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Evidence for dimeric BACE-mediated APP processing

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ABSTRACT

β-Secretase (BACE) is an aspartyl protease, which proteolytically processes amyloid precursor protein, making BACE an interesting pharmacological target in Alzheimer's disease. To study the enzymatic function of BACE, we mutated either of the two aspartic acid residues in the active site of BACE. This rendered BACE functionally inactive without affecting the degree of glycosylation or endosomal localization. In contrast, substituting both active site aspartic acid residues produced a functionally inactive, endoplasmic reticulum-retained and partially glycosylated BACE. Interestingly, co-expression of the two single active site mutants partially restored β-site cleavage of amyloid precursor protein, and the restored activity was inhibited with similar dose-dependency and potency as wildtype BACE by a small molecule inhibitor raised against BACE. In sum, our data suggest that two different active site mutants can complement each other in a partially functional BACE dimer and mediate APP processing.

Introduction

β-Secretase activity is executed by the aspartyl protease BACE, which is distantly related to other aspartyl proteases, such as renin and cathepsin [1,2]. BACE (also referred to as BACE-1, Asp2 or memapsin-2) is a single pass 60 kDa transmembrane glycoprotein that contains a large extracellular domain harboring the active site and a short intracellular domain important for intracellular trafficking [3,4]. BACE is highly expressed in neurons and proteolytically processes the amyloid precursor protein (APP), as well as several other proteins [5–7]. Sequential cleavage of APP by β- and γ-secretase produces the amyloid beta (Aβ) peptide, which accumulates in extracellular deposits in senile plaques in Alzheimer's disease (AD). During intracellular maturation, BACE is extensively posttranslationally modified in the secretory pathway by phosphorylation, *N*-glycosylation and palmitoylation [3,8,9]. Structural data reveal that BACE harbors a large active site with two catalytic aspartate residues at position 93 and 289 [10]. It has been suggested that BACE dimerizes, and that dimerization is important for activity [11,12]. BACE2 is a closely related homolog to BACE, but does not contribute to amyloid peptide production, as it executes APP cleavage at a different site, as compared to BACE (for review see [15]).

Because of the key role of β-secretase in APP processing and Aβ generation, it is an attractive idea to develop therapeutic approaches that reduce β-secretase activity for therapeutic intervention in AD. It is therefore important to obtain a detailed molecular understanding of BACE function and an important question is the relative importance of dimerization and intracellular localization for activity. Also, we have limited knowledge about the precise requirements for the active site aspartic acid residues in the dimeric state of BACE, which may be of direct relevance for generation of BACE inhibitors. To address these issues, we have in this report studied the relationship between BACE enzymatic activity, intracellular localization and dimerization in a BACE-deficient cellular background, to eliminate any effects of endogenous BACE. We show that the BACE dimer is catalytically active in the cell, and suggest a novel mode for BACE activity, where two BACE molecules carrying different active site mutations can complement each other, and partially restore a proteolytic processing activity which is sensitive to BACE inhibitors.

Materials and methods

Antibodies and reagents. Antibodies used for immunoblotting and immunocytochemical experiments were rabbit anti-BACE-1 (EE-17, Sigma–Aldrich), mouse anti-BACE MAB5308 (Chemicon) and rabbit anti-GRP94 (ab3674, Abcam). Brefeldin A (BFA) and Monensin were obtained from Sigma–Aldrich and used at 1 and 3 μg/ml, respectively. Compound 24 is a low molecular weight BACE inhibitor, the synthesis of which has been described [20].

BACE knockout mice and culture of BACE-deficient mouse embryonic fibroblasts. The generation of the BACE knockout mice has been

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described [19]. All animal experiments were performed in accordance with ethical permits from the North Stockholm Committee. BACE knockout mouse embryonic fibroblasts (MEFs) were derived by dissection of 13.5-day-old *BACE* knockout mouse embryos, and maintained in Dulbecco's Minimal Essential Medium supplemented with 10% fetal bovine serum and Penicillin/Streptomycin (Invitrogen). All transfections were performed with LipofectAMINE Plus reagent as described by the manufacturer (Invitrogen).

cDNA constructs. EGFP-EEA1 has been previously described [21]. Generation of the BACE mutants is described in the Supplementary Information.

Immunocytochemistry. Cells were grown on glass cover slips for all experiments. Briefly, 36–48 h post transfection, cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.2% Triton X-100 in PBS, which was followed by incubation with 5% BSA in PBS for 30 min to block unspecific binding. The anti-BACE MAB5308 (1:300) and rabbit anti-GRP94 (1:500) were used as primary antibodies and incubated overnight at 4 °C. For co-localization with EEA1, cells were co-transfected with the BACE constructs and an EGFP-tagged EEA1 (EGFP-EEA1). Coverslips were washed three times with PBS followed by incubation for 1 h with the indicated combinations of secondary antibodies, Alexa 546- and 488-conjugated goat anti-mouse and goat anti-rabbit antibodies (Molecular Probes). After thorough washing in PBS, coverslips were mounted and visualized in a Zeiss LSM510 META confocal microscope.

PNGase F and endo-H treatment. The deglycosylation assay was performed 48 h post transfection. Cell lysates containing 20 μg of total protein were subjected to an overnight incubation with endo-H (Endoglycosidase H) or PNGase F (*N*-glycosidase F) according to the manufacturer's instructions (New England BioLabs). The reaction was stopped by the addition of the SDS sample buffer and analyzed by Western blot, as described in the Supplementary Information.

Pharmacological analysis of intracellular trafficking. BACE$^{-/-}$ MEF cells, cultured in 24 well plates and transfected with different APP and BACE constructs, were incubated with different concentrations of the BACE inhibitor compound 24 (Fig. 4) [20]. Briefly, 24 h post transfection, cell medium was removed and fresh medium containing various concentrations of the BACE inhibitor was added to the cells. 24 h later, 25 μl of each incubation were analyzed for either sAPPα or sAPPαswe. The sAPPα and sAPPβ assays are described in the Supplementary Information.

Results

Effects of active site mutations on BACE dimerization

To study the effect of mutations in the active site, we replaced each of the aspartic acid residues 93 and 289 with alanine residues, generating the D93A and D289A constructs, respectively. We also generated a construct where both aspartic acid residues were replaced with alanine residues (D93A/D289A) (Fig. 1A). We first explored the effects of the various mutations on dimerization. To eliminate effects of endogenous BACE, we introduced the mutant

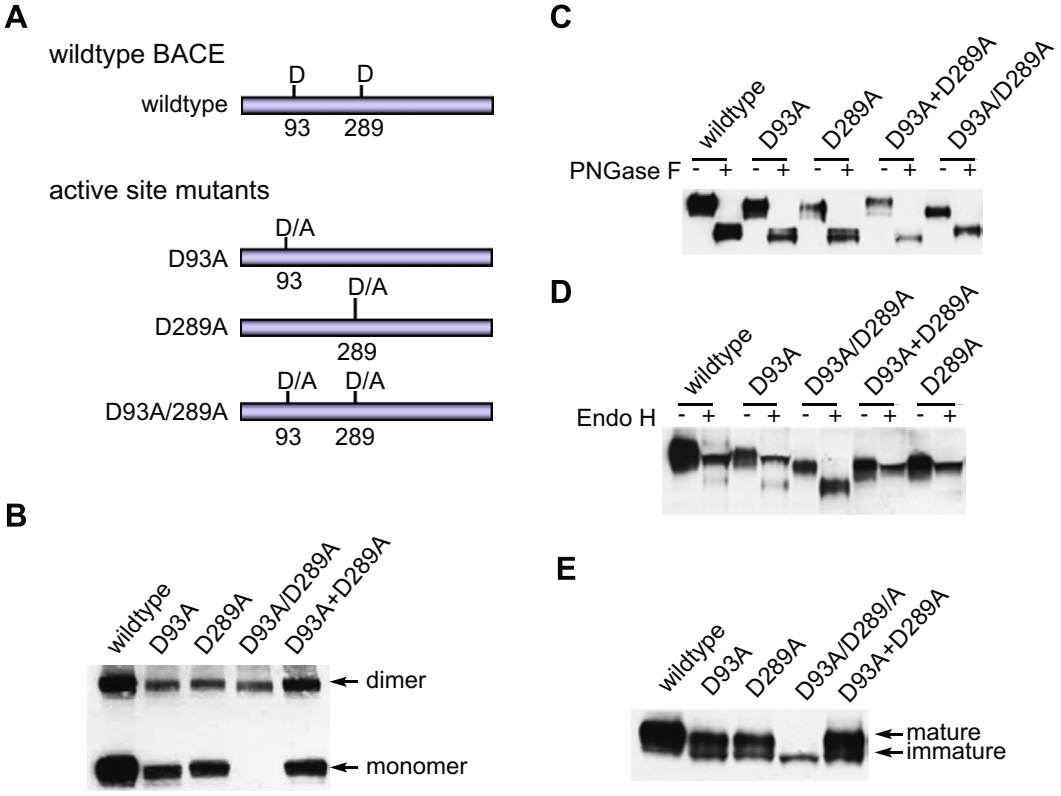

Fig. 1. Effects of active site BACE mutations on the proportion of monomeric and dimeric BACE and on the glycosylation status. (A) Schematic depiction of wildtype BACE and the three active site mutants D93A, D289A and D93A/D289A. (B) Analysis of the proportion of monomeric and dimeric BACE from the active site BACE mutants, transiently expressed in BACE$^{-/-}$ MEF cells. D93A + D289A denotes co-expression of D93A and D289A. The BACE monomer and dimer bands migrate at 60 and 120 kDa, respectively. (C–E) Lysates from BACE$^{-/-}$ MEF cells, transiently transfected with BACE and the different BACE mutants were: (C) treated with PNGase F; (D) treated with Endo-H; or (E) left untreated prior to SDS–PAGE and Western blot analysis using antibodies directed against BACE. PNGase F treatment (C) resulted in more rapid migration of all expressed BACE constructs, suggesting that all BACE proteins are glycosylated. Endo-H treatment (D) resulted in faster migration of the D93A/D289A mutant, suggesting that this protein is partially glycosylated. In the untreated samples (E) D93A, D283A and D93A + D283A migrate as two bands, whereas D93A/D289A appear as one, more rapidly migrating band, corresponding to an immature species. The experiments were conducted 3 times with similar results and the data from one representative experiment is presented.

constructs into primary mouse fibroblasts obtained from a BACE-deficient mouse embryo [19]. Wildtype BACE migrated as both a monomer and dimer in Western blot analysis under nonreducing conditions (Fig. 1B), which is in keeping with previous studies [8,9]. Individual mutations of the aspartic acid residues in the active site did not alter the balance between dimeric and monomeric BACE, as compared to wildtype BACE, whereas the double mutant (D93A/D289A) predominantly migrated as a dimer. Co-expression of D93A and D289A resulted in a monomer versus dimer distribution close to that observed for wildtype BACE (Fig. 1B). These results suggest that the catalytic residues of BACE have an impact on BACE dimerization.

Effects of active site and hydrophobic stretch mutations on BACE maturation and intracellular trafficking

As part of its maturation BACE becomes glycosylated during intracellular trafficking [3]. We therefore tested the glycosylation status of the different mutants by enzymatic digestion with *N*-glycosidase F (PNGase F), which cleaves asparagine (N)-linked high mannose and complex oligosaccharides from glycoproteins, and with Endoglycosidase H (Endo-H), which cleaves N-linked hybrid or high mannose oligosaccharides, but not complex oligosaccharides. Endo-H-sensitivity thus discriminates between proteins trapped in the ER, which are Endo-H-sensitive, and those that have proceeded to the Golgi apparatus, where they become Endo-H-resistant. As for the dimerization studies, we transiently expressed the BACE constructs in the BACE$^{-/-}$ cells followed by Western blot analysis under denaturing conditions. All mutants were PNGase F-sensitive (Fig. 1C), confirming N-linked glycosylation. With regard to the Endo-H-response, wildtype BACE was, as expected [3], Endo-H-resistant (Fig. 1D), and migrated predominantly as the mature, slow-migrating, form (Fig. 1E). The D93A and D289A mutants, as well as co-expressed D93A and D289A behaved similar to wildtype, i.e. they were Endo-H-resistant and migrated as a mixture of mature and immature forms (Fig. 1D and E). In contrast, the D93A/D289A mutant exhibited Endo-H-sensitivity (Fig. 1D) and more rapid migration, corresponding to the immature form (Fig. 1E).

Since advanced glycosylation takes place in the Golgi apparatus, we hypothesized that the partial loss of advanced glycosylation observed in some mutants might reflect impaired intracellular trafficking and ER-retention. To address this, we determined the intracellular localization of the various BACE mutants co-transfected with marker proteins for various intracellular localizations. The experiments revealed that wildtype BACE co-localized extensively with the endosomal marker EEA1 (Fig. 2A) and with the Golgi marker gm130 (data not shown), but not with the ER marker GRP94 (Fig. 2B). This is in keeping with a previous report on an endosomal localization for BACE [1]. The single active site mutants, D93A and D289A, were also largely co-localized with EEA1, whereas, in contrast, the double active site mutant D93A/D289A co-localized predominantly with GRP94 rather than with EEA1, indicative of ER-retention (Fig. 2A and B). Taken together, the glycosylation and immunocytochemistry experiments suggest that the impaired advanced glycosylation pattern displayed by the D93A/D289A construct is a result of impaired trafficking in the secretory pathway and retention in the ER.

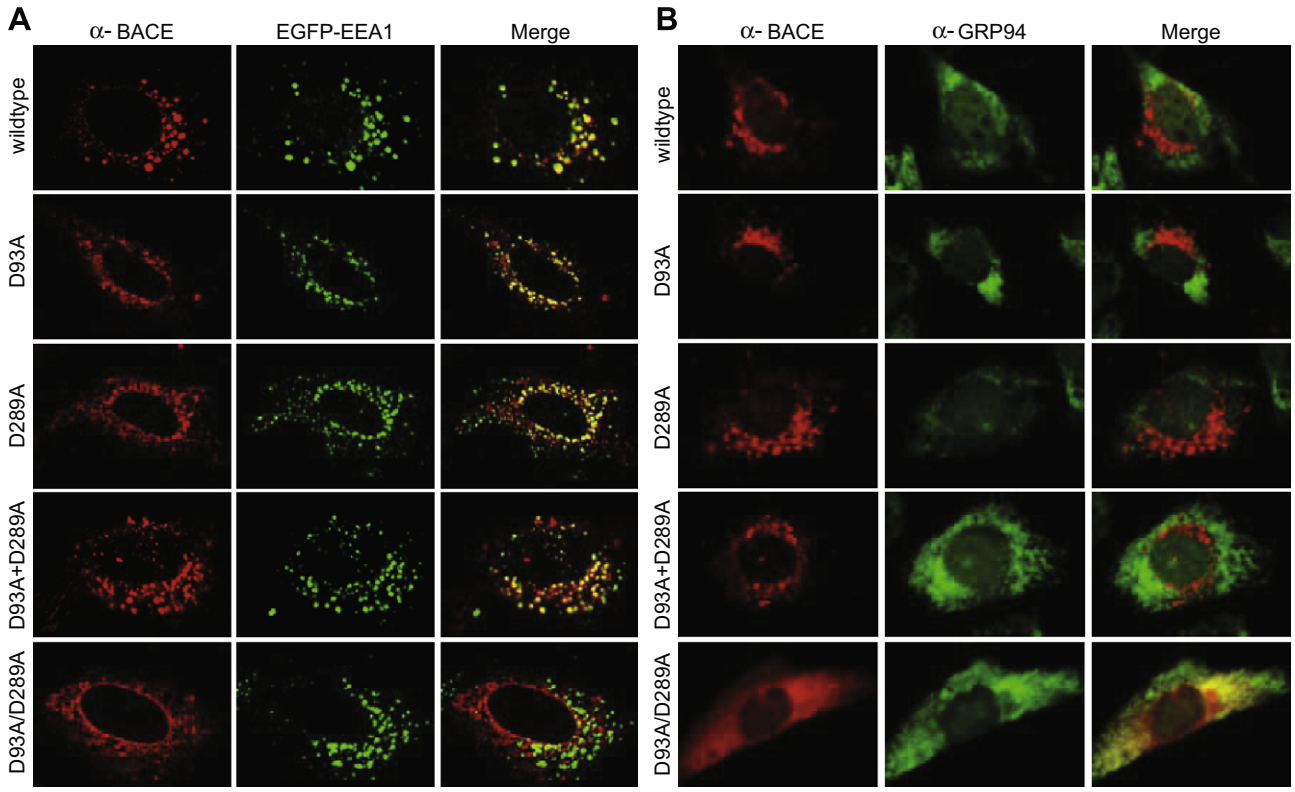

Fig. 2. Analysis of the cellular distribution of the different BACE mutants. BACE$^{-/-}$ MEF cells, transiently expressing BACE and different BACE mutants were subjected to immunocytochemistry using antibodies directed against BACE (in red) and the early endosomal protein EEA1 (A) or the ER marker GRP94 (B) (in green). The D93A/D289A mutant co-localized extensively with GRP94. In contrast, wildtype BACE, D93A, D289A and co-expressed D93A and D289A co-localized with EEA1. The experiment was conducted 3 times with similar results and the data from one representative experiment is presented.

Co-expression of the two active site single mutants D93A and D289A partially restores APP processing activity

In the next series of experiments we set out to address the effects of the different mutations on BACE activity. To this end, we co-expressed the different constructs with either wildtype APP or an APP carrying the Swedish mutation (APPswe, Lys/Asn, Met/Leu at position 670/671, numbering based on the APP695 form) in the BACE-deficient cells (Fig. 3A). The amount of sAPPβ and sAPPβswe released into the medium was recorded using an antibody-based electrochemiluminescence assay (MSD technology). As expected, given the BACE-deficient background, no sAPPβ species could be detected in non-transfected cells or in cells transfected with either APP or APPswe, using antibodies specific for the neoepitope of sAPPβ or sAPPβswe. Cotransfection of wildtype BACE-1 with APP or APPswe resulted in a large increase in the

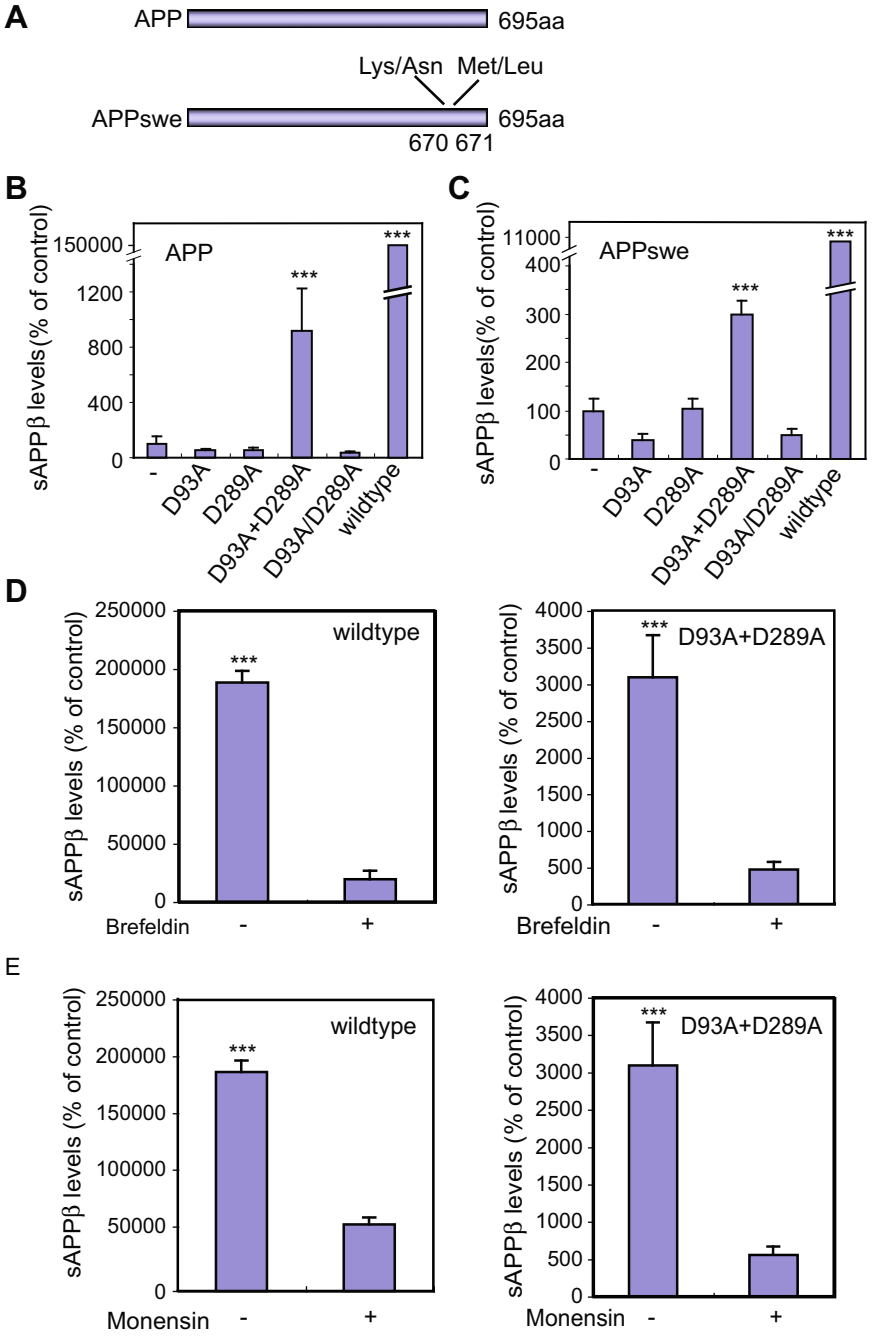

Fig. 3. Analysis of BACE- and BACE mutant-mediated β-secretase cleavage of APP substrates. (A) Schematic depiction of the APP and APPswe, the substrates used to assess β-secretase activity. The APPswe mutant carries two amino acid substitutions at position 670/671 (Lys to Asn, Met to Leu, respectively). (B and C) $BACE^{-/-}$ MEF cells were transiently transfected with APP (B) or APPSwe (C), either alone or in combination with the various BACE mutants. Conditioned media was sampled 36 h post transfection and examined for sAPPβ content using a MSD assay. Data is presented as% of control (APP transfected only, set to 100%). (D and E) $BACE^{-/-}$ MEF cells, transiently cotransfected with APP and either wildtype BACE or a combination of D93A and D289A, were exposed to brefeldin (D) or monensin (E) 24 h after transfection. After a 24 h exposure to the drugs, sAPPβ levles were examined in the conditioned media using the MSD sAPPβ assay. sAPPβ levels from cells cultured in absence of monensin or brefeldin was set to 100% in each case. The experiments were conducted 3 times with triplicates of each transfection reaction. The data presented are the average of the means from the 3 different experiments +/− SD. Values are significant at $^{***}p < 0.001$ and $^{**}p < 0.01$ (Student's *t*-test) as indicated in the figure.

sAPPβ and sAPPβswe levels (Fig. 3B and C). In contrast, expression of any of the three active site mutation constructs (D93A, D289A and D93A/D289A) did not yield any significant increase in sAPPβ levels (Fig. 3B). However, co-expression of D93A and D289A yielded an approximately 9-fold induction over control in sAPPβ release (Fig. 3B). A similar tendency was observed also for processing of APPβswe (Fig. 3C). A partial, but significant, rescue of processing activity was observed by co-expression of D93A and D289A, but not by the active site mutants expressed individually (Fig. 3C). These results show that co-expression of two BACE molecules with different active site mutations can partially restore processing activity, indicating that they functionally complement each other.

Pharmacological inhibition of intracellular trafficking blocks BACE enzymatic activity

We used the sAPPβ release assay to further analyze the correlation between cellular localization and enzymatic activity. As wildtype BACE and co-expression of D93A and D289A resulted in at least partial enzymatic activity, coupled with endosomal localization (Figs. 2 and 3A–C), we asked whether localization to endosomes in these cases was a requirement for function. We used two inhibitors of intracellular trafficking, brefeldin and monensin, and the data show that brefeldin substantially reduced the activity of wildtype and co-transfected D93A and D289A (Fig. 3D). A similar reduction was observed in both cases also by monensin treatment (Fig. 3E). No apparent toxicity was observed following the brefeldin of monensin treatments (data not shown). This suggests that pharmacological retention early in the secretory pathway reduces BACE activity.

Effects of BACE inhibitors on wildtype and co-expressed single active site mutants

As the two different single active site mutations complemented each other when expressed simultaneously we wanted to explore how they responded to pharmacological inhibition of BACE. We therefore examined the effect of a small molecule BACE inhibitor (compound 24) [20] (Fig. 4A) on release of sAPPβ upon expression of wildtype BACE or co-transfected D93A and D289A. In wildtype BACE-transfected cells the IC50 value of compound 24 was 480 nM (Fig. 4B). For co-transfected D93A and D289A, the potency of compound 24 was slightly higher, IC50 = 120 nM, but still in the same range as for the wildtype BACE-expressing cells (Fig. 4B). The effect of compound 24 on cell viability was addressed with the Vialight ATPase assay, and no signs of cellular toxicity were detected within the concentration interval examined (data not shown). These data suggest that inhibitor 24, raised against the active site of one BACE polypeptide, in a dose dependent manner and with

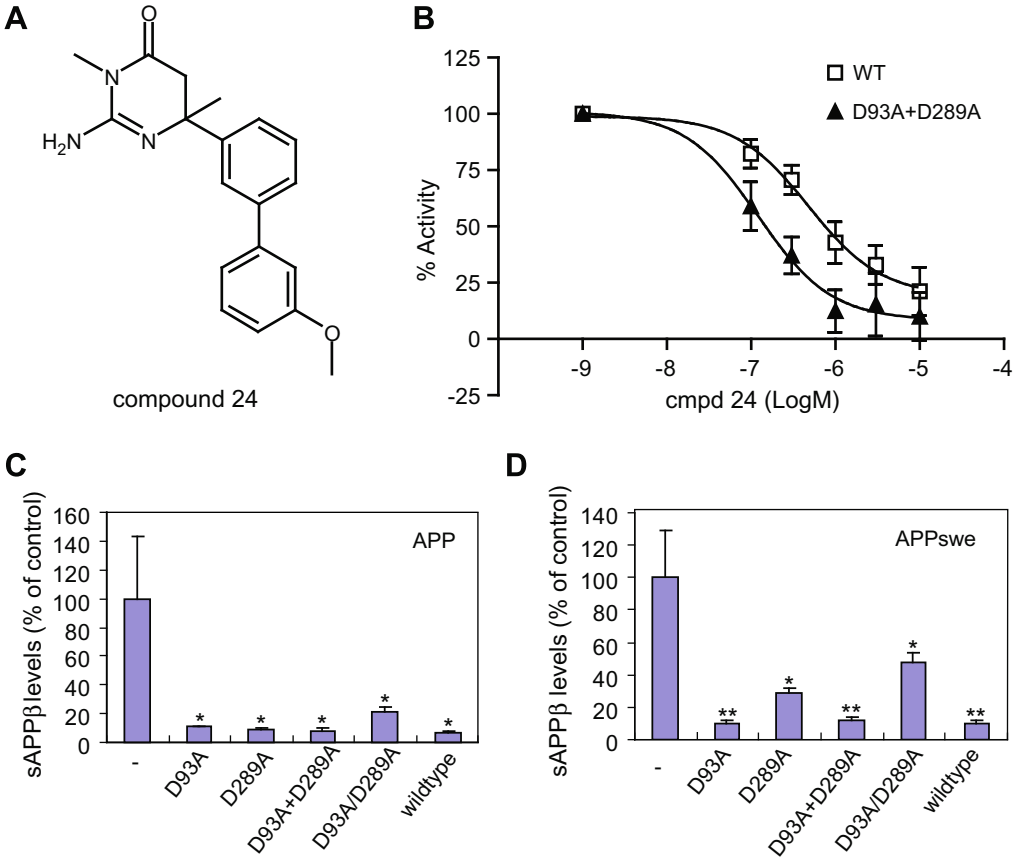

Fig. 4. Inhibition of BACE dimer-catalyzed APP processing with a small molecule inhibitor directed against monomeric BACE. (A) Structure of compound 24. (B) BACE$^{-/-}$ MEF cells were transiently transfected with APP and wildtype BACE (open rectangles) or the D93A and D289A constructs together (filled triangles). Twelve hours post transfection, the BACE inhibitor compound 24 was added. 16 h post inhibitor addition, sAPPβ levels were determined. Data are expressed as% of control where 100% is the sAPPβ level in the absence of β-secretase inhibitor exposure. The data are plotted according to the non-linear regression model and IC50 for wildtype BACE and D93A + D289A transfected cells was determined to 480 and 120 nM, respectively. (C and D) BACE$^{-/-}$ MEF cells were transiently transfected with APP (C) or APPswe (D) alone (−) or cotransfected with each of the BACE constructs, as indicated. Conditioned medium was collected 36 h post transfection and examined for sAPPα content using a MSD assay. The data are displayed as% of control (APP or APPswe only transfected set to 100%). The experiments were conducted 3 times with triplicates of each transfection reaction. The data presented are the average of the means (C and D) or +/− SD (A–C) from the 3 different experiments.

high potency inhibits the activity of two co-expressed BACE mutants, expressing different single active site mutations.

Processing-deficient BACE mutants affect α-secretase cleavage of APP

APP is processed not only by β- and γ-secretase, but also by α-secretase, and the cleavage sites for α- and β-secretase cleavage are located close to each other in the juxtamembrane region. It has been suggested that α- and β-secretase compete for APP [22], and in the light of this, we decided to study whether the various BACE mutations could interfere with α-secretase cleavage. We analyzed the levels of sAPPα, which is generated as a result of α-secretase cleavage, in cells transfected with wildtype BACE or the different mutants. In the BACE-deficient cells transfected with APP or APPswe alone, robust sAPPα levels were observed (Fig. 4C and D), significantly higher than in non-transfected cells (data not shown). In contrast, transfection of wildtype BACE caused a major decrease in sAPPα levels for both APP or APPswe (Fig. 4C and D). Interestingly, expression of all three active site mutants (D93A, D289A and D93A/D289A) caused a remarkable decrease in sAPPα levels. This indicates that the active site mutants, despite impaired catalytical activity, are still able to bind to APP and compete with α-secretase.

Discussion

The data in this report support earlier reports showing that BACE form dimers, and also provide novel evidence that a BACE dimer mediates cellular β-site cleavage of APP in post ER compartments. This notion is based on the partial restoration of enzymatic activity by simultaneous expression of constructs carrying different single active site mutations (D93A and D289A), but not by expression of D93A or D289A alone or by expression of a construct carrying both active site mutations (D93/289A). The mechanism behind this partially rescued activity is not yet understood, but as the catalytic Asps, based on structural data, appear to be buried deep in the active site, direct cooperation between the single remaining Asp in each active site seems less likely. The effect of the Asps may instead be to stabilize binding to APP, which may also be dimeric or oligomeric [8], and that one Asp residue would be sufficient for execution of the partial enzymatic activity. Irrespective of how the Asp mutations complement each other, these findings, combined with an earlier demonstration that differentially tagged BACE proteins can be co-immunoprecipitated [9], provide strong evidence for BACE dimerization.

The functionally active constructs, i.e. wildtype BACE and co-expressed D93A and D289A, co-localized with that of the early endosome marker EEA1. This is in line with an earlier suggestion that endosomes would provide a favourable milieu for the enzymatic activity of BACE, because of the low pH optimum for BACE [1]. Furthermore, when localization to endosomes was inhibited for wildtype BACE and co-expressed D93A and D289A, by the use of brefeldin and monensin, activity was lost, indicating that a relocalization of BACE from endosomes to the ER impaired the functional activity.

It is interesting to note that activity of the co-expressed D93A and D289A mutants was sensitive to a BACE inhibitor to a similar extent as wildtype BACE. The inhibitor, compound 24, inhibits the activity of recombinant, presumably monomeric, BACE extracellular domain [20] and binds to the active site according to X-ray crystallography data (data not shown). It therefore appears that at least some inhibitors raised against the active site of monomeric BACE retain the ability to interfere with BACE activity reconstituted from one or two of the differentially mutated monomers. Our data furthermore suggest that binding to APP can be separated from activity. The proximity between the α- and β-secretase cleavage sites argues that α- and β-secretases can be competing for binding. This notion is supported by the finding that wildtype BACE strongly reduced production of sAPPα when introduced into the BACE-deficient cell line. That the functionally inactive D93A, D289A and D93/289A mutants exhibited a similar negative effect on α-secretase activity indicates that they also bind to APP, but without executing proteolytic processing.

In conclusion, our data extend previous observation on BACE posttranslational modifications and intracellular localization, and we propose a novel mechanism for dimeric BACE, where two differentially mutated BACE molecules can restore a partially functional enzyme when co-expressed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.064.

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