

## Human BACE Forms Dimers and Colocalizes with APP\*

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**$\beta$ -Site APP-cleaving enzyme (BACE) is a membrane-bound aspartyl protease with no strict primary preference for cleavage. The molecular mechanisms that link the  $\gamma$ -secretase multicomponent amyloid precursor protein (APP) processing complex to biochemical properties of BACE generating the N terminus of the amyloid  $\beta$ -peptide have not, as yet, been identified. We found that in human brain tissue, BACE occurred as a dimer. The overall stability of the BACE homodimer was based on intermolecular interactions that were not affected by high salt, nonionic detergents or reducing conditions. BACE homodimers could only partially be separated even under strong denaturing conditions and revealed dramatic differences in the surface charge distribution compared with the monomer. In contrast, the soluble ectodomain of truncated BACE revealed a seemingly lower avidity to the prototypic aspartate protease inhibitor pepstatin and exclusively occurred in the monomeric form. Immunocytochemical studies colocalized APP and BACE in the plasma membrane of cells expressing endogenous levels of BACE and overexpressing APP. In cells that were cotransfected with APP and a putative active site D289A mutant of BACE, colocalization persisted. Remaining enzyme activity was found to be attributable to the mutant protease. Accordingly, inactivation of the carboxyl-terminal active site motif of BACE without an impairment of overall enzyme activity suggests that the enzyme may act as a dimer. Thus, homodimerization of BACE may help the enzyme to acquire specific mechanisms to associate with its substrates to exert catalytic activity.**

The amyloid precursor protein (APP)<sup>1</sup> of Alzheimer's disease is the only source of the amyloid peptide (A $\beta$ ), the major constituent of the characteristic amyloid plaques (1–3). APP is part of a superfamily from which 16 homologous amyloid precursor-like proteins and APP species homologues are derived (4, 5). APP and amyloid precursor-like proteins are substrates for two proteolytic activities designated  $\beta$ -secretase and

$\gamma$ -secretase (6–8). The molecular apparatus for the  $\gamma$ -site cleavage is quite complex and requires the presence of presenilin-1, which is believed to be the  $\gamma$ -secretase itself (9–11).

The  $\beta$ -site APP-cleaving enzyme (BACE) initiates A $\beta$  formation by cleaving at the amino terminus to generate the truncated ectodomains of APP and amyloid precursor-like proteins (8, 12–15). In addition to APP family members, other substrates discovered for BACE are the sialyltransferase ST6Gal-I, the P-selectin glycoprotein ligand-1, and A $\beta$  itself (16–18). No A $\beta$  peptides can be detected in mice with a homozygous deletion of *bace*, demonstrating the essential role in the generation of this peptide (19, 20). On the other hand, the A $\beta$  load correlates with increased  $\beta$ -secretase activity in sporadic Alzheimer's disease patients (21, 22). The elevated BACE mRNA and protein expression *in vivo* is not due to mutations in the ORF but is rather suggested to occur by a change in the translation efficiency (23, 24).

BACE is synthesized as a single type I transmembrane protein, which is subsequently modified by glycosylation, phosphorylation, palmitoylation, and a furin-like cleavage to produce mature BACE (25–28). BACE has been shown to be partially raft-localized, and a small fraction of APP has also been shown to be in lipid rafts (29–31). The cytoplasmic tail of BACE has been demonstrated to be essential for normal trafficking (32). In human SH-SY5Y neuroblastoma cells expressing endogenous or low levels of transfected BACE, the transmembrane domain of BACE is required for the optimal generation of the C-terminal fragment of APP (15). Removal of the transmembrane domain changes the appropriate processing of the substrate APP at its  $\beta$ -site and inhibits C-terminal fragment formation (15). BACE lacking its transmembrane domain remains inactive in the endoplasmic reticulum when retained by a specific signal (33).

Wild-type APP is not a preferred substrate for truncated BACE lacking the transmembrane domain, but a number of reports have documented that BACE cleaves wild-type APP *in vivo* (12, 19, 20, 34). A possible explanation is that truncated recombinant BACE has a loose substrate specificity and displays poor kinetic constants toward its known substrates as shown for APP wild-type and APP Swedish (35). BACE as part of a multiprotein complex could be more active than the soluble monomer, and oligomerization may play a critical role for enzyme-substrate interactions. Here, we report a novel possible mechanism for the regulation of BACE activity including dimerization *in vivo*, colocalization of BACE with APP and a functional elucidation of the BACE active sites. Inactivation of one-half of the enzyme by mutating one of the active sites reveals that the enzyme may act as a dimer, which will be critical for future drug design.

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<sup>1</sup> The abbreviations used are: APP, amyloid precursor protein; sAPP, secreted APP; BACE,  $\beta$ -site APP-cleaving enzyme; BACE-NT, C-terminal truncated BACE; PBS, phosphate-buffered saline; mAb, monoclonal antibody; HIV, human immunodeficiency virus.

## MATERIALS AND METHODS

**Characterization of Native BACE from Human Brain and of Soluble BACE**—Human brain tissue was obtained from the German Brain Bank "Brain-Net."

Human post-mortem brain tissue samples were homogenized with five volumes of 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2% Nonidet P-40, containing a protease inhibitor mixture (Complete tablets; Protease Inhibitors Technical Guide; Roche Applied Science). Aliquots of the homogenate were directly subjected to SDS-PAGE or used to enrich BACE by column chromatography.

After incubation for 30 min at 4 °C, samples were centrifuged at  $10,000 \times g$  for 10 min. The supernatant was applied onto a column of Q-Sepharose (column size HR10/10, FPLC; Amersham Bioscience) equilibrated with buffer A (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, without protease inhibitors). Proteins were eluted with a linear gradient of buffer A to buffer B (800 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40) within 30 min at a flow rate of 1 ml/min. Likewise, the conditioned medium from transfected HEK293 cells expressing the truncated BACE-NT derivative generated by inserting stop codons after amino acid 454 (32) was loaded onto the Q-Sepharose column and eluted as described before. Eluted fractions were analyzed for BACE content by Western blotting using the polyclonal C-terminal BACE GM168 for human BACE or monoclonal BSC-1 (35) for truncated BACE-NT as primary antibodies.

**BACE Expression Constructs**—A full-length BACE construct in pBluescript II SK(+) (Stratagene) was generated by PCR to add an EcoRI at the 5'-end and a XhoI site at the 3'-end. The forward primer used was B1-fwd (5'-GCGCGAATTCATGGCCCAAGCCCTGCCCT-3'), and the reverse primer was B1-rev (5'-CGCGCTCGAGTCACTTCAGCAGGGGAGATGT-3'), respectively. Site directed mutagenesis of BACE-D289A mutant was performed by PCR using a mismatch forward primer (5'-GACAAGAGCATTGTGGCCAGTGGCACCACC-3') and a T7 standard reverse primer. In a second amplification step, the generated "megaprimer" was used with the T3 standard primer on the same template. The second PCR product and pBluescript vector were digested with EcoRI and XhoI and then ligated. The generated constructs were digested with NotI and XhoI, and the BACE encoding region was ligated with expression vector pCDNA3.1/zeo(+). The truncated BACE construct (BACE-NT) for transfection of SH-SY5Y cells was generated by using the reverse primer (5'-GGATATCGCGGCCCGCTAGACAT-AGGCTATGGTTCATGAGGGTTGACTCATC-3'). PCR was performed together with BACE forward primer B1-fwd and full-length pBluescript BACE as template. The PCR product and pCDNA3.1/zeo(+) BACE were digested with EcoRI and NotI and then ligated.

**Cell Culture and Stable Transfection**—SH-SY5Y cells (ATCC number: CRL-2266) were stably transfected with pCEP4/c-Myc-tagged APP695 or pCEP4 using LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol with 4 µg of vector in 50% confluent 10-cm dishes. Medium was replaced after 24 h, and transfectants were selected using 200 µg/ml hygromycin or hygromycin and Zeocin-selective medium after 48 h. Stably transfected cells were subjected to cotransfection with pCDNA3.1/zeo(+) BACE and BACE-D289A with LipofectAMINE plus as described above.

**Immunoprecipitation and Immunoblotting**—Conditioned media from cotransfected SH-SY5Y cells (APP-BACE wild type and APP-BACE-D289A) were clarified by centrifugation and incubated with 3 µl/ml polyclonal antibody 879 (Novartis) and Protein A-Sepharose (Amersham Biosciences). After a 4-h incubation at room temperature with continuous end-over-end rotation, samples were centrifuged and repeatedly washed, and the precipitate was denatured and then subjected to 8% Tris/glycine PAGE (Anamed). After separation, proteins were transferred to nitrocellulose membranes utilizing a tank blot system (Bio-Rad). Filters were incubated with the monoclonal antibody 22C11 and the goat anti-mouse horseradish peroxidase conjugate (Promega), and bands were visualized using the ECL detection system (Amersham Biosciences).

Immunoblots were quantified by measuring the relative optical densities and areas of the corresponding bands using a computerized image analysis system (AlphaEase FC Software Version 3.2.1; Alpha Innotech Corp.).

**Affinity Chromatography**—BACE-containing fractions from Q-Sepharose were pooled and buffer-exchanged into 0.1% Nonidet P-40 and 1 mM MnCl<sub>2</sub> by 1:5 dilution with PBS. Lentil lectin-Sepharose (Sigma) was added and gently mixed by end-over-end rotation for at least 4 h. The lectin-Sepharose was repeatedly washed with 1× PBS and eluted with 150 mM NaCl; 10 mM Tris-HCl, pH 7.5; methyl-α-

glucopyranoside, methyl-α-mannopyranoside, *N*-acetylglucosamine, D-galactose (500 mM each); and 50 mM EDTA.

Fractions eluting from the Q-Sepharose or the lectin column were directly applied to an affinity column of pepstatin A-agarose (Sigma). The affinity column was repeatedly washed with 1× PBS and eluted with free pepstatin (88% purity grade, 0.5 mg/ml in 50% methanol/PBS) or by 70% acetic acid. Alternatively, Q-Sepharose fractions were applied to heparin-Sepharose (Sigma) and eluted with 300–700 mM NaCl in 10 mM Tris-HCl, pH 7.5.

**Anti-BACE Antibodies**—A peptide corresponding to the pro-segment of BACE (amino acids 22–45) was conjugated to keyhole limpet hemocyanin and used as antigen for production of the rabbit polyclonal antibody GM190 (32). Other peptides derived from the protease domain of BACE (amino acids 64–100, 106–139, and 279–318) and from the C-terminal domain (amino acids 482–501) were directly used as antigens for the generation of rabbit polyclonal antibodies including GM168.

The mouse monoclonal antibody BSC-1 directed against the ectodomain of BACE (35) was obtained from Dr. M. Brockhaus, and the polyclonal 879 was from Novartis. The mAb 5308 was obtained from Chemicon International (Temecula, CA). The epitope is the last 21 amino acids of the C terminus of rat BACE and shows no reactivity to BACE-2 by Western blot analysis (Chemicon).

**Two-dimensional Gel Electrophoresis**—BACE was analyzed after Q-Sepharose and size exclusion chromatography on isoelectric focusing gels with an immobile pH gradient (pH 3–10, 13 cm, IPGphor Isoelectric Focusing System; Amersham Biosciences). Protein was resuspended in 125 µl of isoelectric focusing sample buffer containing 8 M urea, 2 M thiourea, 2% (w/v) Chaps, 1% (w/v) Triton X-100, 1% (w/v) dithiothreitol, 10 mM Tris base, 2% (v/v) Pharmalyte, pH 3–10, and 2 mM Peabloc SC. The isoelectric focusing was carried out as described (36). Prior to SDS gel electrophoresis, the strips (gels) were incubated in a solution of 90 mM dithiothreitol, 6 M urea, 30% (v/v) glycerine, 2% (w/v) SDS, 50 mM Tris-HCl, pH 8.8, for 10 min and subsequently in a solution of 260 mM iodoacetamide in the same buffer for 10 min. SDS-PAGE was performed in 12.5% polyacrylamide gels. The second dimension was run until the bromophenol blue front reached the end of the gel. Gels were subjected to Western blotting with GM168.

**Electron Microscopic Immunolocalization**—COS-7 cells were stably transfected with c-Myc-tagged APP695 and grown on glass coverslips to 70–90% confluence. Coverslips were rinsed in PBS containing 1 mM MgCl<sub>2</sub> at 37 °C, and cells were fixed for 30 min in 2.0% freshly prepared formaldehyde in PBS, pH 7.4, dipped into PBS, and washed twice for 5 min with PBS, containing 50 mM ammonium chloride. Cells were incubated with primary antibodies (polyclonal antibody 40090 raised against APP homodimers was diluted 1:100 in PBS) for 4–6 h at room temperature and were incubated after three washes with PBS with the respective secondary goat anti-rabbit antibody coupled to 5-nm gold particles to detect APP. For double labeling of ectopically expressed APP and endogenous BACE (recognized by monoclonal antibody BSC-1 diluted to 100 µg/ml), a goat anti-mouse antibody conjugated to 10-nm gold particles was used. Secondary antibodies were diluted 1:3 in PBS and incubated overnight at RT. After three washes in PBS, cells were fixed for electron microscopy in 2.5% glutaraldehyde (0.05% sodium cacodylate, pH 7.2, 50 mM KCl, 1.25 mM MgCl<sub>2</sub>, 1.25 mM CaCl<sub>2</sub>) for 10 min at 4 °C followed by 30 min in 2% OsO<sub>4</sub>. Dehydration, embedding, thin sectioning, and staining were performed as described (37). Electron micrographs were taken with a Zeiss EM 10A electron microscope.

**Immunofluorescence and Confocal Laser-scanning Microscopy**—Cells were treated and fixed as described for electron microscopy and incubated with the first antibody for 1 h at room temperature using the following dilutions of primary antibodies in PBS: 1:100 for 40090 and 100 µg/ml for mAb 5308. After three washes with PBS, COS-7 cells were incubated for 1 h with the secondary goat anti-rabbit antibody coupled to fluorescein isothiocyanate (Dianova) at a dilution of 1:20 in PBS or the goat anti-mouse antibody coupled to Cy3 (Dianova) in PBS. SY5Y cells incubation was performed with goat anti-mouse antibody coupled to Cy2 (Dianova) and goat anti-rabbit antibody coupled to Cy3, each diluted 1:400. Nuclei were stained with propidium iodide added to the diluted secondary antibodies. Coverslips were rinsed in PBS three times, followed by rinsing in double distilled water and 100% ethanol and were mounted with fluoromount. Immunofluorescence was performed by confocal laser scanning with a Leica DM IRBE or a Leica DM IRB apparatus for the standard technique.



## RESULTS

**BACE Is a Dimer in Human Brain**—BACE defines a novel family of aspartic acid proteases by possessing a carboxyl-terminal and a transmembrane domain. These carboxyl-terminal extensions are absent in single chain eukaryotic aspartic proteases. To study the significance of the extra domains, the expression pattern of endogenous BACE on the protein level was analyzed under *in vivo* conditions. We used a polyclonal antibody raised against the C-terminal peptide of BACE. This antibody was specific for BACE as characterized by Western blotting. Human brain specimens were homogenized using a combination of detergent extraction and Dounce homogenization. Immunoblotting analyses of the  $10,000 \times g$  supernatant fraction (containing the detergent-soluble extract) and of the  $10,000 \times g$  pellet (containing the detergent-insoluble extracts) were performed with C-terminal or pro-peptide domain-specific BACE antibodies. Western blot analysis revealed the appearance of two polypeptides, each migrating as doublets of  $\sim 135$  and  $\sim 120$  kDa in the soluble extract and an additional band of  $\sim 110$  kDa, which was exclusively found in the pellet (Fig. 1A). The well characterized antibody GM190 to the pro-peptide domain of BACE detected the same bands of  $\sim 135$  and  $\sim 110$  kDa but was unable to recognize the  $\sim 120$ -kDa form, which accordingly corresponds to an oligomeric BACE complex lacking the pro-peptide domain (Fig. 1A). This indicates that BACE already dimerizes before maturation and pro-peptide cleavage.

The majority of BACE accumulated in the pellet with an apparent molecular mass of  $\sim 110$  kDa, a molecular weight corresponding to the dimeric unmodified molecule from the endoplasmic reticulum (32). In addition to the oligomeric forms of BACE, we also consistently found smaller amounts of a  $\sim 65$ -kDa polypeptide in the  $10,000 \times g$  supernatant, representing the monomeric form of holo-BACE (Fig. 1A). Notably, the level of this form was highest in the soluble extract. In the presence of reducing agent, BACE migrated as the characteristic doublet band (38).

The presence of SDS-stable dimers of BACE was also analyzed within the crude homogenate of various regions of human brain (Fig. 1B). In the different brain regions analyzed, the majority of BACE immunoreactivity is found in Alzheimer's disease-vulnerable regions, such as the frontal cortex, occipital cortex, and hippocampus. A much lower portion was observed in nonvulnerable regions, such as rostral pons, medulla oblongata, and cerebellum. This finding suggests that the enzyme is the crucial factor for  $A\beta$  production independent of the supply of substrate. This is in agreement with increasing BACE activity levels with aging in the brain of different species that were suggested to be due to age-related posttranslational changes, an allosteric modulation of BACE activity (39), or to the formation of a high complex of BACE that is more active than the monomer (31).

**Characterization of BACE from Human Brain Tissue**—To achieve an enrichment of BACE and a separation of monomers and putative dimers, Q-Sepharose chromatography was performed (Fig. 2A). The pattern of BACE from human tissue separated by anion exchange chromatography showed immunoreactivity in an ascending salt gradient between 550 and 800 mM NaCl (Fig. 2A). Smaller species started to elute first together with the monomer migrating uniquely as a  $\sim 62$ -kDa band and the dimer as a  $\sim 120$ -kDa band. Separation of monomeric and dimeric BACE was achieved by a gradient elution at high salt (Fig. 2A), whereas monomers and dimers as well as initially co-fractionated dimers could be separated without showing any dissociation of subunits during purification at high salt concentrations. The quantification of dimeric and monomeric BACE by measuring the relative optical densities

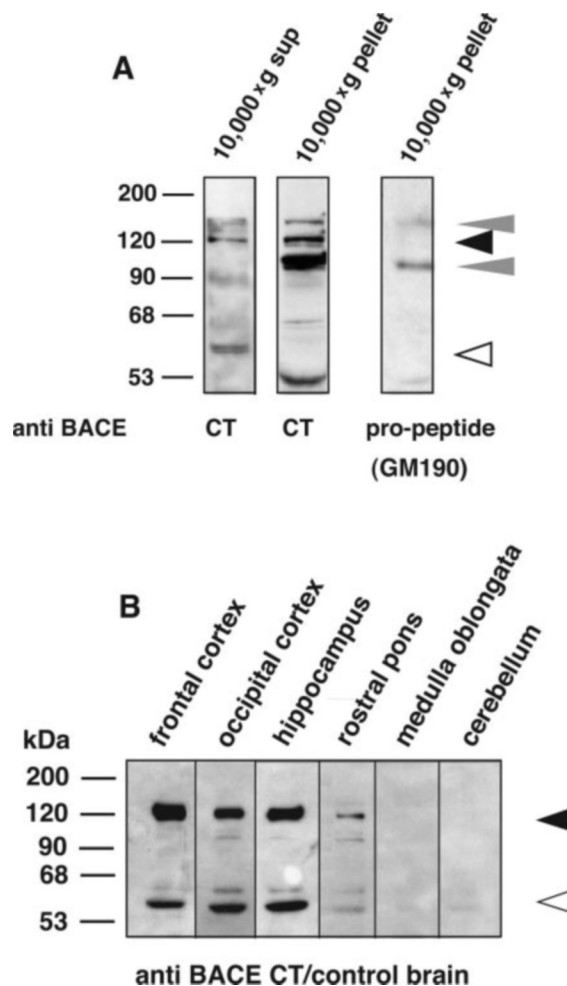
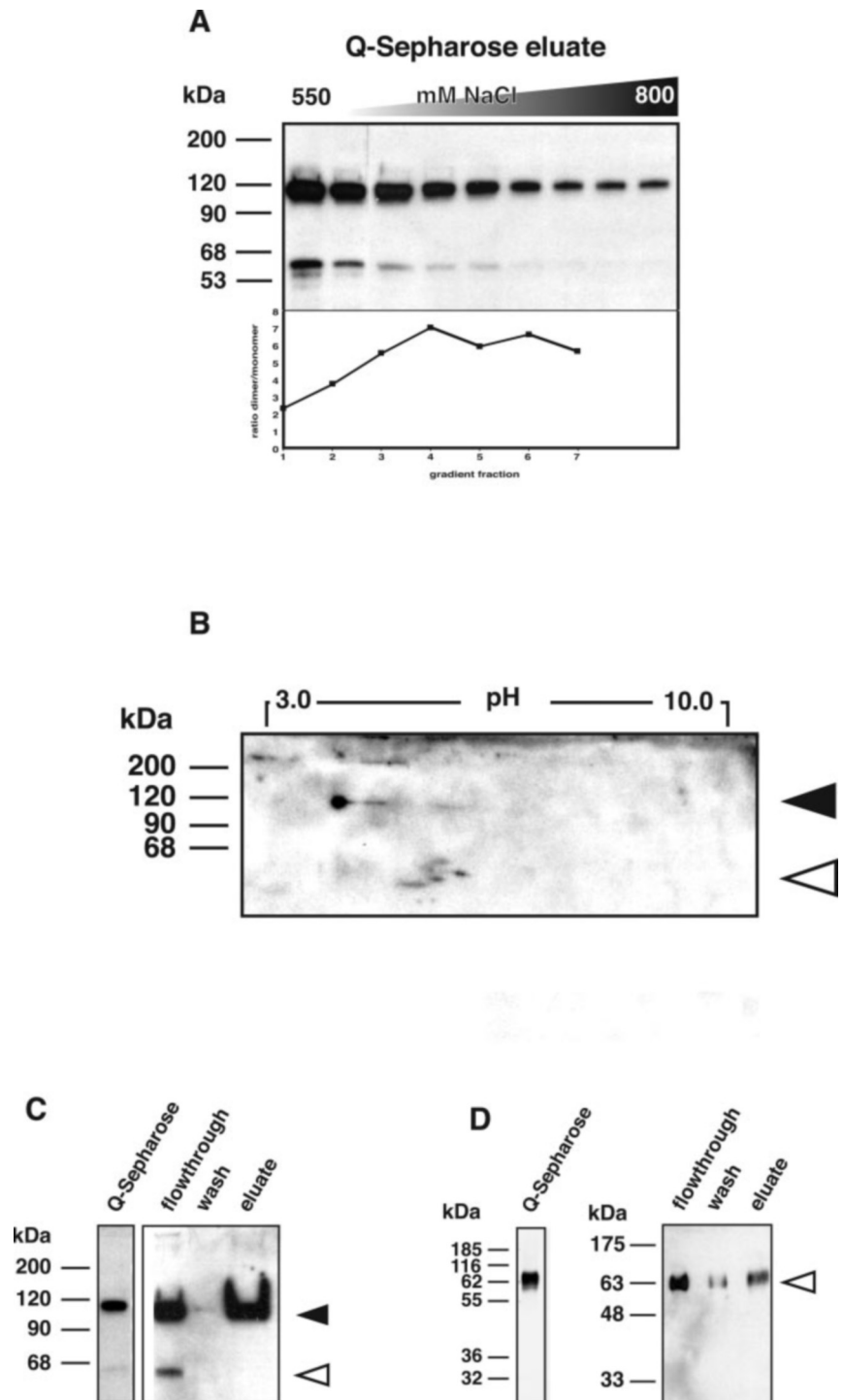


FIG. 1. A, Western blot of detergent-solubilized BACE ( $10,000 \times g$  supernatant (*sup*)) and detergent-insoluble BACE ( $10,000 \times g$  pellet). Detection was performed with the C-terminal polyclonal antiserum GM168 (CT) and the pro-peptide antibody GM190. The closed small arrow marks dimeric BACE that is partially solubilized after the detergent treatment. The large gray arrows (top) point to immature dimeric forms of BACE ( $\sim 110$  kDa) and mature BACE forms ( $\sim 135$  kDa), which both still contain the pro-peptide as evidenced by GM190 reactivity. Monomeric BACE (open arrow) was detected as a less prominent band and exclusively observed in the supernatant. A specific enrichment of pro-peptide species of immature BACE was detected in the  $10,000 \times g$  pellet, whereas mature dimeric BACE was found in the soluble and the pellet fraction (closed arrow). B, Western blot of total brain homogenate of various regions with the C-terminal polyclonal antiserum GM168.

and the areas of the bands revealed a 3-fold ratio increase of dimer/monomer during the salt gradient elution (Fig. 2A). This together with the delayed elution indicated a major shift in surface charge distribution correlating with a stable oligomeric state of BACE.

To further determine the stability and the differences in surface charge distribution, those fractions that were identified by immunoblotting to contain exclusively the SDS-resistant  $\sim 120$ -kDa dimer of BACE were pooled and subjected to two-dimensional electrophoresis (Fig. 2B). Dimers could at least partially be converted into monomers. BACE appeared as a spot at pI 4.0 with other charge species tailing from the theoretically expected pI 4.7 to 5.0. Upon sample treatment with urea and high concentrations of SDS, BACE could only partially be monomerized and was observed as scattered spots around  $\sim 65$  kDa, slightly shifting to pI 5.0 in the monomeric form (Fig. 2B). The overall stability of the BACE dimer suggests strong intermolecular interactions, which are affected

**FIG. 2. Characterization of human BACE.** Detergent-solubilized proteins were applied to a Q-Sepharose column, eluted with a linear gradient from 0.15 to 0.8 M NaCl, and fractions were evaluated for their content of BACE by immunoblotting with polyclonal anti-BACE GM168 after SDS-PAGE on a 12.5% polyacrylamide-resolving gel and transfer to nitrocellulose. BACE was detected in fractions eluting between 550 and 800 mM NaCl (A), with monomeric (~62-kDa) and dimeric (~120-kDa) forms eluting first together before higher salt concentrations allowed us to separate the dimer from the monomer. The NaCl concentration (ranging from 550 to 800 mM) is indicated at the top. The partial conversion of solubilized human BACE dimers into monomers and the pI of both isoforms were analyzed by two-dimensional gel electrophoresis (B). The closed arrowhead marks the position of the BACE dimer, and the open arrowhead points to the charge isoforms of BACE monomers as observed by Western blotting with GM168. The deviation of the monomer from the theoretical isoelectric point of 4.7 and the scattered appearance may result from extensive posttranslational modifications that may be otherwise hidden within the dimer interface. C and D, binding of BACE to pepstatin-agarose (C and D). Partially purified human BACE dimers (C) or soluble truncated BACE monomers (D) containing fractions eluted from Q-Sepharose were assayed for BACE content by Western blotting using the C-terminal specific polyclonal antibody GM168 or the monoclonal BACE antibody BSC-1 for soluble BACE. Most of human BACE eluted from pepstatin-agarose as a dimer (C), whereas the majority of soluble BACE lacking the transmembrane and the cytoplasmic domains is found as a monomer in the flow-through fraction (D). The closed arrowheads mark the position of the dimer, and the open arrowheads indicate the position of the monomer, respectively. Note that the majority of BACE from human brain elutes as a dimer, whereas soluble BACE exclusively exists as a monomer (compare C and D).



neither by high salt nor nonionic detergents and are resistant to reducing conditions, strong denaturing conditions of boiling in SDS, and the presence of urea. Most likely, the dramatic differences in surface distribution also imply that hydrophobic regions must be buried within the interior of the protein dimer. Obviously, this difference is enhanced by dimerization of BACE.

**Affinity Chromatography of BACE Dimers and BACE-NT Monomers**—BACE dimers enriched by ion exchange chromatography bound to lentil lectin-Sepharose, indicating that BACE has *N*-linked carbohydrate chains with  $\alpha$ -D-glucose and  $\alpha$ -D-mannose residues and with high affinity to heparin, thus further supporting the positive charge geometry as observed before (data not shown; Fig. 2B). To gain information on the

specificity of a possible BACE-inhibitor interaction, ion exchange chromatography-enriched BACE was incubated with pepstatin A-agarose beads. Under saturating conditions using a Q-Sepharose fraction containing BACE dimers (Fig. 2C), the ~62-kDa band appeared together with the dimer in the flow-through fraction of the proteinase inhibitor column, indicating that BACE dimers were partially monomerized during the incubation with pepstatin A (Fig. 2C). The BACE dimer was selectively purified in the eluate fraction (Fig. 2C). Whereas the least specific inhibitor for soluble BACE was reported to be pepstatin (35), also truncated BACE (BACE-NT) obtained from BACE-NT HEK293 cell culture supernatant and enriched by ion exchange chromatography was observed to bind to pepstatin A-agarose beads (Fig. 2D). Nevertheless, it possessed a

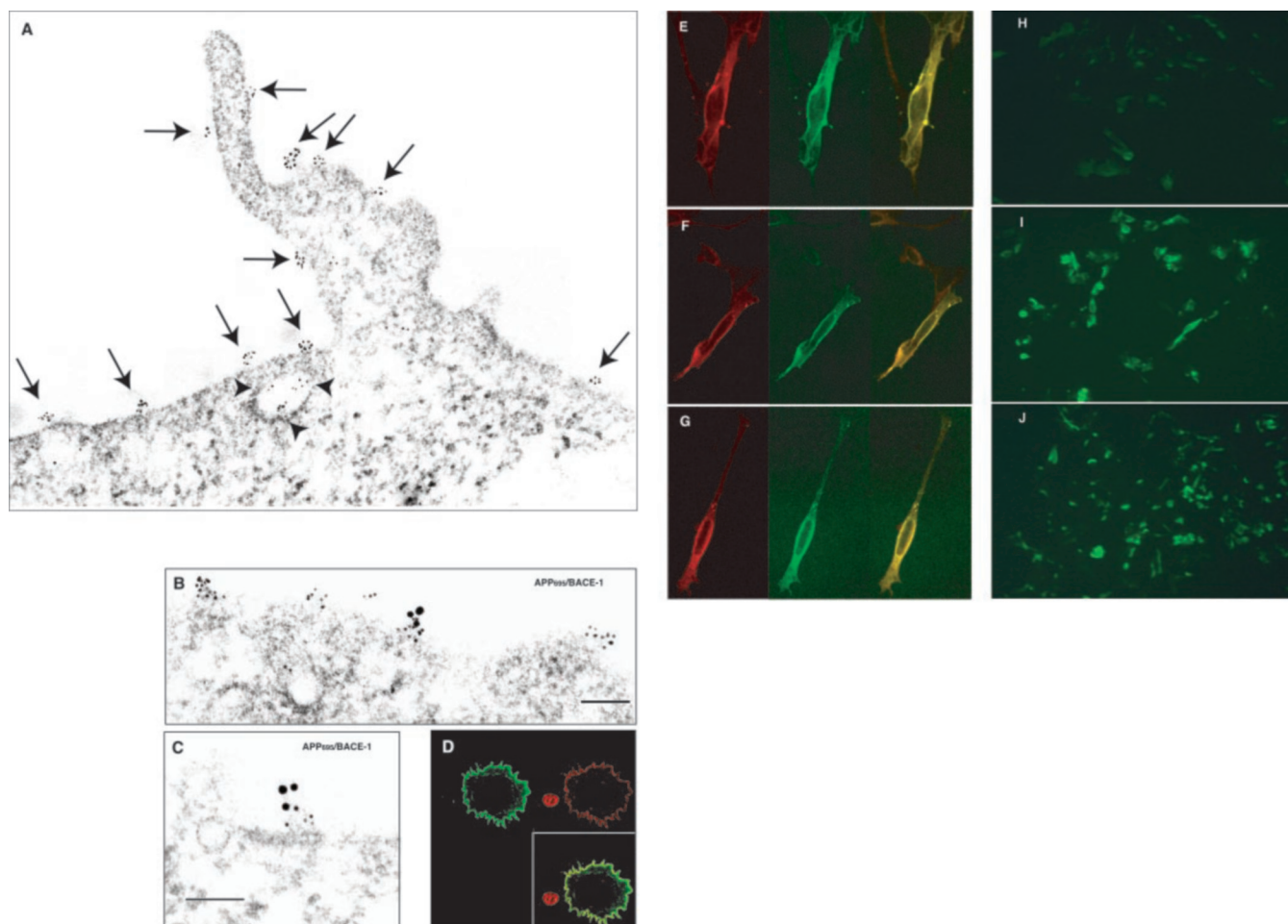


FIG. 3. *A–D*, immunoelectron microscopy and immunofluorescence of APP and BACE with APP<sub>695</sub> stably transfected COS-7 cells. Surface labeling occurred in patchlike structures (*large arrows*) and in cytoplasmic vesicles (*small arrows*) (*A*). A co-localization of APP with endogenous BACE occurred in membrane patches (*B* and *C*). APP<sub>695</sub>, 5-nm gold particle; BACE, 10-nm gold particle; bar, 100 nm. Colocalization of overexpressed APP and endogenous BACE is shown by immunofluorescence (*D*). APP was visualized with the primary antibody 40090 raised against APP homodimers followed by fluorescein isothiocyanate-conjugated secondary anti-rabbit antibody (*green*). BACE was detected by mAb 5308 followed by Cy3-conjugated secondary anti-mouse antibody (*red*). Fluorescence was performed by confocal microscopy, and *yellow fluorescence* represents digitally merged images and indicates the extent of co-localization of BACE and APP immunoreactivity (*D*). *E–J*, the detection of APP and BACE in SH-SY5Y cells was performed by confocal microscopy (*E–G*). The staining of overexpressed APP<sub>695</sub> and endogenous BACE is shown in *E*, staining of overexpressed APP<sub>695</sub> and overexpressed BACE D298A mutant is shown in *F*, and staining of overexpressed APP<sub>695</sub> and overexpressed BACE wild type is shown in *G*. APP was visualized with primary antibody 40090 followed by Cy3-conjugated secondary antibody (*red*). BACE detection was performed with primary monoclonal antibody mAb 5308 and secondary Cy2-conjugated antibody (*green*). A standard fluorescence technique was used to visualize BACE immunoreactivity of endogenous BACE (*H*), of overexpressed BACE D289A mutant (*I*), and of overexpressed BACE wild type (*J*) in the low magnification view (*H–J*). The images are representative of 3–6 cell images/field.

seemingly lower avidity to pepstatin A than human BACE, since the signal was present in the wash fraction and highest in the flow-through fraction (Fig. 2*D*). When the immunoblot shown in Fig. 2*C* was reprobed with two monoclonal antibodies, 22C11 recognizing N-terminal APP and W0–2 against A $\beta$  residues 1–11 (40), secreted APP $\beta$  (sAPP $\beta$ ) was found to co-purify on pepstatin A-agarose beads (data not shown).

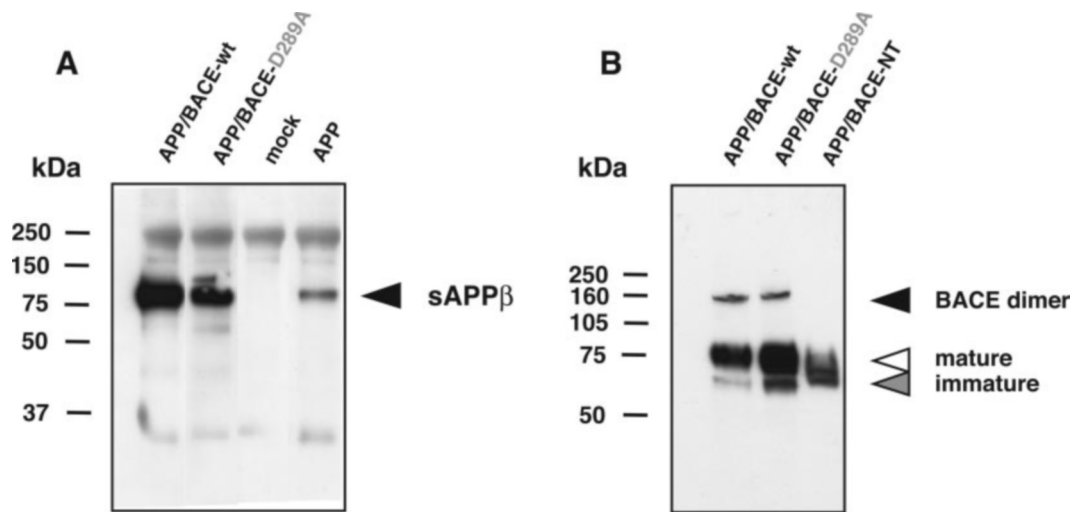
Interestingly, BACE-NT was exclusively observed as a monomer during the purification procedure. Together with the finding that dimers were absent in the eluate of BACE-NT, it can be concluded that human BACE dimerization is physiological, occurs *in vivo*, and is not generated through high local concentrations when bound to Sepharose or agarose beads.

**Colocalization of BACE and APP**—There is not much known about the preferences of membrane-bound BACE for substrate and inhibitor binding. If cleavage of integral membrane APP by BACE occurred, endogenous BACE and overexpressed APP should co-localize at the cell surface. Whereas monoclonal antibodies directed against A $\beta$  residues 1–10 seem to be suitable for cell surface immunostaining of APP, polyclonal antisera to

native APP, either purified from human tissue (41) or dimeric APP from yeast cells (42), are even superior. Immunogold electron microscopy was used to study the membrane localization of APP and endogenous BACE in COS-7 cells stably expressing exogenous APP<sub>695</sub> with a polyclonal antibody (40090) directed against the N-terminal domain of APP homodimers. APP could be stained in microdomain structures on the external surface projecting outward from the cell surface. Thus, APP was localized in groups or patches on the external surface of the transfected COS-7 cells (Fig. 3*A*). Since recent work has shown BACE to be partially raft-localized (29, 43) and a small fraction of APP has also been shown to be present in lipid rafts (44, 45), either the polyclonal antibody used preferentially recognized APP in rafts, or the patch-like structures are novel specific regions of cell membranes.

Double labeling using small (APP) and large (BACE) gold particles clearly identified the presence of exogenous APP and endogenous BACE in most of the patchlike structures on the plasma membrane (Fig. 3, *B* and *C*). BACE always localized together with exogenous APP at the same structures, but APP





**FIG. 4. Processing of APP by the BACE mutant D289A.** sAPP $\beta$  was detected by immunoprecipitation from APP/BACE wild type and APP/BACE-D289A-co-transfected and APP-transfected SH-SY5Y cells with the sAPP $\beta$ -specific polyclonal antibody 879 and detected with the monoclonal antibody 22C11 (A). In control SH-SY5Y cells (lane mock), sAPP $\beta$  was not detectable, whereas in APP-transfected cells, APP was processed to sAPP $\beta$  by endogenous activity of BACE (lane APP). Note that almost equal amounts of sAPP $\beta$  could be immunoprecipitated and detected from APP/BACE wild type- and APP/BACE-D289A mutant-transfected cells, indicating that the mutant was functional through the remaining active site residue Asp<sup>93</sup>. BACE dimer detected with the monoclonal BSC-1 antibody in lysates from SH-SY5Y cells migrates as a ~140–160-kDa band on a 12% Tris/glycine gel, and the monomer has an apparent molecular mass of ~70 kDa (immature) to ~75 kDa (mature) (B). Note that in lysates of APP/BACE wild type-transfected and in APP/BACE-D289A mutant-co-transfected cells, the dimer was present, but it was absent in APP/BACE-NT-transfected cells.

labeling was also found outside of these structures. These are approximately  $\leq 70$  nm in size, similar to rafts, contained most likely the “APP-BACE complex,” and are clearly different from caveolae, which are morphologically defined as flask-shaped, noncoated invaginations of the plasma membrane (46).

The localization of APP and BACE in COS-7 and in SH-SY5Y cells was also examined by double-staining fluorescence (Fig. 3, D–G). Antibodies were directed against recombinant native APP dimers and to the C terminus of BACE. In COS-7 cells, the antibody to BACE (red) revealed a partial colocalization (yellow) with plasma membrane APP (green) (Fig. 3D). The staining of SH-SY5Y cells showed a pronounced cell surface expression of endogenous BACE (green) and transfected APP695 (red) (colocalization is shown in yellow in Fig. 3E), transfected mutant BACE with the carboxyl-terminal uncommon active site motif DSGT modified into ASGT (green) and transfected APP695 (red) (colocalization is shown in yellow in Fig. 3F), and transfected wild-type BACE (green) and transfected APP695 (in red) (colocalization is shown in yellow in Fig. 3G). In the low magnification view, a considerable difference in staining intensity between vector-transfected (Fig. 3H) and BACE-transfected SH-SY5Y cells was observed using standard immunofluorescence techniques (Fig. 3, I and J). This observation indicates that BACE-D289A was expressed equally well compared with the wild-type enzyme, which endorses the use of nontagged BACE constructs. Since confocal microscopy is not suitable for quantification, differences in signal intensity of endogenous and overexpressed BACE were determined by standard fluorescence microscopy (Fig. 3, H–J).

Interestingly, by far most of APP accumulated on the plasma membrane, implying that full-length APP is normally present at the plasma membrane recognized by a rabbit antiserum to native recombinant APP dimers purified from yeast cell culture supernatant. This demonstrates that APP and BACE strongly interact within the cell membrane, and the interaction is not significantly impaired if one of the putative active site motifs (residue Asp<sup>289</sup> within the carboxyl-terminal active site motif DSGT) is mutated.

**Processing of APP by Putative BACE Dimers**—To further address the question of whether or not BACE dimers can have

enzymatic activity, we analyzed a mutant construct based on the following hypothesis. *In vivo*, the N-terminal DTGS active site motifs of dimeric BACE could be used instead of the carboxyl-terminal active site motif DSGT within a single polypeptide chain (see Figs. 4 and 5). Accordingly, site-directed mutagenesis was performed to generate the BACE-D289A mutant and to transfect SH-SY5Y cells. This aspartic peptidase then represents a single domain and should no longer be active in the monomeric form, because two active site motifs are needed within a single polypeptide chain except when the defect is rescued by a second molecule with another N-terminal DTGS active site motif.

Indeed, enzymatic activity of mutant BACE-D289A in comparison with wild-type BACE and control cells was measured by specifically detecting the soluble cleavage product sAPP $\beta$  from culture supernatants of APP co-transfected cells using the neopeptide-specific polyclonal antibody 879 (Fig. 4A). There was no gross change in BACE expression and its enzymatic activity between wild-type and mutant BACE, as is evident from sAPP $\beta$  products that accumulated over 3 days in the supernatant. In contrast, in control cells expressing APP with endogenous BACE levels, sAPP $\beta$  was clearly diminished and could not be detected under the conditions used in vector-transfected cells. Thus, we conclude that the BACE-D289A mutant was functional through the N-terminal motifs after dimerization, as shown in Fig. 5. BACE expression and dimerization of BACE was analyzed in cell lysates of SH-SY5Y cells transfected with APP/BACE wild type, APP/BACE-D289A, and APP/BACE-NT, the last also employed as an *in vitro* assessment for complex formation (Fig. 4B). Similar levels of BACE dimers could be detected in wild-type and mutant-transfected cells but were absent in BACE-NT-transfected cells.

#### DISCUSSION

Very little is currently known about the cellular mechanisms regulating the activity of BACE. The findings of the present study relate to the general problem of understanding APP secretase(s) interaction, which is of paramount importance for therapeutic approaches. A polyclonal antibody against the C terminus of BACE revealed the presence of SDS-stable dimers

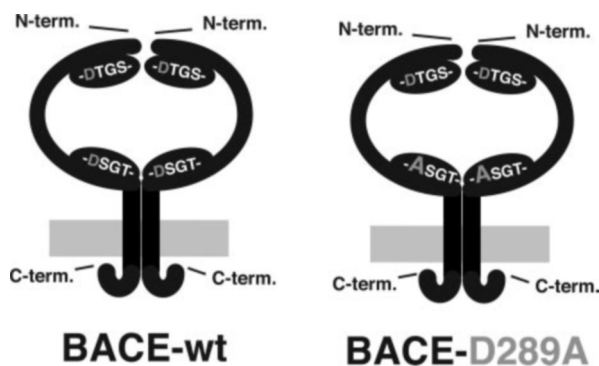


FIG. 5. **Possible active site motifs of BACE.** The BACE polypeptide sequence contains two active site motifs that are characteristic for aspartic acid proteases and represents the first example of a transmembrane aspartic acid protease with a short cytosolic tail. Oligomerization of BACE mainly occurs through the transmembrane and the cytoplasmic domains. The carboxyl-terminal active site motif of BACE is DSGT (mutated to ASGT in BACE-D289A) instead of DTGS within the pepsin family. According to our hypothesis and similar to retroviral aspartic acid proteases, BACE could be active as a homodimeric enzyme by using two DTGS motifs of the dimer instead of a DSGT and one DTGS of the same chain. This mechanism could represent an evolutionary link between ancient homodimeric enzymes and single polypeptide chain eukaryotic aspartic acid proteases, such as renin and cathepsins.

of BACE in human brain. BACE dimers were also detected by a pro-peptide antibody and by a monoclonal antibody in SH-SY5Y cells. Obviously, BACE dimers preexisted under physiological conditions in tissue and were not formed during the detergent lysis, since truncated BACE from cell culture supernatant remained stable in its monomeric form through the different chromatographic analyses.

The biochemical features of BACE observed upon detergent solubilization and purification from human brain were consistent with that of a dimeric integral membrane protein. This observation together with the partial conversion of dimeric BACE into monomers from human brain during isoelectric focusing electrophoresis further confirmed the existence of BACE homodimers *in vivo*. Previously suspected findings on homodimerization of recombinant and transfected BACE were indirectly reported when small amounts of BACE were observed to migrate as higher molecular weight species reflecting some degree of dimerization (38). Also, when expressed with a C-terminal hexahistidine tag in Sf9 cells, ectodomain forms of BACE were secreted as proenzymes and occurred exclusively as monomers. Purified full-length BACE showed an additional band at 110 kDa, corresponding to a dimer (35). When membrane fractions were washed with sodium carbonate, this procedure revealed the presence of BACE dimers in HEK293 BACE-transfected cells, although not referred to by the authors (47). More recently, a putative dimer has also been reported to occur in BACE transfected HEK293 cells, which was insensitive to reducing agents and under strong denaturing conditions (48). Also, a high molecular weight complex of BACE was reported to be more active than the monomer and found enriched in lipid rafts fractions from brain membranes (31). Currently, it is believed that BACE dimers are assembled in the endoplasmic reticulum (49), although it is not much known how SDS-resistant dimers are formed. Since we and others provide increasing evidence that SDS-resistant BACE dimers exist (31, 48), a potential discrepancy in the stability of BACE dimers between published reports and a parallel study by Westmeyer *et al.*<sup>2</sup> might be explained by the use of antibodies directed

against different domains, which may directly or indirectly contribute to the homophilic binding.

The co-localization of APP and endogenous BACE in COS-7 cells in the newly described patchlike structures by electron microscopic studies and of mutant BACE and APP in SH-SY5Y cells as revealed by immunostaining indicate a physical association of full-length BACE with APP in the plasma membrane. This suggests that the plasma membrane is either the site where BACE dimerization is manifested or the main site of BACE activity itself. This is an important consideration, since pro-BACE can produce significant quantities of C99 in the endoplasmic reticulum, and also the expression of the soluble form can lead to enhanced levels of A $\beta$  (25).

In agreement with the data provided by a parallel study,<sup>2</sup> we conclude that the ectodomain may be sufficient for dimerization but the unusual C-terminal transmembrane domain helps to dimerize BACE *in vivo* and to colocalize with APP within the cellular membrane. Thus, BACE dimerization may serve to regulate the enzymatic activity on a post-translational level. Consistent with the membrane association of endogenous BACE is the compartmentalization of BACE into noncaveolar lipid rafts that has recently been published for different types of recombinant BACE (29, 43).

A homodimer-induced redistribution of transmembrane BACE is reminiscent of retropepsins. These retroviral proteases are homodimeric enzymes, such as the integrase, the proteinase, and the reverse transcriptase (50–52). As we observed for human BACE, the dimeric form of HIV proteinase is strongly stabilized against dissociation, which is immeasurably low (52). The oxidizable amino acids Met<sup>95</sup> at the C terminus of HIV-II and Cys<sup>95</sup> of HIV-I have been identified as key residues within the dimer interface (49). A sequence alignment of the amino acid sequence of HIV-II and of BACE shows that a sequence motif including Met residue 95 (ICALFM) has some homology to the transmembrane domain of BACE (LTALGM), which also contains a Met residue. Thus, BACE, like other eukaryotic aspartic acid proteases, has a single polypeptide chain but may have adopted the mechanism from evolutionary more ancient retroviral single lobe proteases to acquire a specific catalytic activity by oligomerization through the transmembrane and the cytoplasmic domain, which is different from the activity of soluble BACE. In addition, the predominant role of the BACE prodomain appears to be in efficient folding and trafficking of the protein through the endomembrane system rather than regulating activity (54).

A still enigmatic problem for drug development is the unexplained extended substrate pocket in soluble BACE, which displays poor kinetic constants toward its normal APP substrate and Swedish mutant APP (35). Activity analysis of the soluble proenzyme revealed little similarity with respect to the substrate preference *in vivo* (25, 35, 54, 55). Here, we postulate the appealing hypothesis that *in vivo* two N-terminal DTGS active site motifs of dimeric BACE molecules could be used instead of the carboxyl-terminal active site motif DSGT within a single polypeptide chain (Fig. 5). This is strongly supported by the mutant construct BACE-D289A, which retained its activity as observed from the cleavage product sAPP $\beta$  and is similar to wild-type APP in SH-SY5Y-transfected cells. Together with our finding of a tight binding of dimeric BACE and a loose binding of BACE-NT to pepstatin, this would also explain why monomeric BACE-IgG fusion protein was not significantly inhibited by pepstatin (12). More evidence for dimeric BACE displaying a higher affinity toward a Swedish APP-like substrate than monomeric BACE-NT was noted in a cellular approach when kinetic analysis of natively purified BACE dimer revealed a higher turnover rate than soluble monomeric

<sup>2</sup> G. G. Westmeyer, M. Willem, S. F. Lichtenthaler, G. Lurman, G. Multhaup, I. Assfalg-Machleidt, K. Reiss, P. Saftig, and C. Haass, submitted for publication.

BACE-NT. These results raise interesting questions about the functional role of BACE and the notable difference from other aspartyl proteases that the BACE active site is more open and less hydrophobic.

Taken together, a correct orientation between BACE and APP as a substrate within the membrane bilayer is most likely required for BACE activity *in vivo*. The sequence of the BACE transmembrane domain alone may not fully determine BACE protease specificity but the specific assembly of the complex. Thus, the transmembrane domain represents a novel target for modulating secretase activity in APP processing. Regulating subcellular localization of BACE by influencing dimerization may provide an alternative route to control substrate preference *in vivo* and the turnover rate. Moreover, catalytic activity of BACE may be regulated by oligomerization through the cytoplasmic domain as it has been shown to occur for the multimeric complexes of membrane-type 1 matrix metalloproteinase (49). Thus, enhanced BACE activity may not only be regulated by the stability of the mRNA or at the level of translation but also through the homophilic interaction of BACE.

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