Fig. 2B, the similar results for Aβ in these treated cells are not shown). This result implies that BACE1 functions as the predominant intracellular β-secretase, and the presence of its endogenous levels is sufficient to mask the action of BACE2 on the cleavage of APP at the β-secretase site. To explore this possibility further, 125.3 cells were pretreated with BACE1–2as oligomer for 24 h to reduce BACE1 mRNA levels as much as 80%. After this pretreatment, these cells...
were then treated with different specific antisense oligomers against BACE2 in the continuing presence of BACE1–2as oligomer. After an additional 48–72 h of treatment, the levels of secreted Aβ were quantified by enzyme-linked immunosorbent assay. Although the oligomer BACE1–2as reduced Aβ40 dramatically, further addition of BACE2 oligomers in these pretreated cells did not further decrease the Aβ levels (Fig. 2C). Again, the ratio of Aβ42/Aβ40 is not changed under these treated conditions (data not shown). A 50–80% reduction of BACE2 transcripts in these double-treated cells was confirmed by TaqMan (data not shown). Thus, the antisense results suggest that only reduction of BACE1 mRNA, but not the BACE2 mRNA, inhibits the production of the Aβ peptides.

BACE1 and BACE2 Have Distinct Cellular Localization—Our antisense experiments suggest that BACE1 and BACE2 serve different functions within cells. As shown previously, overexpression of BACE1 in APP-expressing cells results in the accumulation of β-secretase cleavage products CTF99 and sAPPβ (6–10). Our initial experiments indicated that increased production of BACE2 in these cells did not noticeably enhance production of CTF99 or sAPPβ (see below), contrary to the published observations (19, 23). To resolve this discrepancy, the expression level and intracellular localization of BACE2 under these experimental conditions were studied in more detail.

To visualize and compare the cellular localization of BACE1 and BACE2 within the transfected cells, we employed GFP as a reporter tag fused to the C terminus of either full-length BACE1 or BACE2 protein. All the constructs used in the localization studies are illustrated in Fig. 3. As shown previously, transient expression of low levels of BACE1-GFP in human neuroblastoma SHEP cells revealed that the fluorescence is more confined to the perinuclear regions, mainly in the later Golgi and the TGN (Fig. 4, A–1). We have shown previously that this pattern mimics the localization of endogenous BACE. 5 On the contrary, transient expression of a similar low dosage of BACE2-GFP in SHEP cells exhibited a more diffuse fluorescent signal, seemingly present in all the secretory compartments (Fig. 4, B–1). A comparison of stable cell lines overexpressing either BACE1 or BACE2 confirmed that the localization of these two homologs is distinct (Fig. 4, A–2 and B–2). The majority of BACE1 is still in the later Golgi/TGN, although a small portion is also visible in the endoplasmic reticulum and endosomes and on the cell surface. Quantification of biotinylated cell surface BACE1 indicated that less than 10% is actually localized on the plasma membrane, 2 consistent with the modest fluorescent signal on the surface. In contrast, a substantial amount of BACE2-GFP was present on the cell surface in addition to the intracellular localization. This suggests that a significant portion of BACE2-GFP localized to the cell surface after post-translational maturation. During the preparation of this manuscript, Hussain et al. (24) confirmed significant expression of BACE2 on the cell surface through biotinylation experiments.

To localize intracellular pools of BACE2 precisely, we employed the double-immunofluorescence labeling approach. Experiments were performed with a stable cell line expressing low levels of BACE2-GFP to avoid distorted localization patterns. We found that BACE2 expression clearly overlapped with p58 and β-COP (early and later Golgi markers) in addition to syntaxin 6 (a TGN marker) (Fig. 5). Whereas, BACE1 was shown predominantly in the TGN having minimal overlap with early Golgi markers such as p58 and GM130 under similar transfection conditions. The punctate signals close to the cell surface are typical for proteins found in early endosomes and are more obvious in cells expressing BACE2 than BACE1. Taken together, these data demonstrate that BACE2 has a more diffuse intracellular localization than BACE1, consistent with the observations by Hussain et al. (19).

The Transmembrane Domain But Not the Cytoplasmic Tail Is Required for the Intracellular Localization of BACE2—To determine whether the transmembrane domain or the C-terminal cytoplasmic tail of BACE2 influences its cellular localization, we generated two fusion proteins with truncation of either the C-terminal tail (BACE2 ΔC-GFP) or the transmembrane domain plus the C-terminal tail (BACE2ΔT M C-GFP) (illustrated in Fig. 3). Expression of GFP fused to a BACE1 signal peptide sequence exhibited a ubiquitous localization pattern (Fig. 6A). Transient transfection of the BACE2 ΔC-GFP construct displayed green fluorescence similar to BACE2-GFP (Fig. 6B), suggesting that the C-terminal tail is not a major factor in BACE2 cellular trafficking. However, removal of both the C-terminal domain and the transmembrane domain resulted in a diffuse fluorescent signal over the entire cytoplasm (Fig. 6C). Some punctate vesicle staining probably related to
the secretory vesicles was also visible. Expression of similarly truncated BACE1 C-GFP in SHEP cells produced a significantly weaker green fluorescent signal in the cytoplasm (data not shown), suggesting that soluble BACE1 is more readily secreted from cells than soluble BACE2. To support this further, production of secreted BACE1 or BACE2 in Chinese hamster ovary cells consistently yields a 10-fold higher levels of BACE1 than BACE2 in the conditioned medium (data not shown). It has been demonstrated that the removal of the C-terminal tail of BACE1 defers its maturation (25). The kinetics of BACE2 maturation have not yet been fully elucidated. It is likely that these two proteins undergo different post-translational modifications during maturation that may affect their rate of secretion versus the amount being retained intracellularly.

The Transmembrane Domain of BACE1 and BACE2 Determines their Cellular Localization Fate—Previously, we showed that the transmembrane domain of BACE1 functions as a Golgi/TGN retention signal. To confirm this observation and increase the localization of BACE2 to the TGN where the majority of APP processing at the β-site occurs, we grafted the transmembrane domain and cytoplasmic tail of BACE1 onto the C terminus of the BACE2 catalytic domain to generate a construct BACE2TMC1-GFP (illustrated in Fig. 3). Confocal microscopy of BACE2TMC1-GFP showed that the majority of the signal was confined to the perinuclear region (Fig. 7 E), a typical Golgi localizing pattern. Immunofluorescent labeling of marker proteins indicated that the BACE2TMC1-GFP signal had minimal overlap with the early endosome marker EEA1 (Fig. 7, D–F) but was well overlapped with the TGN marker of syntaxin 6 (Fig. 7, J–L). A reverse chimeric construct BACE1TMC2 containing the BACE1 catalytic domain fused to the BACE2 transmembrane domain and cytoplasmic tail was also generated and similarly expressed in SHEP cells. Immunofluorescent staining of the SHEP cells expressing BACE1TMC2 protein (Fig. 7, B and H) indicated that it was...
localized in a pattern similar to BACE2-GFP (Fig. 5). The immunofluorescent signal of BACE1TMC2 is partially overlapped with signals of both EEA1 and syntaxin 6 (Fig. 7, A–C and G–I), resembling the localizing pattern of BACE2 (Fig. 5). In addition, a construct (BACE2TM1C2-GFP) swapping only the transmembrane domain of BACE1 was also generated, and the cellular localization of BACE2TM1C2-GFP is similar to BACE2TMC1-GFP (data not shown). Taken together these results indicated that the transmembrane domain of BACE1 may be responsible for trafficking to the later Golgi/TGN compartments.

**BACE2 Functions More Like an Alternative α-Secretase in Cells**—An *in vitro* enzymatic assay with either BACE1 or BACE2 suggests that both enzymes preferentially cleave a Swedish mutant peptide substrate over wild type. However, these two enzymes behave differently in cells. Expression of BACE1 in a stable cell line expressing the Swedish mutant APP leads to an increase of the β-secretase cleavage product CTF99 and a reduction of the ectodomain sAPPα, a product produced by α-secretase cleavage (Fig. 8A, BACE1). Overexpression of BACE2 in the same stable cell line did not noticeably increase the production of CTF99, but instead increased the levels of sAPPα (Fig. 8A, BACE2). Considering the results from *in vitro* enzymatic assays of purified BACE2, increased production of sAPPα may reflect the preferential processing of intracellular APP at two sites (Aβ-Phe19 and Aβ-Phe20) adjacent to the α-secretase site. Because we have shown that BACE2TM1-GFP is essentially localized similar to BACE1, we then expressed this construct in the same APP-overexpressing cell line. Interestingly, the cells expressing moderate levels of BACE2TM1-GFP did not increase the accumulation of CTF99, but did increase the levels of sAPPα as did wild-type BACE2 (Fig. 8A). On the other hand, cells expressing BACE1TMC2 did increase the levels of CTF99 and reduce that of sAPPα, suggesting that the intracellular protease specificity of these two endopeptidases depends on their catalytic domain instead of the concentration within their residing intracellular vesicles. The addition of a GFP tag to the C terminus of BACE2

**FIG. 7.** The transmembrane domains of BACE1 and BACE2 determine their intracellular localization patterns. SHEP cells were transfected with either BACE1TMC2 or BACE2TM1C1-GFP for 48 h and then fixed for immunostaining. Antibody B280 was used for recognizing the BACE1TMC2 chimera, and Alexa-488-labeled secondary anti-rabbit IgG was used for visualizing the green fluorescent signal. Texas red-labeled antibodies against early endosomal marker (EEA1) and TGN marker (syntaxin 6) were used to locate early endosomal and TGN structures. Bar, 10 μm.
did not change its protease activity (Fig. 8A, compare BACE2-GFP with BACE2). Removal of the transmembrane domain of BACE2 reduces its processing of APP adjacent to the α-secretase site, whereas the deletion of cytoplasmic tail alone does not change APP processing patterns by BACE2 (Fig. 8A, compare BACE2ΔTMC-GFP with BACE2ΔC-GFP). This reduction in APP processing within the β-peptide region by BACE2ΔTMC-GFP cannot be attributed to insufficient expression of this construct in cells, because expression of the nonmembrane-anchored BACE2ΔTMC-GFP protein was clearly detectable both by Western (Fig. 8A) and immunofluorescent staining (shown in Fig. 6C). This suggests that membrane docking may promote an optimal interaction between BACE2 and the APP substrate.

Typically, β-secretase cleaves Swedish mutant APP much more efficiently than wild-type APP. We therefore compared the processing of APP variants in a SHEP cell line stably expressing BACE2-GFP. As shown in Fig. 8B, the processing patterns are similar among cells transiently expressing wild-type APP, Swedish mutant APP, or London mutant APP (Val661 → Ile661) (Fig. 8B), suggesting that high levels of BACE2 in these cells did not preferentially process one APP variant over the others. In the control lane, BACE1 processes Swedish mutant APP to produce higher levels of CTF99, as confirmed with antibody 6E10. This result may argue against the possibility that BACE2 could function as a cellular β-secretase. Therefore, the substrate preferences observed in vitro are not fully reconstituted in cells. A band that migrates faster than CTF83 (presumably CTF79) is only present in BACE2-expressing cells but not in control cells expressing Swedish mutant APP and BACE2. Farzan et al. (23) reported similar patterns to those shown here and referred to this faster migrating band as CTF79.

Consistent with the previous reports by others (18–19, 23), we also observed a dramatic reduction of secreted Aβ peptides in all the cells expressing high levels of exogenous BACE2 (data not shown). Thus, BACE2 seems to possess little β-secretase activity in these cells and more likely serves as an alternative α-secretase to exclude the formation of Aβ.

**DISCUSSION**

BACE1 and BACE2 share similar structural organization: each is a single chain aspartyl protease having both a DTG and DSG active site motif, a single type I transmembrane domain, and a short cytoplasmic tail. Even though they are two members of a unique transmembrane-spanning subfamily of aspartyl proteases, BACE1 and BACE2 display distinct cellular localization patterns and intracellular protease specificity.

The intracellular localization of endogenous or low amounts of exogenous BACE1 is more restricted to the later Golgi and TGN and minimally detectable in the early Golgi compartments. A small portion of BACE1 moves to the early endosomes or the plasma membrane probably through a process that regulates a phosphorylation on Ser498 near its C-terminal tail (13). BACE2 has a more diffuse localization pattern and is present throughout the Golgi compartments even at low expression levels. The transmembrane domains of BACE1 and BACE2 govern their cellular localization patterns.

Like many mammalian aspartyl proteases, both BACE1 and BACE2 are synthesized with a prodomain that is removed during post-translational modifications. BACE1 has never been shown to autoprocess its prodomain, whereas BACE2 may at least in part undergo self-cleavage to remove its prodomain (Ref. 24 and this study). It appears that BACE1 and BACE2 may go through different processes to regulate their optimal activity within cellular compartments.
BACE2 is mapped to chromosome 21q22.3, a Down’s Syndrome critical region (8, 20–22). Because Down’s Syndrome inherited subjects showed excessive Aβ deposition in their brains at relatively early ages because of the presence of an extra copy of chromosome 21, we and others paid particular attention to the possibility of BACE2 as an alternative β-secretase or γ-secretase. BACE1 was identified simultaneously as the β-secretase by several groups (6–10), but the role of BACE2 in APP processing remains unclear. In vitro enzymatic assays in this and other studies (19, 23) indicate that purified BACE2 cleaves β-secretase peptide substrates containing either wild-type or Swedish mutant sequences. Similar to BACE1, BACE2 preferentially cleats the Swedish mutant APP substrate in vitro. In fact, purified cathepsin D and E also cleave the Swedish mutant peptide substrate efficiently (26–27). In vitro assays also showed that BACE2 could cleave an α-secretase peptide substrate at residues AβPhe19 and AβPhe20, whereas BACE1 could not cleave the same peptide substrate (Ref. 23 and this study). Although in vitro studies suggest that BACE2 could be a potential β-secretase, its role as a β-secretase in cell-based assays is less convincing, similar to the study with cathepsin D (28). Hussain et al. (19) demonstrated that overexpression of BACE2 (also called Asp1) in SK-N-SH cells increased the production of CTF99 (called CTF9) and sAPPα. Farzan et al. (23) showed that BACE2 had a marginal effect on C99 production. In this study, we showed that the exogenous introduction of either wild-type BACE2 or a chimera containing the BACE2 protease domain into an HEK-293 derivative cell line expressing high levels of human Swedish mutant APP did not increase the β-secretase cleavage product CTF99, but did increase secreted fragments recognized by the 6E10 antibody. The lack or inefficiency of BACE2 β-secretase activity within these cells can not be attributed to low expression levels, because a similarly expressed chimera containing the BACE1 activation domain cleaved APP at the β-secretase site effectively (Fig. 8A). It is unclear whether the discrepancy is caused by differences among cell lines used by different groups. Nonetheless, overproduced BACE2 in a stable APP-expressing cell line did not have a discriminate processing of various APP forms including the Swedish mutant form (Fig. 8B), contrary to the observed cleavage by BACE1. Furthermore, the overexpression of BACE2 in APP-expressing cells was consistently shown to profoundly reduce Aβ formation (Refs. 18–19 and 23 and this study), a process that is more consistent with the cleavage of Phe19 and Phe20 near the α-secretase site. It is likely that the spacial alignment of BACE2 in cells preferentially recognizes the sequences at Phe19 and Phe20 instead of the β-secretase site. Our in vitro enzymatic assay also indicated that BACE2 cleaved the α-secretase peptide substrate containing Phe19 and Phe20 faster than the β-secretase substrate containing the wild-type sequence, although the Swedish mutant peptide seemed to be a better in vitro substrate. Because the Swedish mutant APP was identified only in two Swedish families (29) and our cellular assay did not show Swedish mutant as a preferred substrate over wild-type APP for BACE2 (Fig. 8B), it is less likely that BACE2 plays a significant role as a β-secretase in a normal situation. Farzan et al. (23) showed that BACE2 effectively cleaved the Flemish mutant APP at the β-secretase site. This shift may be related to the fact that the cleavage of its intracellular favorite site at Phe19 and Phe20 within the Aβ domain was suppressed because of the mutation of Ala21 → Gly21 in Flemish APP form.

On the other hand, BACE2 does not seem to be a major cellular α-secretase, because substantial reduction of BACE2 transcripts by specific antisense oligomers could not affect the levels of secreted Aβ peptides. Recently, members of the ADAM family (a disintegrin and metalloprotease) are shown as α-secretases (30–32). The reduction of endogenous BACE2 alone may not be sufficient to override the action by the other possible α-secretases. It is likely that APP is not the only cellular substrate of BACE2. Identification of other natural substrates of BACE2 will shed light on its bona fide physiological functions. Recently, BACE1-deficient mice were generated, and the homozygous BACE1–/–APP mouse nearly abolished the production of Aβ peptides even though BACE2 was still present (33–35). Apparently, the natural low levels of BACE2 expressed in the mouse neurons cannot complement the action by BACE1. Altogether, BACE1 seems to be a dominant intracellular β-secretase, and the blocking of BACE1 may be an effective drug target (14). BACE2 may have limited cellular β-secretase activity, and revealing its physiological function may have to wait until the knockout of BACE2 in mice.

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