Several lines of evidence implicate lipid raft microdomains in Alzheimer disease-associated β-amylloid peptide (Aβ) production. Notably, targeting β-secretase (β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1)) exclusively to lipid rafts by the addition of a glycosylphosphatidylinositol (GPI) anchor to its ectodomain has been reported to elevate Aβ secretion. Paradoxically, Aβ secretion is not reduced by the expression of non-raft resident BACE1-4C/A (C474A/C478A/C482A/C485A)). We addressed this apparent discrepancy in raft microdomain-associated secretion of membrane-tethered C-terminal fragment (CTF), termed α-secretase (1). γ-Secretase cleavage of +11 β-CTF and α-CTF generates N-terminally truncated Aβ-related peptides found in cerebral spinal fluid and media conditioned by cultured cells, termed +11 Aβ and p3, respectively (8–10).

In cultured cells, BACE1 localizes to late Golgi/TGN and endosomes at steady state, and a small percentage of BACE1 cycles between the cell surface and endosomes (11, 12). BACE1 processing of APP is observed in multiple compartments, including ER, Golgi apparatus, TGN, and endosomes (13–15). Subcellular localization of BACE1 is cell type-dependent and insensitive to subcellular localization of APP or the pathogenic KM/NL mutant. We conclude that the apparent elevation in Aβ secretion by BACE1-GPI is mainly attributed to preferential cleavage at the β-site and failure to detect +11 Aβ species secreted by cells expressing WTBACE1.

Alzheimer disease is an age-associated neurodegenerative disorder pathologically characterized by the cerebral deposition of 38–42-amino acid-long β-amylloid peptides (Aβ) in senile plaques. Aβ is generated by the sequential proteolysis of a large type I transmembrane protein, termed amyloid precursor protein (APP), by BACE1 (β-site APP-cleaving enzyme 1) and γ-secretases (1). BACE1 is a transmembrane aspartyl protease that cleaves APP within the ectodomain at major cleavage site between Met536 and Asp597 (β-site or +1 cleavage site) (2–5). BACE1 processing results in the release of the large APP ectodomain (β-cleaved soluble APP (sAPPβ)) and the generation of membrane-tethered C-terminal fragment (CTF), termed β-CTF (6). In a subsequent step, β-CTF is cleaved within the transmembrane domain by γ-secretase to release Aβ peptides (7). In addition to the β-site, BACE1 can also cleave APP within the Aβ domain between Tyr606 and Glu607 (β'-site or +11 cleavage site), releasing sAPPβ' and +11 β-CTF. In an alternate processing pathway, APP can be cleaved within the Aβ domain between Lys16 and Leu17 by a set of enzymes, termed α-secretases, which release sAPPα and generate α-CTF (1). γ-Secretase cleavage of +11 β-CTF and α-CTF generates N-terminally truncated Aβ-related peptides found in cerebrospinal fluid and media conditioned by cultured cells, termed +11 Aβ and p3, respectively (8–10).

In cultured cells, BACE1 localizes to late Golgi/TGN and endosomes at steady state, and a small percentage of BACE1 cycles between the cell surface and endosomes (11, 12). BACE1 processing of APP is observed in multiple compartments, including ER, Golgi apparatus, TGN, and endosomes (13–15). Subcellular localization of BACE1 contributes to the relative efficiency of APP cleavage at β'-site (13). For example, ER retention of BACE1 enhances APP β cleavage, whereas targeting of BACE1 to TGN increases cleavage at β'-site (13). Furthermore, overexpression of BACE1 promotes cleavage at
β′-site (2, 14, 15). Neurons express high levels of BACE1, and consistent with a correlation between BACE1 expression levels and the efficiency of β′ cleavage, cultured primary neurons predominantly secrete +11 Aβ species (10). Neurons cultured from BACE1 knock-out embryos lack secretion of +1 and +11 Aβ species (5).

Multiple lines of evidence suggest the involvement of cholesterol- and sphingolipid-rich membrane microdomains, termed lipid rafts, in amyloidogenic processing of APP (for a review, see Ref. 16). A subset of BACE1 associates with lipid raft domains (17, 18). Targeting BACE1 lumenal domain to lipid rafts by the addition of a GPI anchor has been reported to increase Aβ production (19). Recently, we found that S-palmitoylation at 4 Cys residues (Cys474/Cys478/Cys482/Cys485) in juxtamembrane domain is required for lipid raft association of BACE1 (20). Surprisingly, displacing BACE1 from the lipid rafts by the Ala mutation of these 4 Cys residues had no influence on APP processing and Aβ production in cultured cell lines (20). This later finding is in contrast to the data from the analysis of BACE1-GPI chimera, which was thought to increase Aβ production due to its ability to exclusively associate with lipid rafts (19). To resolve this conundrum, we further explored the mechanism by which the BACE1-GPI elevated Aβ production. We found that, unlike WTBACE1 and BACE1-4C/A (C474/478/482/485A), overexpression of BACE1-GPI increased cleavage at β′-site but not at β′-site, resulting in the secretion of increased levels of intact Aβ. However, extensive analysis of cells expressing WTBACE1, non-raft-localized S-palmitoylation-deficient BACE1-4C/A, and predominantly raft-associated BACE1-GPI showed similar levels of sAPPβ secretion into the conditioned medium, establishing no difference in the efficiency of APP processing. Thus, our data reveal that the apparent increase in Aβ production by BACE1-GPI previously attributed to raft-associated APP processing is a result of compromised cleavage at β′-site and the inability to experimentally detect +11 Aβ species.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—Plasmids encoding mouse BACE1-GPI and C-terminally FLAG epitope-tagged WTBACE1 and BACE1-4C/A cDNA have been described (20). BACE1 cDNAs without the epitope tag were generated by PCR. The cDNA encoding BACE1-hephaestin chimera (BACE1-HEP) was constructed by overlap PCR by replacing the transmembrane domain and cytosolic tail of BACE1 with that of mouse hephaestin (Image Clone 1546111, Open Biosystems). Plasmids encoding human BACE1-GPI (with GPI anchor region cloned from carboxypeptidase M) and the corresponding WTBACE1 were provided by Dr. Nigel M. Hooper, University of Leeds, UK (19). Plasmids encoding BACE1-GPI (with GPI anchor region cloned from CD59) and the corresponding WTBACE1 encoding human BACE1-GPI (with GPI anchor region cloned from flotillin-2 (BD Biosciences), and anti-V5 (Invitrogen). Polyclonal antibodies specific for sAPPβ and sAPPβ were kindly provided by Dr. Maria Z. Kounnas, NeuroGenetic Pharmaceuticals Inc., San Diego, CA. Monoclonal antibodies (mAbs) 26D6 and B436 (epitope within residues 1–12 of Aβ) react with the N-terminal region of Aβ and also recognize sAPPα (20). mAb B608 (epitope within residues 30–40 of Aβ) reacts specifically with Aβ40. The following mAbs were purchased from commercial sources: 82E1 (IBL International) (epitope within residues 1–16 of Aβ), 4G8 (Covance) (epitope within residues 17–24 of Aβ), flotillin-2 (BD Biosciences), and anti-V5 (Invitrogen).

**Immunofluorescence Microscopy**—Cells cultured on poly-l-lysine-coated coverslips were fixed using 4% paraformaldehyde. BACE1 antisemur 7523 was diluted in PBS containing 3% BSA and 0.2% Tween 20 and incubated with fixed cells at room temperature for 2 h. Images were acquired on a Leica SP5 confocal microscope using a 100× objective and processed using ImageJ software.

**Protein Analyses**—Cells were lysed as described previously, and aliquots of lysates were separated on 16% Tris-Tricine gels and immunoblotted with CTM1 or CT15 antiserum (30). In some cases, mAb 26D6 or 82E1 was also included with CTM1 for the simultaneous detection of +1 β-CTFs and all APP CTFs. The blots were developed with IR800 anti-rabbit and IR680
anti-mouse secondary antibodies, and the signal intensities were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). For the simultaneous detection of sAPPα and sAPPβ, aliquots of conditioned medium were fractionated on 4–20% SDS gels and probed with mAb 26D6 along with rabbit polyclonal sAPPβ8 WT or sAPPβ8mut antibody. For the analysis of Aβ, aliquots of conditioned medium were separated on 16% Tris-Tricine gels and probed with mAb 82E1. Metabolic and pulse labeling using [35S]Met/Cys were performed essentially as described (26).

Full-length APP and APP CTFs were immunoprecipitated from cell lysates using CTM1 antibody. Aβ and p3 fragments were immunoprecipitated from the conditioned medium using mAb 4G8.

For the analysis of cell surface BACE1, subconfluent cells were surface-biotinylated using NHS-SS-biotin (Pierce) as described previously (26). Biotinylated proteins were captured using streptavidin beads (Pierce), analyzed along with an aliquot of total cell lysate by immunoblotting with BACE1 antibody, and quantified using the Odyssey Infrared Imaging System.

Lipid Raft Fractionation—Cells were lysed in a buffer containing 0.5% Lubrol WX (Lubrol 17A17, Serva) at 4 °C, and lysates were fractionated by discontinuous flotation density gradients as described previously (31). Fractions containing lipid rafts were identified by immunoblotting with flotillin-2 antibody.

Aβ ELISA—Conditioned media were collected 48 h after plating the cells, and the levels of secreted Aβ1–40 were quantified by ELISA as described previously using mAb B608 and the Odyssey Infrared Imaging System.

RESULTS

BACE1-GPI Expression Elevates Aβ Production without Altering sAPPβ Levels—To gain insights into the reported elevation of Aβ production by BACE1 targeted to lipid rafts via a GPI anchor (19), we generated a BACE1-GPI construct by replacing the transmembrane domain and cytoplasmic tail of mouse BACE1 with the GPI anchor region from human placental alkaline phosphatase (PLAP) (Fig. 1A). Although WTBACE1 largely localized to intracellular organelles, modification by the addition of GPI enhanced BACE1 targeting to the cell surface as visualized by immunofluorescence staining and quantified by surface biotinylation experiments (Fig. 1, B and C). Furthermore, as is the case with other GPI-anchored proteins (32), >80% of BACE1-GPI was recovered in fractions enriched for the lipid raft marker flotillin-2. In comparison, only 40% of WTBACE1 was found in these fractions (Fig. 1D).

As expected, S-palmitoylation-deficient BACE1-4C/A mutant was mainly recovered in fractions containing non-raft proteins. Thus, the addition of PLAP GPI anchor mediates efficient targeting of BACE1 to lipid rafts as reported (19).

We measured amyloidogenic processing of APP in the context of differential BACE1 localization in lipid rafts by transfecting HEK293 cells stably overexpressing APPWT with plasmids encoding WTBACE1, BACE1-GPI, or BACE1-4C/A (Fig. 2A). To assess the levels of secreted Aβ, we performed immunoblot analysis of conditioned media using an antibody raised against the N terminal of Aβ.

Insights into BACE1-GPI Processing of APP
Insights into BACE1-GPI Processing of APP

FIGURE 2. BACE1-GPI expression increases Aβ secretion without concomitant increase of sAPPβ levels in medium. A, HEK293 cells stably expressing APPWT were transiently transfected with the indicated BACE1 expression plasmids. Expression of BACE1 and APP FL in cell lysates was analyzed by immunoblotting cell lysates with antibodies BACE1-N and CTM1, respectively. Mat and imm indicate mature and immature APP FL species, respectively. The levels of secreted Aβ in the conditioned medium were analyzed by Western blotting with mAb 82E1, and the quantification was plotted. B, the levels of sAPPβ and sAPPα in the conditioned medium were analyzed by immunoblots by simultaneously probing the blots with polyclonal sAPPβ WT antibody and mAb 26D6, respectively. C, the levels of APP CTFs were analyzed by probing blots of cell lysates simultaneously with mAb 82E1 and CTM1 to detect +1 β-CTFs and all APP CTFs, respectively. D, HEK293 cells were transiently co-transfected with Na/β2 along with empty vector (Vec), WTBACE1, or BACE1-GPI. The levels of β2 CTFs were analyzed by immunoblotting with anti-V5 antibody. Note that BACE1 overexpression decreases the levels of Na/β2 (β2 FL) as it is converted to β2 CTF.

whereas displacing BACE1 from raft domains fails to exert any effect on Aβ production (19, 20).

Being puzzled by these apparently contradicting findings on Aβ production by BACE1 processing of APP in lipid rafts, we decided to examine the levels of secreted sAPPβ, which is a more direct measure for BACE1 cleavage of APP. We used a LI-COR Biosciences two-color imaging system to simultaneously detect sAPPβ and sAPPα on blots using mAb 26D6 and rabbit polyclonal sAPPβ antibody, respectively. Results from Western blot analysis show that overexpression of WTBACE1, BACE1-GPI, or BACE1-4C/A markedly elevated sAPPβ levels in the medium compared with that of vector control cells. Interestingly, the levels of sAPPβ as well as sAPPα in the medium were quite similar in all cases (Fig. 2B). Therefore, it is clear that cells expressing BACE1-GPI secrete severalfold higher levels of Aβ relative to those expressing WTBACE1 despite secreting similar levels of sAPPβ in the medium.

BACE1-GPI Cleaves APP Preferentially at β Site—Because the level of sAPPβ in the medium reflects BACE1 activity, the above results indicate that the efficiency of APP cleavage by BACE1-GPI appears indifferent from that of WTBACE1 and BACE1-4C/A. To better understand the underlying mechanism by which BACE1-GPI expression elevates Aβ production by 6-fold without an increase in APP processing per se, we examined the profile of APP CTFs in cell lysates. We used an APP C-terminal antibody, CTM1, to detect all APP CTFs: +1 and +11 β-CTFs derived by BACE1 cleavage and α-CTF derived by α-secretase cleavage (at +16 residue of Aβ). Simultaneously, we used mAb 82E1 antibody in the same blots to specifically detect +1 β-CTF. Compared with naïve 293 cells, stable APPWT cells transfected with an empty vector had high levels of α-CTF; only trace levels of β-CTFs were detectable. The steady-state levels of +1 β-CTFs markedly increased following expression of WTBACE1 (Fig. 2C). As reported in the original report of Vassar et al. (2), we also observed a marked increase in +11 β-CTFs following expression of WTBACE1 (Fig. 2C). Indeed, the levels of +11 β-CTFs were clearly higher than those of +1 β-CTFs in cells transfected with WTBACE1. Surprisingly, overexpression of BACE1-GPI markedly elevated +1 β-CTFs but not +11 β-CTFs (Fig. 2C). This differential cleavage of APP at β-site could be related to preferential raft localization of BACE1-GPI, although non-raft-localized BACE1 mutant BACE1-4C/A showed an APP CTF profile nearly identical to that of WTBACE1 (Fig. 2C). Together, the analyses of sAPPβ and APP CTFs indicate that cleavage site selectivity, but not the overall efficiency of APP cleavage, is influenced by GPI anchoring of BACE1.

The above findings also indicate that addition of GPI anchor to BACE1 may have introduced a partial loss of BACE1 cleavage efficiency, which resulted in compromised cleavage of APP at β-site. We tested whether this notion is true by examining the cleavage efficiency of another well characterized BACE1 substrate, Na/β2. As described previously by Kovacs and co-workers (33), co-expression of Na/β2 and WTBACE1 increased β2 CTFs compared with vector-transfected cells with a concomitant decrease in the levels of full-length Na/β2 (Fig. 2D). Co-expression of BACE1-GPI also increased the levels of β2 CTFs but to a lesser extent compared with WTBACE1. Thus, when compared with WTBACE1, GPI anchoring of BACE1 introduces subtle differences in the efficiency of cleavage of APP, Na/β2, and likely other substrates.

Reduced Cleavage at β’-Site by BACE1-GPI Is Independent of Expression Level—To further characterize BACE1 cleavage of APP at β- and β’-sites and rule out the possibility that compromised cleavage at +11 cleavage site of BACE1-GPI is cell type dependent, we transiently co-transfected COS cells with APPWT and BACE1. COS cells co-expressing BACE1-GPI showed significantly lower levels of +11 β-CTFs compared with those expressing WTBACE1 or BACE1-4C/A (p < 0.001), whereas the levels of +1 β-CTFs were comparable (Fig. 3, A and B).

High level expression of BACE1 is known to promote APP cleavage at β’-site (14). Therefore, we investigated whether the observed reduced cleavage at β’-site by BACE1-GPI is related...
to its expression level by titrating the amount of transfected WT BACE1 and BACE1-GPI plasmids (i.e., transfection of 62.5, 125, 250, or 500 ng of plasmid DNA/dish). Under these conditions of transient BACE1 expression, \(\beta\)-secretase processing of APP was the most prominent species. Still, increase in \(\beta\)-secretase-derived CTFs could be readily observed. In the case of WTBACE1, we observed a dose-dependent increase in \(\beta\)-CTFs that correlated with the level of BACE1 expression. Consistent with previous reports, \(\beta\)-CTFs became more predominant at higher levels of WTBACE1 expression (Fig. 3C). Similarly, we observed a dose-dependent increase in \(\beta\)-CTFs in the case of BACE1-GPI, but \(\beta\)-CTFs were barely detectable even at high BACE1-GPI expression (Fig. 3C). These results indicate that marked loss of cleavage at \(\beta\)-site by BACE1-GPI is not related to the level of expression of this chimera.

Cleavage of APP at \(\beta\)-Site Reduces Levels of \(+1\) \(\beta\)-CTF Generated by Cells Expressing WTBACE1—Previous studies have shown that \(+1\) \(\beta\)-CTFs can be generated from BACE1 cleavage of APP FL as well as \(+1\) \(\beta\)-CTFs (C100) (15). We considered the possibility that WTBACE1 sequentially cleaves APP at \(\beta\)- and \(\beta\)-sites but that BACE1-GPI only cleaves at \(\beta\)-site. To test whether this was the case, we co-transfected COS cells with APP M596V (\(\beta\)-site mutant) or E607V (\(\beta\)-site mutant) (15). As expected, there was a com-

![Figure 3](image1.png)

**Figure 3.** Reduced cleavage at \(\beta\)-site of APP by BACE1-GPI in COS cells. A, COS cells were transiently co-transfected with APPWT and the indicated BACE1 constructs. The levels of APP CTFs were analyzed by immunoblotting with CTM1 antibody. B, \(\beta\)-CTF and \(+1\) APP CTF signal intensities were quantified from three independent experiments, and means \(\pm\) S.E. were plotted. C, reduced cleavage at \(\beta\)-site of APP by BACE1-GPI is not dependent on BACE1 expression level. COS cells were co-transfected with APPWT (1 \(\mu\)g) and increasing concentrations of WTBACE1 or BACE1-GPI expression plasmids. BACE1 expression and the levels of APP CTFs were analyzed by immunoblotting with BACE1-NT and CTM1 antibodies. Vec, vector. *, \(p < 0.05\).

![Figure 4](image2.png)

**Figure 4.** BACE1-GPI exhibits impairment of independent as well as sequential cleavage at \(\beta\)-site of APP. A, COS cells were transiently co-transfected with C100 and the indicated BACE1 constructs. The levels of APP CTFs were analyzed by Western blotting with CTM1 antibody. B, the identity of APP CTFs was confirmed by co-transfection of COS cells with APP \(\beta\)-site (APPM596V) and \(\beta\)-site (APP607V) cleavage mutants and with empty vector (Vec) or WTBACE1. APP CTFs were analyzed by probing simultaneously with mAb 26D6 and CTM1 to detect \(+1\) \(\beta\)-CTFs and total APP CTFs, respectively. C, COS cells were transiently co-transfected with the indicated APP and BACE1 plasmids. Aliquots of cell lysates were probed with BACE1-NT and CTM1 antibodies. The levels of sAPP\(\beta\) and sAPP\(\alpha\) in the conditioned medium were analyzed by immunoblotting simultaneously with sAPP\(\beta\)-WT antibody and mAb 26D6, respectively.
Insights into BACE1-GPI Processing of APP

FIGURE 5. BACE1-GPI displays impaired β'-site of both APPWT and APPSwe—A, HEK293 stable pools co-expressing APPWT or APPSwe and the indicated BACE1 constructs were analyzed by immunoblotting. Cell lysates were probed with BACE-NT and CTM1 antibodies. The levels of sAPPβ and sAPPα in the conditioned medium of APPWT were analyzed by probing simultaneously with sAPPβ antibody and mAb 26D6, respectively. Polyclonal sAPPβ antibody (specific for APPSwe cleaved at β-site) was substituted in place of sAPPβ WT for the analysis of media from APPSwe cells. B–E, analysis of APP processing and Aβ secretion by metabolic labeling. HEK293APPWT cells stably expressing the indicated BACE1 constructs were pulse-labeled for 15 min or continuously labeled for 3 h with [35S]Met/Cys. APP FL and APP CTFs were immunoprecipitated from the cell lysates with CTM1 antibody and analyzed by phosphorimaging. Secreted Aβ was analyzed by immunoprecipitation of media conditioned by cells that were labeled for 3 h using mAb 4G8 (epitope 17–24 of Aβ). C, ELISA quantification of conditioned media of stable 293APPWT/APPWT pools was performed using mAb B608 capture (epitope 30–40 of Aβ) and mAb B436 detection (epitope 12–12 of Aβ). Values represent mean ± S.E. of three independent experiments. **, p < 0.01. ** and D, metabolic analysis of Aβ secretion in N2a695.13 (D) or N2aSwe.10 (E) pools stably expressing the indicated BACE1 constructs. Cells were metabolically labeled with [35S]Met/Cys for 3 h and analyzed by immunoprecipitation of lysates (APP FL) or conditioned medium (secreted Aβ and related peptides) followed by phosphorimaging. BACE1 expression was confirmed by immunoblotting with BACE1-NT antibody. Vec, vector.

Complete absence of +1 β-CTFs in cells overexpressing APPM596V mutant, and generation of +11 β-CTFs was completely abolished in cells overexpressing APPP607V mutant (Fig. 4B). When these cleavage site mutants of APP were co-expressed with BACE1 constructs, it was clear that BACE1-GPI preferentially cleaved APP at the β-site and did not resort to cleaving at β'-site even when the β-site was mutated (M596V mutant) (Fig. 4C). Moreover, when processing at β'-site was abolished by APPP607V mutation, WTBACE1 generated higher levels of +1 β-CTFs as compared with BACE1-GPI. Consistent with this observation, there was a clear increase in the levels of sAPPβ in the media conditioned by cells co-expressing APPP607V and WTBACE1 as compared with BACE1-GPI (Fig. 4C). The complete absence of sAPPβ in the medium from cells expressing APPM596V demonstrates the specificity of sAPPβ antibody. These findings indicate that when both β- and β'-sites are available WTBACE1 cleavage of APP at β'-site reduces the levels of +1 β-CTF generated.

In the experiments described above, BACE1 plasmids were transiently expressed in HEK293 cells stably expressing APPWT or co-transfected with APPWT into COS cells. To confirm these results and obviate potential concerns related to transient protein overexpression, we generated pools of HEK293APPWT cells stably expressing WTBACE1, BACE1-GPI, or BACE1-4C/A (Fig. 5A). Consistent with the results obtained from transient transfection, stable expression of WTBACE1 and BACE1-4C/A increased +1 and +11 β-CTFs. Stable expression of BACE1-GPI increased +1 β-CTF but again failed to increase +11 β-CTF (Fig. 5A). Analysis of conditioned medium showed a comparable increase of sAPPβ in each of the BACE1 pools compared with the vector pool (Fig. 5A). In agreement with previous reports (20, 34), stable overexpression of BACE1 reduced the levels of mature APP FL and α-CTF compared with the vector pool, although there was a minor difference in the extent of the reduction in independent stable BACE1-GPI pools (Fig. 5A).

BACE1-GPI Exhibits Similar Reduction of Cleavage at β'-Site toward APPWT and APPSwe—To examine whether BACE1-GPI differentially regulates processing of APP in the secretory pathway, we used the familial Alzheimer disease-linked APPSwe, which undergoes BACE1 processing in the secretory pathway as early as during transit of nascent APP though the Golgi apparatus (26). In stable pools of HEK293APPswe cells, overexpression of either WTBACE1 or BACE1-GPI markedly elevated +1 β-CTFs with a concomitant reduction in α-CTFs relative to that of vector control cells (Fig. 5A). In addition, there was an increase in +11 β-CTFs in cells overexpressing WTBACE1 but not BACE1-GPI (Fig. 5A). Similar to the results we obtained from HEK293APPWT pools, we noticed slightly higher levels of mature APP FL in cells expressing BACE1-GPI pools relative to that of WTBACE1. Because stable WTBACE1 and BACE1-GPI pools were generated from the same parental HEK293APPswe clone, this difference does not reflect the levels of APP expression but rather reflects a subtle disparity in the extent to which APP is processed. Together, these results indicate that BACE1-GPI cleaves both APPWT and APPswe preferentially at β-site, whereas WTBACE1 cleaves both at β- and β'-sites.

Next, we analyzed the levels of sAPP in the medium from HEK293APPswe pools stably expressing WTBACE1 or BACE1-GPI. Probing with sAPPβ antibody specific for secreted APPswe revealed the expected increase in sAPPβ level in either case. However, we failed to see a difference in sAPPβ levels between WTBACE1 and BACE1-GPI cells (Fig. 5A). Probing with 26D6
antibody revealed that overexpression of either WTBACE1 or BACE1-GPI markedly reduced sAPP\(\alpha\) levels in the medium (Fig. 5A); the magnitude of decrease was greater in the case of WTBACE1 pool. Thus, stable expression of BACE1-GPI does not increase amyloidogenic processing of APP\(_{\text{swc}}\) as compared with WTBACE1.

**Cleavage at \(\beta\)-Site Reduces Levels of Intact \(\text{A}\beta\) Secreted by Cells Expressing WTBACE1**—We were interested to see whether the observed difference in the levels of +1 versus +11 \(\beta\)-CTFs generated by cells expressing WTBACE1 and BACE1-GPI is reflected in \(\text{A}\beta1–40\) and \(\text{A}\beta1–40\) levels secreted in the medium. Therefore, we carried out [\(\text{\textsuperscript{35}S}\)]Met/Cys metabolic labeling in HEK293APP\(_{\text{swc}}\) pools described above. Pulse labeling with [\(\text{\textsuperscript{35}S}\)]Met/Cys revealed that synthesis of APP was similar between these cell lines (Fig. 5B). Continuous labeling showed a complete reduction in mature APP FL in WTBACE1-expressing cells. In the case of BACE1-GPI, there was a detectable level of mature APP FL consistent with the data obtained from Western blot analysis. Analysis of APP CTFs immunoprecipitated using an APP C-terminal antibody, CTM1, revealed a robust and comparable increase in +1 \(\beta\)-CTFs in WTBACE1 and BACE1-GPI-expressing cells (Fig. 5B). In addition, we could readily detect +11 \(\beta\)-CTFs in cells expressing WTBACE1 but not in BACE1-GPI-expressing cells consistent with the immunoblotting data.

Next, we examined \(\text{A}\beta\) levels in the medium by immunoprecipitation using mAb 4G8, which can capture both intact \(\text{A}\beta\) and +11 \(\text{A}\beta\) species. To our surprise, WTBACE1 cells secreted less \(\text{A}\beta\) compared with vector control cells but instead secreted high levels of +11 \(\text{A}\beta\) (Fig. 5C). In contrast, BACE1-GPI-expressing cells secreted high levels of \(\text{A}\beta\) and only small amounts of +11 \(\text{A}\beta\). These results suggest that efficient cleavage at the \(\beta\)-site by WTBACE1 results in +11 \(\beta\) production at the expense of intact \(\text{A}\beta\). To confirm these results, we quantified \(\text{A}\beta1–40\) in the conditioned medium by ELISA. Results showed that overexpression of WTBACE1 in HEK293APP\(_{\text{swc}}\) cells markedly reduced the levels of secreted \(\text{A}\beta1–40\) in the conditioned medium (1.5-fold less than vector control), whereas expression of BACE1-GPI elevated \(\text{A}\beta\) secretion (1.5-fold higher compared with vector control) (Fig. 5C).

We also conducted a set of experiments in mouse N2a neuroblastoma cells. For this, we retrovirally infected N2a695.13 cells (which stably expresses human APPWT) with WTBACE1, BACE1-GPI, or BACE1-4C/A and generated stably transduced pools. By [\(\text{\textsuperscript{35}S}\)]Met/Cys metabolic labeling, we observed that overexpression of WTBACE1 or BACE1-4C/A resulted in reduced \(\text{A}\beta\) secretion concomitant with an increase of +11 \(\text{A}\beta\) secretion as compared with vector pool (Fig. 5D). Identical results were obtained in N2aAPPSwe.10 pools (which stably express human APP\(_{\text{swc}}\) stably transduced with WTBACE1 (Fig. 5E). In both cell types, stable expression of BACE1-GPI elevated \(\text{A}\beta\) secretion with little or no increase in secretion of +11 \(\text{A}\beta\) (Fig. 5, D and E). Thus, increased \(\text{A}\beta\) secretion by BACE1-GPI expression is mainly due to reduced cleavage of APP at \(\beta\)-site as compared with WTBACE1. Based on the results detailed above, we conclude that reduced cleavage at \(\beta\)-site of BACE1-GPI is cell type-independent. Moreover, N-terminal truncation of +1 \(\beta\)-CTF by further cleavage at \(\beta\)-site contributes to the apparent difference in the levels of intact \(\text{A}\beta\) secreted by cells expressing WTBACE1 and BACE1-GPI (supplemental Fig. S1).

**BACE1-GPI Chimeras with Different GPI Anchors Elicit Reduced Cleavage at \(\beta\)-Site**—The GPI anchor region in the BACE1-GPI construct used in our present study is derived from the human PLAP sequence. The amino acid sequence of the GPI anchor motifs from PLAP (this study), carboxypeptidase M (CP[\(\text{\textsuperscript{dase}}\)] (19), and CD59 (21) appended to the ectodomain of BACE1 are depicted in black boxes. An asterisk denotes the site where the GPI anchor is attached. B, COS cells were transiently co-transfected with APPWT and the indicated BACE1 constructs, and the levels of APP CTFs were analyzed by Western blotting with CTM1 antibody. C, HEK293 cells stably expressing APPWT were transiently transfected with the indicated BACE1-GPI chimera and corresponding WTBACE1 expression plasmids. BACE1 expression was analyzed by immunoblotting cell lysates with BACE1-NT antibody. The levels of sAPP\(\beta\) and sAPP\(\alpha\) in the conditioned media were analyzed by simultaneously probing immunoblots with polyclonal sAPP\(\beta\), mAB and mAb 26D6, respectively. TM, transmembrane domain; Vec, vector.
Insights into BACE1-GPI Processing of APP

**Figure 7.** Replacing transmembrane and cytoplasmic domains of BACE1 does not affect relative efficiencies of β- and β’-site cleavage. A, the schematic structure of BACE1-HEP chimera. The predicted transmembrane domains (TM) of BACE1 and hephaestin are shaded. B, COS cells were transiently co-transfected with APPWT and the indicated constructs. The levels of APP CTFs were analyzed by immunoblotting with CTM1 antibody to visualize APP CTFs. D and E, signal intensities of +1 and +11 β-CTFs from the blots depicted in B and C were quantified. The relative levels of individual APP CTFs were plotted as a percentage of total. Vec, vector.

Reduced β’-Site Cleavage by BACE1-GPI Is Not Related to Cellular Distribution of APP—Cellular localization of BACE1 has been shown to influence the relative efficiency of BACE1 cleavage at β- and β’-sites. Retention of BACE1 in the ER or TGN compartments increases the cleavage at β- or β’-site of APP, respectively (13). We wanted to examine whether BACE1-GPI also displays this subcellular location-specific β- and β’-site cleavage efficiency. Because it is not feasible to add ER or TGN targeting signals to the C terminus of BACE1-GPI, we resorted to targeting APP to ER and TGN by adding the ER and TGN retention signals to the C terminus of APP. In addition to these ER and TGN targeting mutants of APP, we also included the endocytosis-defective mutant APPNPTY in this study (Fig. 8A). We examined APP metabolism in COS cells co-transfected with APPER, APPTGN, or APPNPTY along with vector, WTBACE1, or BACE1-GPI. ER retention of APP resulted in increased cleavage by WTBACE1 at β-site of APP in comparison with a previous study (13), whereas TGN retention of APP did not alter the relative cleavage at β- and β’-sites as compared with APPWT (Fig. 8B). However, neither of these APP mutants influenced cleavage by BACE1-GPI at β’-site. Moreover, retention of APP at the surface by deletion of the internalization motif (ΔNPTY) did not have any effect on the relative levels of +1 and +11 β-CTFs (Fig. 8B). As expected, there was a clear reduction in α-CTFs and sAPPα when APP was retained in the ER (Fig. 8B). On the other hand, α-CTFs and sAPPα were increased in cells transfected with APPΔNPTY, which is known to have higher steady-state residence at the...
plasma membrane. The levels of sAPPβ in the conditioned medium corresponded to that of APP β-CTFs with one exception that cells expressing APPER secreted undetectable levels of sAPPβ (Fig. 8B). This latter finding is consistent with an earlier study where reduced sAPPβ secretion in cells expressing APPER was reported (36). Therefore, our results indicate that cellular localization of APP does not influence cleavage by BACE1-GPI at β'-site, and the levels of sAPPβ secreted by cells expressing BACE1-GPI are indistinguishable as compared with WTBACE1.

**BACE1-GPI Structure Analysis by Molecular Dynamics Simulation**—We explored potential structural changes introduced in BACE1 ectodomain upon addition of GPI anchor using molecular dynamics simulations. The details of these simulations are provided in the supplementary Methods, and they have recently been successfully used to investigate the substrate specificity of this enzyme (37). The conformational changes in the specific regions at the N terminus (insert A (Gly158-Leu167), 10s loop (Lys9-Tyr18), flap (Val107-Glu129), and third strand (Lys107-Gly117)) and C terminus (insert B (Lys218-Gly221), insert C (Ala251-Pro258), insert D (Trp270-Thr274), insert E (Glu290-Ser295), and insert F (Asp310-Asp317)) in BACE1 ectodomain were studied (Fig. 9A). The flap and third strand play important roles in substrate recognition and positioning (38), and insert F plays a role in substrate entry at the active site (39). The superposition of the most representative structures derived from WTBACE1 and BACE1-GPI simulations explicitly shows that the addition of GPI anchor retains the overall structure of the enzyme but introduces significant changes in the conformations and positions of the flap, third strand, and insert F regions (Fig. 9, A and B). A plot of the root mean square fluctuation of BACE1-GPI chimera also shows that, in comparison with WTBACE1, the flap and the third strand of the enzyme are more flexible (supplemental Fig. S2A).

The Ca(Thr72)-Cβ(Asp32) distance defines the motion of the flap and is considered to be the key parameter in substrate recognition (40). The Thr72 residue is located at the tip of the flap, and Asp32 constitutes the catalytic dyad that is critical for the catalytic functioning of the enzyme. For BACE1-GPI chimera, this distance is computed to be 4.8 Å longer than that for BACE1, i.e. 14.0 ± 1.3 and 18.8 ± 1.2 Å for BACE1 and BACE1-GPI, respectively (supplemental Fig. S2B).

In the x-ray structure of BACE1 (Protein Data Bank code 1W50) (41), the 10s loop is positioned between the third strand and insert F segments. The molecular dynamics simulation shows that the Gln12 residue of the 10s loop mediates interactions between the third strand and insert F. In WTBACE1, the backbone and the side chain of Gln12 (10s loop) form hydrogen bonds with the side chains of Ser113 (third strand) and Glu310 (insert F), respectively (Fig. 9C). However, the addition of GPI anchor to BACE1 was found to destroy the Gln12-Ser113 bond and retain only the Gln12-Glu310 hydrogen bond. The loss of the Gln12-Ser113 bond facilitates the formation of strong hydrogen bonds between the backbone of Asn111 (third strand) and the side chains of Asp311 (insert F) and Glu316 residues (insert F), respectively (Fig. 9D). These structural rearrangements bring the third strand and insert F in close proximity.

**DISCUSSION**

We recently characterized BACE1 S-palmitoylation at 4 Cys residues located in the transmembrane and cytoplasmic boundary and reported that this post-translational modification of BACE1 is required for lipid raft association (20). Because targeting BACE1 to lipid rafts through the attachment of a GPI anchor elevated Aβ secretion, we expected that interfering with raft association of BACE1 by mutation of the palmitoylated Cys residues will lower Aβ secretion. However, displacing BACE1 from lipid raft domains did not affect BACE1 processing of APP and Aβ secretion (20). In this study, we characterized the paradoxical increase in Aβ secretion associated with expression of BACE1-GPI. We used three different BACE1-GPI constructs, including the BACE1-GPI (carboxypeptidase) construct used in previous characterization of raft-associated APP processing (19). We report that expression of BACE1-GPI elevated intact Aβ secretion compared with WTBACE1 and BACE1-4C/A. However, this increase in Aβ secretion by BACE1-GPI was not concomitant with release of sAPPβ. Because the level of cellular BACE1 activity is reflected by the amount of sAPPβ secreted in the conditioned medium, this later finding argues against an overall increase in amyloidogenic processing of APP previously attributed to raft association of BACE1-GPI (19). Instead, our results demonstrate that WTBACE1 and BACE1-4C/A cleaved APP at β- and β'-sites to generate +1 as well as +11 β-CTFs, whereas BACE1-GPI mainly generated +1 β-CTF due to marked loss of APP cleavage at β'-site. Moreover, a significant portion of +1 CTFs was converted to +11 CTFs by β'-site cleavage by WTBACE1, which reduced the levels of intact Aβ secreted by the cells; this conversion occurred at extremely low
efficiency in cells expressing BACE1-GP1, allowing efficient secretion of intact Aβ (supplemental Fig. S1). Thus, although expression of BACE1-GPI elevated secretion of intact Aβ compared with WTBACE1, this difference was not due to enhanced APP processing associated with exclusive raft targeting of BACE1 as was originally described (19).

In accordance with the previous study that used BACE1-GPI chimera (19), we found that replacing the transmembrane domain and cytosolic tail of BACE1 with a GPI anchor motif efficiently targeted BACE1 ectodomain to lipid rafts in HEK293 and mouse N2a neuroblastoma cells. However, we did not observe the reported increase in sAPPβ secretion upon overexpression of BACE1-GPI (19), this difference was not due to enhanced APP processing associated with exclusive raft targeting of BACE1 as was originally described (19).

In accordance with the previous study that used BACE1-GPI chimera (19), we found that replacing the transmembrane domain and cytosolic tail of BACE1 with a GPI anchor motif efficiently targeted BACE1 ectodomain to lipid rafts in HEK293 and mouse N2a neuroblastoma cells. However, we did not observe the reported increase in sAPPβ secretion upon overexpression of BACE1-GPI (19). Our experiments, which were conducted in multiple cell types, consistently showed that cells expressing BACE1-GPI secreted similar levels of sAPPβ compared with those expressing WTBACE1 or BACE1-4C/A. It is important to note that an earlier study by Haass and coworkers (21) independently reported that the level of sAPPβ secretion was indistinguishable between cells expressing WTBACE1 and BACE1-GPI in agreement with our results.

It has been shown that BACE1 cleavage at β'-site of APP depends on the level of BACE1 expression (14). In agreement, the efficiency of cleavage at β'-site correlated well with the level of WTBACE1 overexpression in our studies. However, BACE1-GPI cleaved mainly at β-site of APP with very little cleavage at β'-site even when the chimera was highly overexpressed (Fig. 3). This partial loss of β'-site-specific cleavage by BACE1-GPI was readily apparent in experiments where we expressed APP C100 and a β'-site cleavage mutant (M596V). Whereas WTBACE1 cleaved either protein at β'-site, BACE1-GPI failed to do so. Together, these findings reveal an inherent loss of β'-site recognition by BACE1-GPI chimera (Fig. 4C).

Following initial attachment to proteins, GPI anchors undergo structural remodeling to generate variability in the side chains and lipid moieties (32). Thus, it is possible that the observed difference in sAPPβ secretion between the studies as well as the reduced cleavage at β'-site discovered in the present study is a unique feature associated with the particular GPI anchor region (from PLAP) that we initially used. To formally rule out this possibility, we performed parallel analysis of BACE1 modified with GPI anchors from PLAP, carboxypeptidase M, and CD59 (19, 21). Examination of APP processing by BACE1 chimeras with different GPI anchor motifs revealed that all three chimeras exhibited compromised cleavage at β'-site.

FIGURE 9. Molecular dynamics simulations of WTBACE1 and BACE1-GP1. A, superposition of the most representative structures derived from the WTBACE1 and BACE1-GP1 simulations. The highlighted regions show the 10s loop, flap, third strand, and insert F regions of BACE1-GP1 chimera (purple color) and BACE1 (red color). B, the surface representation of the 10s loop, flap, third strand, and insert F of BACE1 and BACE1-GP1. C, interactions between the amino acid residues of the 10s loop, third strand, and insert F in BACE1 and BACE1-GP1.
Insights into BACE1-GPI Processing of APP

\( \beta'-\)site of APP and in each case secreted similar levels of sAPP\( \beta \) and generated lower levels of +11 \( \beta \)-CTF as compared with WTBACE1 (Fig. 6).

In metabolic labeling experiments conducted in stable HEK293 and N2a cells, it was obvious that there was a significant decrease of full-length \( \alpha \beta \) with a concomitant increase of +11 \( \alpha \beta \) upon overexpression of WTBACE1. An increase in +11 \( \alpha \beta \) secretion was observed with APPWT (which is processed predominantly in endocytic organelles) and APP\( _{S\text{w}} \) (which is processed during transit in the secretory pathway) (Fig. 5, B–E). The mAb 4G8 used for immunoprecipitation of secreted \( \alpha \beta \)-related species in our experiments is capable of reacting with both intact \( \alpha \beta \) and N-terminally truncated +11 \( \alpha \beta \), thus permitting us to visualize this conversion. However, +11 \( \alpha \beta \) was undetectable when antibodies specific to the N-terminal residues of \( \alpha \beta \) were used for detection in immunoblots, immunoprecipitation, or ELISA analysis in this study (Figs. 2A and 5C) as well as in the report by Cordy et al. (19).

In cultured cells, such as the ones used in this study, the majority of WTBACE1 resides in endocytic organelles and TGN at the steady state, and only a subset of BACE1 resides at the cell surface (11, 20, 42, 43). On the other hand, GPI-anchored mammalian proteins (32), including BACE1-GPI, are highly enriched at the plasma membrane and in membrane raft microdomains (Fig. 1). In our studies, there was an ~3-fold increase in cell surface BACE1 in N2a and HEK293 cells stably expressing BACE1-GPI as compared with WTBACE1 (Fig. 1). Thus, the loss of \( \beta'-\)site cleavage observed in cells expressing BACE1-GPI could be related to having limited access to a pool of APP substrate that is dynamically transported in secretory or endocytic vesicles. In addition, the relative efficiency of BACE1 cleavage of APP at \( \beta \)- and \( \beta' \)-sites is influenced by the subcellular compartment in which BACE1 resides (13). For example, retention of BACE1 in ER increases cleavage at \( \beta \)-site, whereas TGN localization promotes cleavage at \( \beta' \)-site (13). These results were faithfully reproduced in WTBACE1 cells by targeting APP to the ER or TGN by means of organelle targeting signals appended to the C terminus. However, targeting APP to TGN failed to promote cleavage at \( \beta' \)-site by BACE1-GPI (Fig. 8). Similarly, expression of APP\( _{\Delta N P T Y} \), a mutant in which transit of APP through the endocytic pathway is inhibited by the deletion of the NPTY internalization motif, also failed to increase BACE1-GPI processing of APP at \( \beta' \)-site (Fig. 8). These results indicate that markedly reduced cleavage at \( \beta' \)-site is intrinsic to this chimera.

The efficiency of cleavage at \( \beta \)- and \( \beta' \)-sites was unaffected by expression of BACE1-HEP, a chimera that contains the transmembrane domain and cytosolic tail of hephaestin fused to BACE1 ectodomain. Thus, the diminished ability of BACE1-GPI to cleave APP at \( \beta' \)-site is not specifically related to the lack of a specific sequence motif encoded within BACE1 transmembrane domain or cytosolic tail sequence. Alternatively, we suggest that dynamic localization of BACE1 in raft and non-raft microdomains confers a certain flexibility to the transmembrane and juxtamembrane region of BACE1, thus allowing the catalytic site to cleave at both \( \beta \)- and \( \beta' \)-sites. Modifying BACE1 with the GPI anchor results in the loss of this structural plasticity, and as a consequence, BACE1-GPI chimera fails to adopt the conformation required to facilitate APP cleavage at \( \beta' \)-site. Molecular modeling of BACE1-GPI chimera supports the idea that the addition of GPI anchor introduces structural changes to BACE1 ectodomain. Specifically, the molecular dynamics simulations show that the GPI anchor introduces significant changes in the positioning of the flap, third strand, and insert F (Fig. 9, A and B). These regions have been implicated in the recognition, positioning (38), and entry (39) of the substrates. The elongation of the Ca(Thr\(^{2+}\))–Cβ(Asp\(^{32}\)) distance by 4.8 Å shows that the flap is substantially more open in BACE1-GPI (supplemental Fig. S2B). This opening could influence the substrate orientation inside the active site and alter the catalytic activity of the enzyme. In BACE1-GPI chimera, the loss of the Glu\(^{12} \)(10s loop)–Ser\(^{113} \)(third strand) hydrogen bond and the formation of additional Asn\(^{111} \)(third strand)–Asp\(^{311} \)(insert F) and Asn\(^{111} \)(third strand)–Gln\(^{316} \)(insert F) bonds brings the third strand and insert F close to each other. This structural rearrangement could also contribute to the observed changes in APP processing. Thus, it is possible that structural alterations introduced in BACE1-GPI chimera position the catalytic site toward the \( \beta \)-site but only allow poor access to \( \beta' \)-site of APP. Indeed, experimental mutagenesis of APP residues located between \( \beta \)- and \( \beta' \)-sites as well as in the juxtamembrane loop region of BACE1 has demonstrated the importance of structural compatibility and spatial orientation for efficient \( \beta \) and \( \beta' \) cleavage of APP (44).

Previous studies have demonstrated independent (de novo cleavage at +11) as well as sequential cleavage (+1 followed by +11 cleavage) at \( \beta' \)-site of APP by BACE1 (14, 15, 45). Results from \( \beta \)-site cleavage mutant (M596V) and APP C100 demonstrate that either mode of +11 cleavage is compromised in cells expressing BACE1-GPI. Finally, when we expressed a \( \beta' \)-site APP cleavage mutant (E607V) so that we could examine processing at the \( \beta \)-site in the absence of confounding sequential cleavage at \( \beta' \)-site, we observed higher levels of sAPP\( \beta \) release and increased generation of \( \beta \)-CTF by cells expressing WTBACE1 as compared with BACE1-GPI (Fig. 4C). This finding clearly demonstrates increased efficiency of \( \beta \)-site cleavage of APP by WTBACE1 relative to BACE1-GPI. Therefore, we conclude that elevated \( \alpha \beta \) secretion by cells expressing BACE1-GPI is mainly contributed by loss of cleavage at \( \beta' \)-site rather than up-regulation of overall APP processing as a result of predominant raft association of BACE1-GPI chimera. While preparing this manuscript, we learned of a pathogenic early onset Alzheimer disease APP mutation at the \( \beta' \) site. Molecular modeling of BACE1-GPI chimera supports the idea that the addition of GPI anchor introduces structural changes to BACE1 ectodomain. Specifically, the molecular dynamics simulations show that the GPI anchor introduces significant changes in the positioning of the flap, third strand, and insert F (Fig. 9, A and B). These regions have been implicated in the recognition, positioning (38), and entry (39) of the substrates. The elongation of the Ca(Thr\(^{2+}\))–Cβ(Asp\(^{32}\)) distance by 4.8 Å shows that the flap is substantially more open in BACE1-GPI (supplemental Fig. S2B). This opening could influence the substrate orientation inside the active site and alter the catalytic activity of the enzyme. In BACE1-GPI chimera, the loss of the Glu\(^{12} \)(10s loop)–Ser\(^{113} \)(third strand) hydrogen bond and the formation of additional Asn\(^{111} \)(third strand)–Asp\(^{311} \)(insert F) and Asn\(^{111} \)(third strand)–Gln\(^{316} \)(insert F) bonds brings the third strand and insert F close to each other. This structural rearrangement could also contribute to the observed changes in APP processing. Thus, it is possible that structural alterations introduced in BACE1-GPI chimera position the catalytic site toward the \( \beta \)-site but only allow poor access to \( \beta' \)-site of APP. Indeed, experimental mutagenesis of APP residues located between \( \beta \)- and \( \beta' \)-sites as well as in the juxtamembrane loop region of BACE1 has demonstrated the importance of structural compatibility and spatial orientation for efficient \( \beta \) and \( \beta' \) cleavage of APP (44).

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26176 JOURNAL OF BIOLOGICAL CHEMISTRY
