LOSS OF CLEAVAGE AT \( \beta' \)-SITE CONTRIBUTES TO THE APPARENT INCREASE IN \( \alpha \beta \) SECRETION BY BACE1-GPI PROCESSING OF AMYLOID PRECURSOR PROTEIN

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Several lines of evidence implicate lipid raft microdomains in Alzheimer’s disease associated \( \beta \)-amyloid peptide (\( \alpha \beta \)) production. Notably, targeting \( \beta \)-secretase (BACE1) exclusively to lipid rafts by the addition of a GPI anchor to its ectodomain has been reported to elevate \( \alpha \beta \) secretion. Paradoxically, \( \alpha \beta \) secretion is not reduced by the expression of non-raft resident \( S \)-palmitoylation-deficient BACE1 (BACE1-4C/A). We addressed this apparent discrepancy in raft microdomain-associated BACE1 processing of amyloid precursor protein (APP) in this study. As previously reported, we found that expression of BACE1-GPI elevated \( \alpha \beta \) secretion as compared with wild-type BACE1 (wtBACE1) or BACE1-4C/A. However, this increase occurs without any difference in the levels of APP ectodomain released following BACE1 cleavage (sAPP\( \beta \)), arguing against overall increase in BACE1 processing of APP per se. Further analysis revealed that wtBACE1 cleaves APP at \( \beta \)- and \( \beta' \)-sites, generating +1 and +11 \( \beta \)-C-terminal fragments (CTF), and secreting intact as well as N-terminally truncated \( \alpha \beta \). In contrast, three different BACE1-GPI chimeras preferentially cleave APP at the \( \beta \)-site, mainly generating +1 \( \beta \)-CTF and secreting intact \( \alpha \beta \). As a consequence, cells expressing BACE1-GPI secrete relatively higher levels of intact \( \alpha \beta \), without increase in BACE1 processing of APP.

Markedly reduced cleavage at \( \beta' \)-site exhibited by BACE1-GPI is cell-type independent, and insensitive to subcellular localization of APP or the pathogenic KM/NL mutant. We conclude that the apparent elevation in \( \alpha \beta \) secretion by BACE1-GPI is mainly attributed to preferential cleavage at \( \beta \)-site and failure to detect +11 \( \alpha \beta \) species secreted by cells expressing wtBACE1.

Alzheimer's disease (AD) is an age-associated neurodegenerative disorder pathologically characterized by the cerebral deposition of 38–42 amino acid-long \( \beta \)-amyloid peptides (\( \alpha \beta \)) in senile plaques. \( \alpha \beta \) is generated by the sequential proteolysis of a large type I transmembrane protein, termed amyloid precursor protein (APP), by BACE1 (\( \beta \)-site APP-cleaving enzyme 1) and \( \gamma \)-secretases (1). BACE1 is a transmembrane aspartyl protease that cleaves APP within the ectodomain at a major cleavage site between Met\(^{596} \) and Asp\(^{597} \) (\( \beta \)- or +1 cleavage site) (2-5). BACE1 processing results in the release of the large APP ectodomain (sAPP\( \beta \)) and the generation of membrane-tethered C-terminal fragment (CTF), termed \( \beta \)-CTF (6). In a subsequent step, \( \beta \)-CTF is cleaved within the transmembrane domain by \( \gamma \)-secretase to release \( \alpha \beta \) peptides (7). In addition to the \( \beta \)-site, BACE1 can also cleave APP within the \( \alpha \beta \) domain.
between Tyr\textsuperscript{606} and Glu\textsuperscript{607} (\(\beta'-\) or +11 cleavage site), releasing sAPP\(\beta'\) and +11 \(\beta\)-CTF. In an alternate processing pathway, APP can be cleaved within the \(\alpha\) domain between Lys\textsuperscript{16} and Leu\textsuperscript{17} by a set of enzymes, termed \(\alpha\)-secretases, which release sAPP\(\alpha\) and generate \(\alpha\)-CTF (1). \(\gamma\)-secretase cleavage of +11 \(\beta\)-CTF and \(\alpha\)-CTF generates N-terminally truncated \(\beta\)-related peptides found in cerebrospinal fluid and media conditioned by cultured cells, termed +11 \(\beta\) 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 \(\beta'-\)site (13). For example ER retention of BACE1 enhances APP \(\beta\)-cleavage whereas targeting of BACE1 to TGN increases cleavage at \(\beta'-\)site (13). Furthermore, overexpression of BACE1 promotes cleavage at the \(\beta'-\)site (2,14,15). Neurons express high levels of BACE1, and consistent with a correlation between BACE1 expression levels and the efficiency of \(\beta'-\)cleavage, cultured primary neurons predominantly secrete +11 \(\beta\) species (10). Neurons cultured from \textit{BACE1} knockout embryos lack secretion of +1 and +11 \(\beta\) species (5).

Multiple lines of evidence suggest the involvement of cholesterol- and sphingolipid-rich membrane microdomains, termed lipid rafts, in amyloidogenic processing of APP (reviewed in (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 \(\beta\) production (19). Recently we found that \(\delta\)-palmitoylation at 4 Cys residues (Cys\textsuperscript{474}/Cys\textsuperscript{478}/Cys\textsuperscript{482}/Cys\textsuperscript{485}) 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 \(\beta\) 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 \(\beta\) 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 \(\beta\) production. We found that unlike wtBACE1 and BACE1-4C/A, overexpression of BACE1-GPI increases cleavage at \(\beta'-\)site but not at \(\beta'-\)site, resulting in the secretion of increased levels of intact \(\beta\). However, extensive analysis of cells expressing wtBACE1, non-raft-localized \(\delta\)-palmitoylation-deficient BACE1-4C/A and predominantly raft-associated BACE1-GPI showed similar levels of sAPP\(\beta\) secretion into the conditioned medium, establishing no difference in the efficiency of APP processing. Thus, our data reveals that the apparent increase in \(\beta\) production by BACE1-GPI previously attributed to raft-associated APP processing is a result of compromised cleavage at \(\beta'-\)site and the inability to experimentally detect +11 \(\beta\) species.

**EXPERIMENTAL PROCEDURES**

c\textit{DNA constructs}—Plasmids encoding mouse BACE1-GPI and C-terminally FLAG epitope-tagged wtBACE1 and BACE1-4C/A (C474/478/482/485A) 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 ID: 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, Leeds (19). Plasmids encoding BACE1-GPI (with GPI anchor
region cloned from CD59) and the corresponding wtBACE1 were provided by Dr. Christian Haass, Ludwig-Maximilians-University, Munich (21). Plasmids encoding APP C100 with ER (C100ER) and TGN (C100TGN) retention signals were provided by Dr. Takeshi Iwatsubo, University of Tokyo, Tokyo (22). APP695 cDNA with ER (APP695 ER) and TGN (APP695 TGN) retention signals were generated by exchanging the C-terminal segments from C100ER and C100TGN into APPwt cDNA. Plasmid APP695ΔNPTY was generated by exchanging the C-terminal region of APP770ΔNPTY cDNA (23) (provided by Dr. S. Sisodia, The University of Chicago, Chicago) into APPwt cDNA. V5-tagged sodium channel β2 subunit (Na,β2) expression plasmid was provided by Dr. Dora Kovacs, Harvard Medical School, Charlestown (24). APP β-site cleavage mutant (M596V; numbering corresponds to APP695 isoform) and β'-site cleavage mutant (E607V) were generated by PCR mutagenesis. All PCR amplified cDNA fragments were verified by sequencing. For retroviral expression, the cDNAs were subcloned into retroviral vector pMXs-puro (provided by Dr. Toshio Kitamura, University of Tokyo, Japan).

Retroviral infections and generation of stable cell lines—Human embryonic kidney (HEK) 293 cells stably expressing c-Myc epitope-tagged APP695wt and APP695Swe were provided by Dr. S. Sisodia, The University of Chicago (25). N2a cells stably expressing c-Myc epitope-tagged APPwt (N2a695.13) and APPSwe (Swe.10) have been described previously (26). BACE1- fibroblasts were provided by Dr. Philip C. Wong, The Johns Hopkins University School of Medicine, Baltimore (5). Retroviral infections were performed as described previously (27) using Plat-E (provided by Dr. Toshio Kitamura, University of Tokyo, Japan) or Phoenix (ATCC) packaging cell lines. Stably transduced pools of HEK293 cells were selected in the presence of 1 μg/ml puromycin and BACE1- fibroblasts and N2a cells were selected in the presence of 4 μg/ml puromycin, respectively.

Antibodies—Rabbit polyclonal antibody BACE1-NT was raised against a synthetic peptide corresponding to residues 46-60 of BACE1. BACE1 antiserum 7523 was provided by Dr. Christian Haass, Ludwig-Maximilians-University, Munich (28). Monoclonal antibodies (mAb) 26D6 and B436 [epitope within residues 1-12 of Aβ] react with the amino-terminal region of Aβ and also recognize sAPPa (20). mAb B608 [epitope within residues 30-40 of Aβ] reacts specifically with Aβ40. The following mAbs were purchased from commercial sources: 82E1 (IBL) [epitope within residues 1-16 of Aβ], 4G8 (Covance) [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 antiserum 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 100X 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 Odyssey Infrared Imaging system (LI-COR Inc., Lincoln, NE, USA). 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 sAPPβwt or sAPPβSwe 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 S-S biotin (Pierce) as described previously (26). Biotinylated proteins were captured using streptavidin beads (Pierce) and analyzed along with an aliquot of total cell lysate by immunoblotting with BACE1 antibody and quantified using 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 for capture and mAb B436 for detection (20). Synthetic Aβ40 peptide (Bachem) was diluted in culture medium to generate the standard curve. Each sample was assayed in duplicate using appropriate dilution of the conditioned media so that the relative luminescent units were in the linear range of the standards included on each plate.

**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 and cytoplasmic tail of mouse BACE1 with the GPI anchor region from human placental alkaline phosphatase (PLAP) (Fig. 1A). While wtBACE1 largely localizes to intracellular organelles, modification by the addition of GPI enhances BACE1 targeting to the cell surface as visualized by immunofluorescence staining and quantified by surface biotinylation experiments (Fig. 1B and C). Furthermore, as it is the case with other GPI-anchored proteins (32), >80% of BACE1-GPI is recovered in fractions enriched for the lipid raft marker flotillin-2. In comparison, only 40% of wtBACE1 were 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-terminus of Aβ and quantified the signal intensities. Results showed that overexpression of wtBACE1 or BACE1-4C/A increased Aβ production by 1.5 fold compared to that of vector control cells (Fig. 2A). However, expression of BACE1-GPI caused a 6-fold increase in Aβ production. The above findings confirm previous reports that targeting BACE1 to lipid rafts by the use of a GPI anchor elevated Aβ production whereas displacing BACE1 from raft domains failed 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 LI-COR two color imaging system to simultaneously detect sAPPα and sAPPβ 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 to 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 several-fold higher levels of Aβ relative to those expressing wtBACE1, despite secreting similar levels of sAPPβ in the medium.

**BACE1-GPI cleaves APP preferentially at the β-site**—Since 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 to naïve 293 cells, stable APPwt cells transfected with an empty vector have 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 and colleagues (2), we also observed a marked increase in +11 β-CTFs following expression of wtBACE1 (Fig. 2C). Indeed, the levels of +11 β-CTFs are clearly higher than that 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 nearly identical APP CTF profile as that of wtBACE1 (Fig. 2C). Together, the analysis of sAPPβ and APP CTFs indicate that cleavage site selectivity, and 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 has resulted in compromised cleavage of APP at β-site. We wanted to test if this notion is true by examining the cleavage efficiency of another well-characterized BACE1 substrate, β2-subunit of the voltage-gated sodium channel (Naβ2wt). As described previously by Kovacs and colleagues (33) co-expression of Naβ2wt and wtBACE1 increased β2 CTFs compared to vector transfected cells with a concomitant decrease in the levels of full-length Naβ2wt (Fig. 2D). Co-expression of BACE1-GPI also increased the levels of β2 CTFs but to a lesser extent compared to wtBACE1. Thus, when compared to wtBACE1, GPI anchoring of BACE1 introduces subtle differences in the efficiency of cleavage of APP, Naβ2wt, and likely other substrates.

**Reduced cleavage at β’-site by BACE1-GPI is independent of expression level**—In order 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 coexpressing BACE1-GPI showed significantly lower levels of +11 β-CTFs compared to those expressing wtBACE1 or BACE1-4C/A (p<0.001), whereas the levels of +1 β-CTFs were comparable (Fig. 3A and B).
High-level expression of BACE1 is known to promote APP cleavage at β'-site (14). Therefore we asked if the observed reduced cleavage at β'-site by BACE1-GPI is related to its expression level, by titrating the amount of transfected wtBACE1 and BACE1-GPI plasmid (i.e transfaction of 62.5, 125, 250 or 500 ng of plasmid DNA/dish). Under these conditions of transient BACE1 expression, α-CTF derived from α-secretase processing of wtAPP is the most prominent species. Still, increase in β-CTFs can be readily observed. In the case of wtBACE1, we observed a dose-dependent increase in +1 and +11 β-CTFs that correlated with the level of BACE1 expression. Consistent with previous reports, +11 β-CTFs became more predominant at higher levels of wtBACE1 expression (Fig. 3C). Similarly, we observed a dose-dependent increase in +1 β-CTFs in the case of BACE1-GPI, but +11 β-CTFs were barely detectable even at high BACE1-GPI expression (Fig. 3C). These results indicate that marked loss of cleavage at β'-site by BACE1-GPI is not related to the level of expression of this chimera.

Cleavage of APP at β'-site reduces the levels of +1 β-CTF generated by cells expressing wtBACE1—Previous studies have shown that +11 β-CTFs can be generated from BACE1 cleavage of APP FL as well as +1 β-CTFs (C100) (15). We considered the possibility that wtAPP sequentially cleaves APP at β- and β'-sites, but BACE1-GPI only cleaves at β-site. To test if this were the case, we co-transfected COS cells with APP C100 fused to signal peptide (22) along with wtBACE1 or BACE1-GPI. Immunoblot analysis of lysates revealed that wtBACE1 readily processed C100 to generate +11 β-CTFs, whereas BACE1-GPI failed to do so (Fig. 4A). Thus, wtBACE1 can cleave C100 at β'-site, whereas BACE1-GPI is severely impaired in its ability to cleave C100 at β'-site.

In order to formally establish the identity of +1 and +11 β-CTFs, we co-expressed BACE1 with APP M596V (+1 site mutant) or E607V (+11 site mutant) (15). As expected, there is complete absence of +1 β-CTFs in cells overexpressing APPM596V mutant, and generation of +11 β-CTFs is completely abolished in cells overexpressing APPE607V mutant (Fig. 4B). When these cleavage site mutants of APP were co-expressed with BACE1 constructs, it is clear that BACE1-GPI preferentially cleaves APP at the β-site, and does not resort to cleaving at the β'-site even when the β-site is mutated (M596V mutant) (Fig. 4C). Moreover, when processing at β'-site is abolished by APPE607V mutation, wtBACE1 generates higher levels of +1 β-CTFs as compared with BACE1-GPI. Consistent with this observation, there is a clear increase in the levels of sAPPβ in the media conditioned by cells co-expressing APPE607V and wtBACE1 as compared with BACE1-GPI (Fig. 4C). 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 comparable increase of sAPPβ in each of the BACE1 pool compared to 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 to 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 towards APPwt and APPSwe—**

In order to examine whether BACE1-GPI differentially regulates processing of APP in the secretory pathway, we employed the FAD-linked “Swedish” variant of APP (APP_Swe), 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 HEK293APP_Swe cells overexpression of either wtBACE1 or BACE1-GPI markedly elevated +1 β-CTFs with 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. Since stable wtBACE1 and BACE1-GPI pools were generated from the same parental HEK293APP_Swe clone, this difference does not reflect the levels of APP expression, rather reflects subtle disparity in the extent to which APP is processed. Together, these results indicate that BACE1-GPI cleaves both APPwt and APP_Swe preferentially at the β-site whereas wtBACE1 cleaves both at β- and β'-sites.

Next we analyzed the levels of sAPP in the medium from HEK293APP_Swe pools stably expressing wtBACE1 or BACE1-GPI. Probing with sAPPβ antibody specific for secreted APP_Swe 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 sAPPα levels in the medium (Fig. 5A); the magnitude of decrease was greater in the case of wtBACE1 pool. Thus, in stable expression of BACE1-GPI does not increase amyloidogenic processing of APP_Swe as compared with wtBACE1.

**Cleavage at β'-site reduces the levels of intact Aβ secreted by cells expressing wtBACE1—**

We were interested to see if the observed difference in the levels of +1 versus +11 β-CTFs generated by cells expressing wtBACE1 and BACE1-GPI is reflected in Aβ1-40 and Aβ11-40 levels secreted in the medium. Therefore, we carried out [35S] Met/Cys metabolic labeling in HEK293APP_Swe pools described above. Pulse labeling with [35S] Met/Cys revealed that synthesis of APP is similar between these cell lines (Fig. 5B). Continuous labeling showed complete reduction in mature APP FL in wtBACE1 expressing cells. In the case of BACE1-GPI, there is detectable level of mature APP FL, consistent with the data obtained from Western blot analysis. Analysis of APP CTFs immunoprecipitated using APP C-terminal antibody, CTM1, revealed robust and comparable increase in +1 β-CTFs in wtBACE1 and BACE1-GPI expressing cells (Fig. 5B). In addition, we could readily detect +11 β-CTFs in cells expressing wtBACE1 but not in BACE1-GPI expressing cells, consistent with the immunoblotting data.

Next we examined Aβ levels in the medium by immunoprecipitation using mAb 4G8, which can capture both intact Aβ and +11 Aβ species. To our surprise, wtBACE1 cells secreted less Aβ compared to vector control cells, but instead secreted high levels of +11 Aβ (Fig. 5C). In contrast, BACE1-GPI expressing cells secreted high levels of Aβ, and only small amounts of +11 Aβ. These results suggest that efficient cleavage at β'-site by wtBACE1 results in +11 Aβ production at the expense of intact Aβ. To confirm these results, we quantified Aβ1-40 in the conditioned medium by ELISA. Results showed that overexpression of wtBACE1 in HEK293APP_Swe cells markedly reduced the levels of secreted Aβ1-40 in the conditioned medium (1.5 fold less than vector control), whereas expression of BACE1-GPI elevated Aβ secretion (1.5 fold higher compared to 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 wt APP) with wtBACE1, BACE1-GPI, or BACE1-4C/A and generated stably transduced pools. By \[^{35}S\]Met/Cys metabolic labeling we observed that overexpression of wtBACE1 or BACE1-4C/A results in reduced Aβ secretion concomitant with an increase of +11 Aβ secretion as compared with vector pool (Fig. 5D). Identical results were obtained in N2aAPPSwe.10 pools (which stably express human APP Swe) stably transduced with wtBACE1 (Fig. 5E). In both cell types stable expression of BACE1-GPI elevated Aβ secretion, with little or no increase in secretion of +11 Aβ (Fig. 5D and E). Thus, increased Aβ secretion by BACE1-GPI expression is mainly due to reduced cleavage of APP at β'-site as compared with wtBACE1. Based on the results detailed above, we conclude that reduced cleavage at β'-site of BACE1-GPI is cell type independent. Moreover, N-terminal truncation of +1 β-CTF by further cleavage at β'-site contributes to the apparent difference in the levels of intact Aβ secreted by cells expressing wtBACE1 and BACE1-GPI (Supplementary Fig. S1).

**BACE1-GPI chimeras with different GPI anchors elicit reduced cleavage at β'-site**—GPI anchor region in the BACE1-GPI construct used in our present study is derived from human PLAP sequence. The GPI anchor sequence used for lipid raft targeting of BACE1 in the previous study was derived from carboxypeptidase M (19). In addition, a third BACE1-GPI chimera has been described, which contains the GPI anchor region from CD59 (21) (Fig. 6A). Since GPI anchors are known to undergo different fatty acid remodeling (32,35), we wanted to rule out the possibility that the type and side group modifications of PLAP GPI anchor region caused the reduction of cleavage at β'-site of BACE1-GPI observed in our experiments. We obtained BACE1-GPI (carboxypeptidase M) and BACE1-GPI (CD59) expression plasmids (and corresponding wtBACE1) from Drs. Nigel M. Hooper and Christian Haass, respectively. We then co-transfected them and BACE1-GPI (PLAP) or the respective wtBACE1 along with APPwt into COS cells and analyzed APP processing in parallel. Interestingly, we observed preferential cleavage of APP cleavage at β-site in all three BACE1-GPI chimeras (Fig. 6B). To extend these findings, we examined sAPP in the media conditioned by HEK293APPwt cells transiently transfected with each BACE1-GPI. Results indicate that cells expressing each BACE1-GPI chimera or corresponding wtBACE1 secrete similar levels of sAPPβ (Fig. 6C). Thus, GPI anchor sequences from three different proteins fused to BACE1 ectodomain compromise cleavage at β'-site of APP, and the BACE1-GPI chimeras process APP at the +1 site with similar efficiency as compared with wtBACE1.

**BACE1 transmembrane and C-terminal domains are dispensable for APP β'-site cleavage**—We wondered whether deletion of transmembrane and cytosolic tail sequence in BACE1-GPI, rather than raft association per se, underlies reduced APP cleavage at β'-site. In order to test this notion, we generated a BACE1 chimera, BACE1-HEP wherein the transmembrane and cytosolic tail of BACE1 were replaced by corresponding sequences of hephaestin, a type 1 protein involved in iron transport and highly expressed in duodenum (Fig. 7A). Like BACE1, hephaestin has a short cytosolic tail, but the domains exchanged between the two proteins have no significant sequence similarity. We found that COS cells co-transfected with APPwt and wtBACE1 or BACE1-HEP accumulate similar steady-state levels of +1 and +11 β-CTFs (Fig. 7B and D). Similar results were obtained from 293APPwt cells stably expressing BACE1-HEP (Fig. 7C and E). Therefore, these results indicate that the primary amino acid sequence of the transmembrane and cytoplasmic domains of BACE1 can be replaced without compromising BACE1 cleavage of APP at β'-site.
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 increase 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. Since 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, APPΔNPTY in this study (Fig. 8A). We examined APP metabolism in COS cells co-transfected with APPER or APPTGN or APPΔNPTY along with vector or wtBACE1 or BACE1-GPI. ER retention of APP resulted in increased cleavage by wtBACE1 at β-site of APP, in agreement with previous study (13), whereas TGN retention of APP did not alter the relative cleavage at β- and β'-sites as compared with wtAPP (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 is clear reduction in α-CTFs and sAPPα when APP is 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 later 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 is 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 supporting information (Supplementary Methods), and they have recently been successfully employed to investigate the substrate specificity of this enzyme (37). The conformational changes in the specific regions at the N-terminus [insert A (Gly158 – Leu167), the 10s loop (Lys9 – Tyr14), the flap (Val67 – Glu77) and third strand (Lys107 – Gly117)] and C-terminus [insert B (Lys218 – Asn221), 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 the substrate recognition and positioning (38) and insert F in its entry at the active site (39). The superposition of the most representative structures derived from wtBACE1 and BACE1-GPI simulations explicitly exhibits 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. 9A and B). A plot of the root-mean-square fluctuation of BACE1-GPI chimera also shows that, in comparison to wtBACE1, the flap and the third strand of the enzyme are more flexible (Supplementary Fig. S2A).

The Cα(Thr72)-Cβ(Asp32) distance defines the motion of the flap and considered to be the key parameter in the 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 the one for BACE1 i.e. 14.0 ± 1.3 Å and 18.8 ± 1.2 Å for
BACE1 and BACE1-GPI respectively (supplementary Fig. S2B).

In the X-ray structure of BACE1 (PDB ID: 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 10s-loop mediates interactions between third strand and F insert. 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 Glu12-Ser113 bond and retain only the Gln12-Glu310 hydrogen bond. The loss of Glu12-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 Gln316 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). Since 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 employed three different BACE1-GPI constructs, including 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 to wtBACE1 and BACE1-4C/A. However, this increase in Aβ secretion by BACE1-GPI was not concomitant with release of sAPPβ. Since the level of cellular BACE1 activity is reflected by the amount of sAPPβ activity, 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 cleave APP at β- and β'-sites to generate +1 as well as +11 β-CTFs, whereas BACE1-GPI mainly generates +1 β-CTF due to marked loss of APP cleavage at β'-site. Moreover, a significant portion of +1 CTF is converted to +11 CTF by β'-site cleavage by wtBACE1, which reduces the levels of intact Aβ secreted by the cells; this conversion occurs at extremely low efficiency in cells expressing BACE1-GPI, allowing efficient secretion of intact Aβ (Supplementary Fig. S1).

Thus, while expression of BACE1-GPI elevates secretion of intact Aβ as compared with wtBACE1, this difference is 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 employed 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 do 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 secrete similar levels of sAPPβ as compared with those expressing wtBACE1 or BACE1-4C/A. It is important to note that an earlier study by Haass and colleagues independently reported that the level of sAPPβ secretion was indistinguishable between cells expressing wtBACE1 and BACE1-GPI, which is in agreement with our results (21).

It has been shown that BACE1 cleavage at β'-site of APP depends on level of BACE1 expression (14). In agreement, efficiency of cleavage at β'-site correlated well with 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 is highly overexpressed (Fig. 3). This partial loss of β-site-specific cleavage by BACE1-GPI is 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 employed. 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 of APP and in each case secreted similar levels of sAPPβ and generated lower levels of +11 β-CTF as compared with wtBACE1 (Fig. 6).

In metabolic labeling experiments conducted in stable HEK293 and N2a cells, it is obvious that there is a significant decrease of full-length Aβ with concomitant increase of +11 Aβ upon overexpression of wtBACE1. Increase in +11 Aβ secretion is observed with APPwt (which is processed predominantly in endocytic organelles) and APPswc (which is processed during the transit in secretory pathway) (Fig. 5 B-E). The mAb 4G8 used for immunoprecipitation of secreted Aβ-related species in our experiments is capable of reacting with both intact Aβ as well as N-terminally truncated +11 Aβ, thus permitting us to visualize this conversion. However, +11 Aβ is undetectable when antibodies specific to the N-terminal residues of Aβ 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 in 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 is ~3 fold increase in cell surface BACE1 in N2a and HEK293 cells stably expressing BACE1-GPI as compared with wtBACE1 (Fig. 1). Thus, loss of β'-site cleavage we observe 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 β- and β'-site is influenced by subcellular compartment in which BACE1 resides (13). For example, retention of BACE1 in ER increases cleavage at β-site, whereas TGN localization promotes cleavage at β'-site (13). These results are 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 β'-site by BACE1-GPI (Fig. 8). Similarly, expression of APPΔNPTY, a mutant in which transit of APP through the endocytic pathway was inhibited by the deletion of NPTY internalization motif also failed to increase BACE1-GPI processing of APP at β'-site (Fig. 8). These results indicate that markedly reduced cleavage at β'-site is intrinsic to this chimera.

The efficiency of cleavage at β- and β'-sites were unaffected by expression of BACE1-HEP, a chimera that contains the transmembrane and cytosolic tail of hephaestin fused to BACE1 ectodomain. Thus, the diminished ability of BACE1-GPI to cleave APP at β'-site is not specifically related to the lack of specific sequence
motif encoded within BACE1 transmembrane or cytosolic tail sequence. Alternatively, we suggest that dynamic localization of BACE1 in raft and non-raft microdomains confers certain flexibility to the transmembrane and juxtamembrane region of BACE1, thus allowing the catalytic site to cleave at both β- and β'-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 β'-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. 9A and B). These regions have been implicated in the recognition, positioning (38) and entry (39) of the substrates. The elongation of the Ca(Thr72)-Cβ(Asp32) distance by 4.8 Å shows that the flap is substantially more open in BACE1-GPI (supplementary 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 Glu12 (10s loop)-Ser113 (third strand) hydrogen bond and the formation of additional Asn111 (third strand)-Asp311 (insert F) and Asn111 (third strand)-Gln316 (insert F) bonds bring 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 towards the β-site but only allows poor access to β'-site of APP. Indeed, experimental mutagenesis of APP residues located between β- and β'-sites as well as juxtamembrane loop region of BACE1 has demonstrated the importance of structural compatibility and spatial orientation for efficient β- and β'-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 β'-site of APP by BACE1 (14,15,45). Results from β-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 β'-site APP cleavage mutant (E607V) so that we could examine processing at the β-site in the absence of confounding sequential cleavage at β'-site, we observe higher levels of sAPPβ release and increased generation of β-CTF by cells expressing wtBACE1 as compared with BACE1-GPI (Fig. 4C). This finding clearly demonstrates increased efficiency of β-site cleavage of APP by wtBACE1 relative to BACE1-GPI. Therefore we conclude that elevated Aβ secretion by cells expressing BACE1-GPI is mainly contributed by loss of cleavage at β'-site rather than upregulation 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’s disease APP mutation at the β'-cleavage site (E607K [APP695 numbering]) that impaired cleavage at β'-site leading to increased Aβ secretion (46), in a manner similar to what we have observed with BACE1-GPI.

REFERENCES


FOOTNOTES

Acknowledgements
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Abbreviations
APP, amyloid precursor protein; BACE1, β-site APP cleaving enzyme 1; Aβ, β-amyloid; CTF, C-terminal fragment; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; PLAP, placental alkaline phosphatase; TGN, trans-Golgi network; FL, full-length; ELISA, enzyme-linked immunosorbent assay; HEP, hephaestin; 4C/A, C474A/C478A/C482A/C485A.
Keywords
Alzheimer’s disease, BACE1, amyloid, amyloid precursor protein, cholesterol, palmitoylation, lipid rafts.

FIGURE LEGENDS

FIGURE 1. **GPI anchoring targets BACE1 to cell surface and lipid rafts.** A, Schematic structure of BACE1 and BACE1-GPI chimera. The predicted transmembrane domain is shaded. Ala substitutions of S-palmitoylated Cys residues are indicated in BACE1 4C/A mutant. GPI anchor motif from PLAP appended to the ectodomain of BACE1 is depicted in a black box. An asterisk denotes the site where GPI anchor is attached. B, *BACE1*−/− fibroblasts stably expressing wtBACE1 or BACE1-GPI were immunostained with BACE1 antiserum 7523. C, N2a cells stably expressing wtBACE1 or BACE1-GPI were labeled by surface biotinylation. Biotinylated proteins were captured on streptavidin beads and the levels of cell BACE1 were examined by Western blot analysis. Signal intensities of surface BACE1 were normalized to input lysate in each case, and the mean ± SEM from three independent experiments were plotted as the fold difference compared to wtBACE1. *, p<0.05. D, Sucrose density gradient fractionation of stably transduced HEK293 cells. Cells were solubilized in 0.5% Lubrol WX and subject to sucrose gradient fractionation to separate membrane raft-associated proteins from non-raft proteins. The distribution of BACE1 and raft marker flotillin-2 were determined by Western blot analysis.

FIGURE 2. **BACE1-GPI expression increases Aβ secretion without concomitant increase of sAPPβ levels in the 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-NT and CTM1, respectively. Mat and imm indicate mature and immature APP FL species. The levels of secreted Aβ in the conditioned 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 sAPPβwt 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 Naβ2 along with empty vector, 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 Naβ2 (β2FL) as they get converted to β2 CTF.

FIGURE 3. **Reduced cleavage at β'-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, β- and +11-APP CTF signal intensities were quantified from three independent experiments and mean ± SEM were plotted. C, Reduced cleavage at β'-site of APP by BACE1-GPI is not dependent on BACE1 expression level. COS cells were co-transfected with APPwt (1 µg) 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.

FIGURE 4. **BACE1-GPI exhibits impairment of independent as well as sequential cleavage at β′-site of APP.** A, COS cells were transiently co-transfected with C100 and 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 β-site (APP569V) and β′-site (APPE607V) cleavage mutants, and with empty vector or wtBACE1. APP CTFs were analyzed by probing simultaneously with mAb 26D6 and CTM1 to detect +1 β-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 sAPPβ and sAPPα in the conditioned medium were analyzed by immunoblotting simultaneously with sAPPβwt antibody and mAb 26D6, respectively.

FIGURE 5. **BACE1-GPI displays impaired β′-site of both APPwt and APPSwe.** A, HEK293 stable pools coexpressing 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 sAPPβwt antibody mAb and 26D6, respectively. Polyclonal sAPPβswe antibody (specific for APPSwe cleaved at β-site) was substituted in place of sAPPβwt for the analysis of media from APPSwe cells. B-E, Analysis of APP processing and Aβ secretion by metabolic labeling. HEK293APPswe stably expressing 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 293APPswe BACE1 pools was performed using mAb B608 capture (epitope 30-40 of Aβ) and mAb B436 detection (epitope 1-12 of Aβ). Values represent mean ± SEM of three independent experiments. ***, p<0.01 D and E, 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.

FIGURE 6. **BACE1 with different GPI anchor motifs exhibit loss of β′-site cleavage efficiency.** A, Schematic structure of wtBACE1 and BACE1-GPI. The amino acid sequence of the GPI anchor motifs from PLAP (this study), carboxypeptidase M (CPdase) (19), and CD59 (21) appended to the ectodomain of BACE1 are depicted in black boxes. An asterisk denotes the site where GPI anchor is attached. B, COS cells were transiently co-transfected with APPwt and 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 with BACE1-NT antibody.
antibody. The levels of sAPPβ and sAPPα in the conditioned media were analyzed by simultaneously probing immunoblots with polyclonal sAPPβ wt antibody and mAb 26D6, respectively.

FIGURE 7. Replacing the transmembrane and cytoplasmic domains of BACE1 does not affect relative efficiencies of β- and β'-site cleavage. A, The schematic structure of BACE1-hephaestin (BACE1-HEP) chimera. The predicted transmembrane domains 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. C, HEK293APPwt cells stably expressing the indicated constructs 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 panels B and C were quantified the relative levels of individual APP CTFs were plotted as a percentage of total.

FIGURE 8. Analysis of BACE1-GPI processing of APP ER, APP TGN, and APPΔNPTY. A, The schematic structure of APP. ER and TGN localization signals appended to the C-terminus of APP are indicated. The predicted transmembrane domain is shaded. B, COS cells were transiently co-transfected with the indicated APP and BACE1 constructs. The levels of APP CTFs were analyzed by immunoblotting with CTM1 (for APPwt, APP ER and APP TGN) or CT15 (for APPΔNPTY) antibodies. The levels of sAPPβ and sAPPα in the conditioned medium were analyzed by immunoblotting simultaneously with sAPPβ wt antibody and mAb 26D6, respectively.

FIGURE 9. Molecular dynamics simulations of wtBACE1 and BACE1-GPI. A, Superposition of the most representative structures derived from the wtBACE1 and BACE1-GPI simulations. The highlighted regions show the 10s loop, flap, third strand, and insert F regions of BACE1-GPI chimera (red color) and BACE1 (blue color). B, The surface representation of the 10s loop, flap, third strand, and insert F of BACE1 and BACE1-GPI. C, Interactions between the amino acid residues of the 10s loop, third strand and insert F in BACE1 and BACE1-GPI.
Figure 1

A

wtBACE1

...DESTLMTIAYVMMAIACALFMLPLCLMVCQWRCLRCLRHQHDDFADDISLLK

BACE1-4C/A

B

BACE1-GPI

...DEST<sup>TTD*AAHPGRSVVPALLPLLACTLLLLLETATAP</sup>

B

Vector

wtBACE1

BACE1-GPI

20 μm

C

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D

Lipid rafts

Top

Wt

GPI

4C/A

Flot-2

Bottom
Figure 3

A

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B

% of total CTFs

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\beta-CTF & 10 & 20 & 30 & * & 10 & 20 \\
+11 CTF & 5 & 15 & 25 & 5 & 15 & 25 \\
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C

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\hline
62.5 & 125 & 250 & 500 & 62.5 & 125 & 250 & 500 \\
\end{array}
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Figure 4

(A) C100

(B) APP CTFs

26D6

β

+11

α

CTM1

γ

APPWT

APP596V

APP607V

Vec  Wt  GPI

Vec  Wt  GPI

Vec  Wt  GPI

BACE1

APP CTFs

sAPPβ

sAPPα
Figure 5

A

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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>+11</td>
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B

15 min pulse

CTFs

mature

immature

APP

β

α

+11

C

3 h continuous

sAPPβ

sAPPα

sAPPβwt

sAPPα

293APPwt

293APP_Swe

D

E

BACE1

APP FL

Aβ

Aβ

p3

+11

Vec | Wt | GPI | 4C/A |
|-----|----|-----|------|

Aβ

p3

+11
Figure 6

A

wtBACE1

BACE1-GPI

...DESTLMTIAYVMAAICALFMLPLCLMVCQWRCLRCLRHQHDDFADDISLLK

...DESTTTD*AAPHGRSVPALLPLLAGTLLLETATAP GPI (PLAP)

...DESTPDHS*AATKPSLFLFLLVSLLHIFFK GPI (carboxypeptidase M)

...DESTASL*ENGGTSLSEKTVLLLVTPLAAAWSLHP GPI (CD59)

B

Vec  Wt  GPI (PLAP)  Vec  Wt  GPI (CPdase)  Vec  Wt  GPI (CD59)

APP CTFs

C

Vec  Wt  GPI (PLAP)  Vec  Wt  GPI (CPdase)  Vec  Wt  GPI (CD59)

BACE1

sAPPβ

sAPPα
Figure 7

A

wtBACE1

...DESTLMTIAYVMAICALFMLPLCLMVCQWRCLRCLRHRHDDFADDSSLK...

...DESTLSSALIAICVLLLLIALALGGVWYQHRQKLRNNRSILDDSFKLLSLKQ

BACE1-HEP

B

APPwt

<table>
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<tr>
<th></th>
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<th>HEP</th>
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C

293 APPwt

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D

% of total CTFs

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E

% of total CTFs

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<th></th>
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Figure 8

A

N \rightarrow TM \rightarrow C

\text{APP}_{\text{wt}} \ldots \text{PEERHLSKMQQNGYENPTYKFFEQMQN}

\text{APP}_{\text{ER}} \ldots \text{PEERHLSKMQQNGYENPTYKFFEQMQNKKLN}

\text{APP}_{\text{TGN}} \ldots \text{PEERHLSKMQQNGYENPTYKFFEQMQNSDYQRL}

\text{APP}\Delta\text{NPTY} \ldots \text{PEERHLSKMQQNGYE----KFFEQMQN}

B

\begin{array}{cccc|cccc|cccc|cccc}
& \text{Vec} & \text{Wt} & \text{GPI} & & \text{Vec} & \text{Wt} & \text{GPI} & & \text{Vec} & \text{Wt} & \text{GPI} & & \text{Vec} & \text{Wt} & \text{GPI} \\
\text{APP}_{\text{wt}} & \text{BACE1} & \text{APP FL} & \text{APP CTFs} & \text{sAPP}\beta & \text{sAPP}\alpha & \text{sAPP}\beta & \text{sAPP}\alpha \\
\text{APP}_{\text{ER}} & & & & & & & & & & & & & & & & \\
\text{APP}_{\text{TGN}} & & & & & & & & & & & & & & & & \\
\text{APP}\Delta\text{NPTY} & & & & & & & & & & & & & & & & \\
\end{array}
Figure 9

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

[Diagram of protein structures with labels and annotations]

[Legend for protein structures]

[Details of specific protein regions and interactions]
Loss of cleavage at β'-site contributes to the apparent increase in Aβ secretion by BACE1-GPI processing of amyloid precursor protein
Kulandaivelu S. Vetrivel, Arghya Barman, Ying Chen, Phuong D. Nguyen, Steven L. Wagner, Rajeev Prabhakar and Gopal Thinakaran

J. Biol. Chem. published online June 3, 2011

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