Membrane-bound BACE1 naturally cleaves its trans-membrane substrate amyloid precursor protein (APP) at the two adjacent \( \beta \)- and \( \beta' \)-sites. Cleavage at these two sites generates the heterogeneous N-terminal end of APP C-terminal fragments that are further processed by \( \gamma \)-secretase to release A\( \beta \)-(1–40/42) or A\( \beta \)-(1–40/42). The significance underlying A\( \beta \)-(11–40/42) in Alzheimer's disease pathogenesis has remained to be experimentally elucidated, but increased production of A\( \beta \)-(1–40/42) has been broadly demonstrated to contribute to amyloid depositions in senile plaques. In this study, we show that the cleavage of APP at the \( \beta \)-site by BACE1 is readily disrupted through limited structural twists, whereas the \( \beta' \)-site is relatively better positioned to gain access to the BACE1 catalytic cavity. Radical insertion or deletion of residues between \( \beta \)- and \( \beta' \)-site also favors cleavage of APP at the \( \beta' \)-site. On the other hand, either lengthening or shortening the loop region of BACE1 has a minor impact on the selective cleavage of APP at these two adjacent sites, but significantly shortening the loop region impairs the ability of BACE1 to process APP at both sites. Thus, processing of APP by BACE1 is clearly dependent on a mutual structural compatibility in addition to the sequence feature. The knowledge gained from this study will potentially offer an opportunity for rational design of small molecule drugs to block the cleavage of APP specifically at the \( \beta' \)-site while not disturbing the functions of other cellular asparyl proteases.

Amyloid peptides (A\( \beta \)),\(^1\) the major components of the amyloid depositions found in senile plaques, are excised from amyloid precursor protein (APP) through sequential cleavage by two endopeptidases: \( \beta \)- and \( \gamma \)-secretases. Elevated levels of A\( \beta \) in human brains have been shown to correlate with cognitive decline (1). Genetic, pathological, and biochemical evidence has proclaimed A\( \beta \) as one of the potential etiological factors for Alzheimer's disease (2–3). Therapeutic interventions strategically targeted to either block A\( \beta \) production or enhance its clearance have begun to show promise in animal models (4).

A type I transmembrane aspartyl protease has been identified to be the \( \beta \)-secretase called BACE1, an acronym for \( \beta \)-site APP cleaving enzyme (5–9). Mammalian aspartyl proteases are typically translated as zymogens, but BACE1 possesses its catalytic activity although its pro domain is still retained (10–13). Full maturation of BACE1 occurs in the Golgi where its pro domain is removed by furin or furin-like convertases (12–15) and a complex sugar moiety is attached. Noticeably, BACE1 activity is independent of these posttranslational modifications. All these features are consistent with the findings that BACE1 is also active in the endoplasmic reticulum compartments where immature BACE1 is translated and properly folded (12, 13, 16–18). Initially, BACE1 is expected to cleave APP only at the \( \beta \)-site for releasing A\( \beta \)-(1–40/42). When BACE1 is overexpressed in cells, an alternative processing of APP at the \( \beta' \)-site (between Tyr\(^{10}\)Glu\(^{11}\) within the A\( \beta \) region) becomes apparent (5). Cleavage of APP at the \( \beta' \)-site seems more robust in rodent species than in humans because more A\( \beta \)-(11–40/42) in rodents has been reported previously (19, 20). Mouse deficiency in BACE1 not only dramatically reduces production of A\( \beta \)-(1–40/42) but also A\( \beta \)-(11–40/42) (21), suggesting that processing of APP at the \( \beta' \)-site occurs naturally. Recent studies have demonstrated that processing of APP at the \( \beta' \)-site by BACE1 is independent of the initial cleavage of APP at the \( \beta \)-site (23, 24). In fact, the preferential cleavage at either site largely relies on the sequence feature (24). In the case of wild type human APP (APP WT), \textit{in vitro} assays with synthetic peptide substrates indicate that the \( \beta \)-site is slightly superior to the \( \beta' \)-site (24). More A\( \beta \)-(1–40/42) is therefore detected by mass spectroscopy than A\( \beta \)-(11–40/42) in cells expressing APP WT, and this phenomenon is more evident in cells expressing Swedish mutant APP (APP SW) for its far better cleavable sequence at the \( \beta \)-site than \( \beta' \)-site. When the protein levels of BACE1 are greatly increased, processing of APP at the \( \beta' \)-site is enhanced, probably due to the sequential processing of both APP and CTF99 substrates by an increased amount of BACE1 (22–24).

With the knowledge that BACE1 is capable of cleaving APP at two adjacent cleavable sites, we then ask whether the structural flexibility between the APP \( \beta \)- and \( \beta' \)-site would contribute to the selective cleavage of a site without an optimal sequence feature. The knowledge gained from this study may potentially be applicable to the discovery of small molecules that potentially block processing at the \( \beta \)-site. In this report, we found that the distal space of the cleavage sites from the membrane is one important factor for cleavage at the \( \beta \)-site by BACE1. However, the structural compatibility between BACE1 and its APP substrate is more important. Cleavage at the \( \beta \)-site by BACE1 is easily disrupted upon twisting the secondary structure near the \( \beta \)-site. These results have revealed impor-
Selective Cleavage of APP at the β- or β’-Site

An unusual product termed CTFβ was indicated based on known cleavages at the β, β’ or α-site. An unusual product termed CTFα, denoted with an arrowhead, C. HM cells were pretreated with 5 μg/ml of BFA for 1 h and then transfected with individual APP constructs for another 48 h in the continuing presence of BFA treatment. The lysates were similarly analyzed by Western blot.

MATERIALS AND METHODS

Cell Lines—Human HEK-293 cells were maintained at 37 °C in a humidified, 5% CO2 controlled atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and glutamine. HEK-293 cells were used to generate a stable cell line (IM) expressing BACE1 under the selection of hygromycin B (100 μg/ml).

Transfection—Transfection was performed using the LipofectAMINE 2000 reagent (Invitrogen). A total of 20 μg of DNA were transfected into cells plated in 10-cm dishes using 80 μl of LipofectAMINE 2000 reagent. DNA and LipofectAMINE solutions were made in a total of 2 ml of Opti-MEMI media and added to each dish containing 8 ml of antibiotic-free Dulbecco’s modified Eagle’s medium.

Western Blot Analysis—Cell lysates were prepared according to Yan et al. (6). Equal amounts of cellular extracts were loaded onto a 4–12% Nupage gel or 16% Tricine gel (Invitrogen), and the proteins were separated by electrophoresis. The protein was then transferred to a nitrocellulose membrane according to standard procedures followed by Western analysis. The primary antibody C8 recognizes the C-terminal nitrocellulose membrane according to standard procedures followed by Western analysis. The primary antibody C8 recognizes the C-terminal

SITE-DIRECTED MUTAGENESIS—Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Palo Alto, CA) according to the standard procedure. APPWT was initially mutated to APP46 (K670N/M671L) or APPISY (V669I/K670S/M671Y/D672E/A673V). IST was incorporated into the β-site (S679I/G680S) to generate APPWT-βIST and APPISY-βIST. Two Pro substitutions (F675P/R676P) were incorporated into APPISY to generate APPISY/2P. All constructs were validated by double strand DNA sequencing.

RESULTS

Abolished Cleavage of the APP β-Site by BACE1 upon Local Conformational Change—Our previous studies with a mutant APP construct termed APPISYEV containing the optimal ISY-EV sequence at both β- and β’-sites have demonstrated that both β- and β’-sites are cleavable by BACE1 (24). This has also been confirmed with a pulse-chase experiment (data not shown). Two possible scenarios may account for the simultaneous cleavage of two adjacent APP sites by BACE1: 1) the APP structure overlapping the β- and β’-site is sufficiently flexible like a random coil so that BACE1-mediated hydrolysis at either site can be accommodated, and 2) the secondary structure spanning this region is relatively rigid, either in a β-turn or α-helix, but allows both the β- and β’-site to be positioned in proximity to the BACE1 catalytic cleft. Because the structure of APP has not been resolved, we intend to differentiate between these possibilities by a mutagenesis approach to distort conformation near the BACE1 cleavage sites. We first used APPISYEV as a template and generated additional APP variant with either insertion or deletion of residues between the β- and β’-site as outlined in Fig. 1A. We reasoned that either insertion or deletion of residues between these two sites should
Selective Cleavage of APP at the β- or β'-Site

A

![Western blot analysis of APP cleavage](image)

**Fig. 2.** Balancing of an optimal BACE1 β-site with a structural twist. A, both APP<sub>WT</sub> and APP<sub>S14AS</sub> were mutated by either insertion of Ala or deletion of Arg-His residues between the β- and β'-site. B, HM cells were transfected with the indicated constructs for 48 h, and the lysates were analyzed by Western blot with antibody C8.

cause a shift of the conformation in this region. As shown in Fig. 1B, the patterns of BACE1-cleaved C-terminal fragments were variable in cells expressing different APP constructs. Insertion of either one or two Ala residues did not noticeably affect the cleavage of APP at these two sites, whereas insertion of four Ala residues almost completely shifted the cleavage to the β'-site. Interestingly, when two residues (Arg-His) in the center of this region were deleted, the cleavage at the β'-site was also predominant (Fig. 2B). Thus, to either shorten or extend the length between β- and β'-site would favor BACE1 to cleave APP at the β'-site.

A Pro residue is known to typically break a α-helical or β-sheeted structure. Theoretically, the substitution (F4P/R5P) in the Aβ region would cause conformational changes in this region. To test further which cleavable subsite will be more sensitive to a structural bending, we generated such a construct named APP<sub>ISYEV</sub> (Fig. 1A). Transfection of this construct in HM cells showed that CTF<sub>β</sub> was again a preferred product that migrated at the expected size on a gel (Fig. 1B). To our surprise, we also observed a fragment that migrated obviously slower than the expected CTF<sub>β</sub> (denoted with an arrowhead in Fig. 1, B and C). We referred to this fragment as CTF<sub>β</sub>-site because it has an undefined N-terminal end. Sequence inspection of a BACE1 cleavable site suggests that an upstream EIS-EV is a potential processing site for generating this CTF<sub>β</sub> (24 and 25). This suggested that a BACE1 inaccessible cleavage site in a normal circumstance could very well be aligned into the BACE1 active cleft upon a structural twist.

To further understand these cleavages in subcellular compartments, we treated cells with either brefeldin A (BFA) or monensin. BFA arrests secretory proteins in the endoplasmic reticulum by blocking the exit of secretory vesicles from the endoplasmic reticulum, whereas monensin locks transport of secretory vesicles in the Golgi. As demonstrated previously (23, 24), processing of APP at the β-site is presumably favored in cells treated with BFA, whereas monensin treatments favor production of CTF<sub>β</sub>. We found that the patterns of aforementioned APP mutants processed by BACE1 in monensin-treated cells were generally similar to the patterns in the untreated transfected cells (data not shown). However, BFA treatment indeed accumulated more CTF<sub>β</sub> than the untreated condition (comparing Fig. 1, B with C) consistent with that seen previously (23, 24). Interestingly, although production of CTF<sub>β</sub> was detectable in BFA-treated cells expressing APP<sub>ISYEV</sub>, the anomalous fragment CTF<sub>β</sub> was still more robustly generated. Clearly, the cleavage of the β-site in APP<sub>ISYEV</sub> was suppressed whereas a totally new product was produced with a local conformational shift. Thus, conformational changes in the Aβ N-terminal region mainly disrupt the cleavage of APP at the β-site.

Given the fact that the template used for the above study contains ISYEV at both β- and β'-sites, it is unclear whether similar insertions or deletions in either APP<sub>WT</sub> or APP<sub>S14AS</sub> would replicate the same selective cleavage. To resolve this, we generated more mutant APP as illustrated in Fig. 2A. Because the β-site in APP<sub>S14AS</sub> is much more superior to the wild type β'-site, we routinely observed CTF<sub>β</sub> as a predominant cleavage product in APP<sub>ISYEV</sub> expressing cells (see lane APP<sub>ISYEV</sub> in Fig. 2B). Remarkably, a significant shift of the processing to the β'-site still occurred in both APP<sub>ISYEV</sub> insertion and deletion variants regardless of the fact that the β-site possesses a much more optimal cleavable sequence than the β'-site in APP<sub>ISYEV</sub> (Fig. 2B). The same shift was also confirmed to occur in variants with wild type cleavage sites (Fig. 2, A and B). Thus, a structurally perturbed superior β-site cannot compete against a well positioned suboptimal β'-site.

Taken together, these data suggested that BACE1 cleavage at the β-site is more easily disrupted than the β'-site if there is a structural change in this region. Natural processing of APP at the β'-site is clearly caused by its favorable orientation together with the presence of the cleavable sequence.

The Extended or Shortened Linker Region Does Not Disrupt Cleavage at the β-Site—Given the fact that a significant structural twist between the β- and β'-site (as caused in APP<sub>ISYEV</sub>) would suppress the cleavage at the β-site by BACE1, it was still unclear whether the similarly suppressed cleavage at the β-site in APP 4A-insertion constructs (APP<sub>ISYEV</sub>+4A/ISY, APP<sub>WT</sub>+4A/WT, or APP<sub>ISYEV</sub>+4A/WT) was primarily caused by the structural or the distal changes of the β-site from the membrane. To differentiate between the changes, we inserted Ala residues between Ala<sup>21</sup> and Glu<sup>22</sup> in the APP<sub>ISYEV</sub> template because it retains cleavage at both sites by BACE1. As a consequence, the distance of the β-site to the membrane in APP<sub>ISYEV</sub>+2A/ISY<sub>21A</sub> would mimic that in APP<sub>ISYEV</sub>+4A/ISY (Fig. 3A). Examinations of these new APP variants actually revealed no changes at both the β- and β'-site in HM cells transfected with APP<sub>ISYEV</sub>+2A/ISY<sub>21A</sub> or APP<sub>ISYEV</sub>+2A/ISY<sub>21A</sub> (Fig. 3B). Moreover, examination of another construct named APP<sub>ISYEV</sub> +2A/ISY<sub>21A</sub> that placed the β-site farther from the membrane by inserting four Ala residues at position 21 also did not indicate a noticeable shift (data not shown).
We also examined APP mutants with shortened linker regions spanning the BACE1 cleavage sites and the residues that anchor APP in the membrane. When four residues (Phe19-Phe20-Ala21-Glu22) were deleted in APP ISY/ISY, patterns of cleaved products were not changed as shown by cells expressing APPISY/ISY/H9004FFAE (Fig. 3C). Therefore, insertion or deletion of a few residues outside the β- and β'-sites does not disrupt APP processing patterns unlike the mutants shown in Figs. 1 and 2. These results suggest that the distance of the β-site from the membrane is not a determining factor for BACE1 to cleave at the β-site, whereas the proper conformation in this region is more important.

The Unfavorable β-Site Is Cleavable by BACE1 if the β'-Site Is Blocked—Having seen the inhibited cleavage at the β-site by BACE1 in APPISY/ISY with similar mutants, it was intriguing to see whether this inhibition is attributable to an absolute inaccessibility of the mutant APP β-site to the BACE1 cleft after a structural twist. Therefore we examined another mutated APP protein named APPWT/4A/KK with a Y10K/E11K substitution in APP WT/4A/WT. Prior analysis of cleavable sequences suggests that the β'-site is blocked by such a substitution (data not shown) or a similar mutation (23), whereas cleavage at the wild type β-site is retained. Utilizing APPWT/4A/KK would allow us to determine whether a structurally twisted β-site is actually accessible to the BACE1 active site. Examination of cells expressing APPWT/4A/KK showed clear cleavage at the β-site, whereas no CTFβ was detectable (Fig. 4A). Moreover, the blocked β'-site was not processed by BACE1 even with monensin treatment, a condition known to
favor the production of CTF_{β}. (Fig. 4B). Thus, if a well pos-
tioned β-site is not cleavable, BACE1 would continue to search
for the structurally twisted β-site for processing. Again, these
data support the idea that the cleavage of APP by BACE1
possesses elasticity despite the dockings of both BACE1 and
APP in the membrane.

Mouse APP Is Better Aligned for Processing at the β- Site by
BACE1—In rodent species, cleavage of APP at the β- site
seems more robust as more Aβ-(11–40/42) is detected (19–20)
and cleavage at the β- site is likely species-specific (21). Notice-
ably, three residues in mouse Aβ differ from the corresponding
human sequence (Fig. 5A). When we mutated these three res-
ides to mouse sequence in human wild type APP and ex-
pressed this mutant construct (mAPP_{WT}) in HM cells, we found
that CTF_{α} was the predominant product (see mAPP_{WT} in Fig.
5B). However, depending on the transfection efficiency, CTF_{α}
could be a major product in cells expressing APP_{WT} (see examples
in lane APP_{WT} in Fig. 5B). This has complicated our
interpretations of differences due to mouse and human APP
β-sites. To determine whether conformation truly affects se-
lective cleavage in the mouse sequence, we mutated only R5G
in APP_{SYVSY} while preserving optimal cleavage sequence at
both sites. Examination of transfected HM cells with this mu-
tant APP (APP_{SYVR} → GlySY) showed a remarkable shift to CTF_{α}
from a dominant CTF_{α} product in APP_{SYVSY} (Fig. 5B).
Although Gly is not a typical breaker of protein secondary struc-
ture, natural mutation of Arg to Gly has noticeably caused
functional changes in many proteins. For example, a homoy-
zygous missense mutation (Arg to Gly) in the critical binding
region of the human EC-SOD gene (SOD3) is associated with
dramatically increased serum enzyme levels (26). A mutation
of Arg_{306} to Gly_{306} results in enhanced S-cone syndrome (27).
A similar Arg_{306} to Gly_{306} mutation in factor V is verified in
young patients with venous thrombosis (28). Thus, the Gly
residue in mouse Aβ may facilitate better alignment of the
β-site of APP with the active cleft of BACE1, and this will
certainly shift preferential cleavage.

The Loop Region of BACE1 Confers Its Mobility toward Its
Substrates—When compared with the other mammalian aspar-
tylic proteases, BACE1 and its homologue BACE2 share unique
features, which are the presence of a transmembrane domain
and a loop region (Fig. 6A). To determine whether the loop
region plays a role in the selective cleavage at APP β- and
β'-sites, we generated BACE1 mutants with this region either
shortened or extended. Knowing that six Cys residues within
the BACE1 catalytic domain are required for proper folding of
active BACE1, we chose to mutate BACE1 between residues
424–453 (Fig. 6A) to leave these Cys unchanged. These con-
structs were transfected in cells stably expressing either AP-
P_{WT} or APP_{SW}, and patterns of C-terminal fragments were
examined. We found that BACE1ΔL5 with a deletion of five
residues within the loop region (Δ446–450) did not disrupt the
normal processing of APP by BACE1 (Fig. 6B). However, a
10-residue deleted BACE1ΔL10 (Δ444–453) did dramatically
decrease the cleavage of both WT and Swedish APP whereas
BACE1+L45, another mutant with 45 residues inserted within
the loop region, did not affect BACE1 activity. Interestingly,
one of these BACE1 variants displayed obvious selective
cleavage toward the β- or β'-site. To further verify this obser-
vation, we co-transfected these BACE1 mutants with the
above mentioned APP variants in HEK-293 cells. As exempli-
fied in the lower panel of Fig. 6B, we found again that these
BACE1 variants displayed no clear selective cleavage at the
β- or β'-site. Thus, the loop region is clearly important for con-
ferring the mobility of BACE1 on the membrane, but the selective
cleavage at the β- or β'-site lies more on the preferential pre-
sentation of these two sites to the BACE1 active cleft.

DISCUSSION

As revealed by the crystal structure, the active cavity of
BACE1 is more open but less hydrophobic than many other
human aspartyl proteases (29). This open structure clearly
permits BACE1 to process APP at two adjacent cleavable sites.
Unlike other aspartyl proteases such as cathepsin D and E,
BACE1 has a type I transmembrane domain that confines its
catalytic domain facing the luminal or cytoplasmic side. Inev-
itably, BACE1 will only process its limited accessible sub-
strates as discussed previously (17). Transfection experiments
suggest that two additional membrane bound proteins, Golgi-resident sialyltransferase (30) and a leukocyte adhesion protein, P-selectin glycoprotein ligand 1 (31), are likely cleaved by BACE1. Noticeably, BACE1 prefers to cleave a site near the membrane: 27 or 17 residues in the respective β- or β'-site, 14 residues in sialyltransferase, and 17 residues in P-selectin glycoprotein ligand 1. We have demonstrated here that the distance of a BACE1 cleavable site is important, but the proper alignment of a cleavable site to the BACE1 active cleft is even more crucial. For example, a potentially cleavable site EIS-EV (31 residues away from the membrane) would be cleaved only if a structural bending existed (see lane APP_{ISY/PSY/ISY} in Fig. 1, B and C). Therefore, substrates cleaved by BACE1 are clearly restricted by a sequence feature and a conformational compatibility.

We hypothesized that BACE1 cleavage at either the β- or β'-site would be affected if there is a local conformational change surrounding these two sites. Although the crystal structure of APP has not yet been revealed, investigation of possible structural changes in the affected region was explored by a site-directed mutagenesis approach. Indeed, we readily observed inhibitions of cleavage at the β-site but not the β'-site in many APP variants discussed in this study. Even though the crystal structure of APP has not been solved, various structures of synthetic Aβ peptides have been revealed. For example, Shao et al. (32) suggest that micelle-bound human Aβ peptide adopt two α-helices from residues Tyr^{10}Val^{24} and Lys^{26}-Ala^{32} whereas Crescenzi et al. (33) observed similar two-helical structures from Ser^{25}Gly^{35} and Lys^{28}Gly^{38}. Based on this study, we would speculate that the N-terminal Aβ region in full-length APP likely possesses an α-helical structure. For example, an insertion of four Ala residues between the β- and β'-site could rotate the original β-scissor bond to a near opposite side, and this helical rotation may cause the twisted β-site to be less accessible to the BACE1 active cleft. Similarly, a deletion of either two or three residues within this region would also cause a similar helical shift, and this might have caused the original β-site in APP deletion mutants to no longer be accommodated by the BACE1 active cleft (Figs. 1 and 2). On the other hand, insertions at residue 21 in Aβ may only extend a secondary structure near the membrane, but the conformation of cleavage sites is not necessarily perturbed.

In most neuritic plaques, Aβ-(1–40/42) are the predominant peptides (34). In this study, we have clearly demonstrated that the cleavage at the APP β-site is more easily disrupted than the β'-site through a structural twist. This implicates that a rational design of small molecular drugs to block access of BACE1 to the β-site is a promising alternative approach to reduce specific Aβ-(1–40/42) production. Even though Aβ-(11–40/42) was shown to form aggregates in vitro and the aggregates are neurotoxic (35), this has not been validated in animal models. Moreover, rodents, which typically have a higher proportion of Aβ-(11–40/42) than humans, do not develop amyloid deposits although other species with the same sequence feature as humans, such as dog or rabbit, do develop amyloid depositions. Mice engineered to express human APP can form amyloid deposits in their brains, but Aβ-(1–40/42) are the predominant peptides although other species with the same sequence feature as humans. Even though Aβ-(1–40/42) was shown to form aggregates in vitro and the aggregates are neurotoxic (35), this has not been validated in animal models. Moreover, rodents, which typically have a higher proportion of Aβ-(11–40/42) than humans, do not develop amyloid deposits although other species with the same sequence feature as humans.

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Processing Amyloid Precursor Protein at the β-Site Requires Proper Orientation to Be Accessed by BACE1

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