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The metabolism of β-amyloid converting enzyme and β-amyloid precursor protein processing ☆

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Abstract

Herein we investigated the processing of β-secretase (BACE), implicated in Alzheimer's disease through processing of β-amyloid precursor protein (βAPP), into smaller metabolites. Four products of ~34, ~12, ~8, and ~5 kDa were identified, none of which were generated autocatalytically. The ~34 and ~12 kDa forms are held together by disulfide bridges. The ~34 kDa form results from two cleavages: an N-terminal processing at $RLPR_{45}\downarrow$ by furin/PC5, and a C-terminal cleavage at $SQDD_{379}\downarrow$ by an unknown enzyme that also releases the C-terminal ~12 kDa product. Microsequencing of the ~8 and ~5 kDa fragments showed that they are the result of processing at $VVFD_{407}\downarrow$ and $DMED_{442}\downarrow$, respectively. Mutagenesis of the identified cleavage sites revealed that the mutants D379A, D379L or D379E block the degradation of BACE into the ~12 kDa product, confirming the importance of Asp_{379}. Notably, the D379E mutant results in higher βAPP derived C99 levels. In contrast, D442A or D442E did not affect the production of the ~8 or ~5 kDa products. The levels of the ~8 and ~5 kDa products are significantly lower in the mutant D407A but less so D407E, likely due to the low efficacy of ER exit of the D407A mutant. Indeed, while co-expression of βAPP with BACE results in enhanced production of $A\beta_{11-40}$, the D407A mutant produces mostly $A\beta_{40}$.

Keywords: β-Secretase; Metabolism; βAPP processing; Mutagenesis; Aβ formation; ER/Golgi intermediate compartment; Microsequencing; Asp cleavage site; Alzheimer's disease

Proteins and peptides that are biologically active are often generated by intracellular limited proteolysis of inactive precursors. Within the secretory pathway such processing is in part afforded by one or more members of the proprotein convertase family, serine proteases related to bacterial subtilisin [1,2]. Recently, it was also demonstrated that enzymes known as secretases could also perform secretory processing. Attention was particularly focused on these enzymes when it was realized that they play a major role in the processing of the amyloid precursor protein βAPP and can regulate the production of the toxic peptide Aβ implicated in the etiology of Alzheimer's disease. Thus, α-secretases are usually metalloproteinases that are involved in the shedding of various membrane-bound proteins into secretable forms, including βAPP into soluble sAPPα [3,4]. The membrane-bound adamalysin (ADAM) family of metalloproteases is believed to constitute the major α-secretases [5]. In contrast, both β- and γ-secretases are needed for the generation of the amyloid peptide Aβ [6]. Initial cleavage of βAPP by the aspartyl protease

☆ *Abbreviations:* aa, amino acid; PC, proprotein convertase; pro, prosegment; mBACE; mouse β-secretase converting enzyme; ER, endoplasmic reticulum; TGN, *trans* Golgi network; sBACE, soluble BACE; $BACE_F$, full length BACE; BACE-Δpro, BACE lacking the prosegment; PMA, phorbol 12-myristate 13-acetate.

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β-secretase (BACE, also called BACE-1 or memapsin-2) [7–9] results in the formation of the membrane-bound C-terminal C99 and C89 fragments, by cleavage at the sequences EVKM↓D (or EVNL↓D, for the Swedish βAPPsw mutant) and DSGY↓E, respectively. Cleavage of C99 by γ-secretase releases either the predominant 40 amino acid (aa) long Aβ species ($A\beta_{1-40}$, referred to here as $A\beta_{40}$) or the longer 42-aa variant ($A\beta_{1-42}$, here called $A\beta_{42}$). Cleavage of C89 by γ-secretase results in the second most abundant N-terminally truncated variants in neurons, $A\beta_{11-40}$ and $A\beta_{11-42}$ [10]. The formation of Aβ and its derivatives occurs at multiple sites in the secretory pathway including the endoplasmic reticulum/intermediate compartment (ER/IC), the Golgi network, and the endosomal/lysosomal pathway, to produce multiple forms of the Aβ peptide that can be either secreted from the cell or remain intracellular [11]. The enzyme γ-secretase is a complex of presenilins PS1 or PS2 and at least three other proteins [11–13]: nicastrin [14], aph-1 [15], and pen-2 [16]. Although it seems that presenilins are indeed necessary for the production of most secreted and intracellular Aβ, particularly that generated in downstream organelles, it was shown recently that a presenilin-independent γ-secretase is active in the ER/IC and is responsible for the production of a portion of intracellular $A\beta_{42}$ [11].

We [17] and others [18,19] have shown that BACE is first synthesized as a zymogen that undergoes cleavage at $RLPR_{45}$↓ by one or more members of the proprotein convertase family, mostly by furin and PC5. Such zymogen cleavage improves ~2-fold the activity of BACE [20]. It also seems that the prosegment (aa 22–45) of BACE is important for the proper folding [20] and efficient exit of proBACE from the endoplasmic reticulum (ER) en route to the *trans* Golgi network (TGN), as BACE-Δpro, lacking the prosegment, is predominantly localized in the ER [17]. It was also observed that a minor amount of BACE can be shed into the medium [17], possibly by ADAM10, and that this processing is upregulated by agents that activate protein kinase C [21]. In our hands we found that in HK293 cells co-expression of βAPPsw with either soluble sBACE or BACE-Δpro resulted in higher levels of $A\beta_{40}$ and less of C99 or $A\beta_{11-40}$. Since different forms of BACE result in distinct Aβ products, we hypothesized that BACE processing may modulate its cellular sorting ability, and hence the production of Aβ [17]. The results of Hussain et al. [21], suggested that blocking the shedding of BACE does not affect the level of secreted Aβ. Recently, it was also reported that BACE undergoes processing by an unknown enzyme at a surface-exposed α-helix at the sequence $TEAL_{228}$↓ A, generating stable N- and C-terminal fragments that remain covalently associated via disulfide bonds. The efficiency of BACE endoproteolysis was observed to depend heavily on cell and tissue type, and the cleaved BACE seems to retain measurable β-secretase activity [22].

In this work we report a different type of non-autocatalytic processing of BACE generating ~34, ~12, ~8, and ~5 kDa fragments, two of which (~34 and ~12 kDa) are held together by disulfide bridges. We identified the processing sites by microsequencing and showed that preventing some of these cleavages by mutagenesis can profoundly affect the cellular sorting of BACE and the production of Aβ.

Materials and methods

Construction of mouse BACE variants by site-directed mutagenesis. The pcDNA3 vector (Clontech) with the mouse (m) BACE cDNA containing the wild type sequence was used as template to introduce mutations [17]. Site-directed mutagenesis using Stratagene (Torrey Pines Road, La Jolla, CA) QuikChange XL site-directed mutagenesis kit was used to generate the recombinant proteins. The *Eco*RI–*Kpn*I fragment of mBACE was subcloned into the cloning vector pUC19 and the mutations were introduced using a set of primers containing the relevant mutations according to the manufacturer's instructions. All mutants were verified by DNA sequencing. After mutagenesis, the fragment was cloned back into the expression vector pCDNA-3.zeo-mBACE-Flag and the proteins were expressed in HK293 and Neuro2A cells. The constructs containing either soluble BACE (sBACE) or full length BACE lacking the prosegment (BACE-Δpro) were previously reported [17].

Transfections and biosynthetic analyses. All transfections were done with $2–4 \times 10^5$ HK293 or Neuro2a cells using Effectene (Qiagen) or Lipofectamine (Invitrogen) and a total of 1–1.5 or 8 μg, respectively, of BACE construct cDNAs were subcloned into the vector pcDNA3. Two days post-transfection, the cells were washed and then pulse-incubated for various times with either 250 μCi/ml [^{35}S]Met/Cys (Perkin–Elmer Life and Analytical Sciences, Woodbridge, Ont., Canada) or 400 μCi/ml [^{3}H]Phe (Amersham Biosciences). Pulse-chase experiments with [^{35}S]Met/Cys were carried out as described previously [17]. The cells were lysed in immunoprecipitation RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (Roche Molecular Biochemicals), after which the lysates and media were prepared for immunoprecipitations [17]. The Flag monoclonal antibody (mAb) used is directed against the Flag-M2 epitope (1/500 dilution; Stratagene). The rabbit polyclonal antisera used at a dilution of 1:200 were: Ab PA1-757 directed against aa 485–501 of human BACE (Affinity Bioreagents, Golden, CO); and anti-β-amyloid antibodies directed against either aa 1–16 of Aβ (APP-711) that recognizes βAPP, Aβ, and C99 [17], or another developed against $A\beta_{40}$ that predominantly recognizes this 16 aa N-terminal peptide (Ab A8326, Sigma, Toronto, Ont., Canada). Immunoprecipitates were resolved by SDS–PAGE (either 8% or 14% Tricine gels) and autoradiographed.

Microsequencing analyses. Transfected HK293 cells were pulse-labeled with 250 μCi/ml [^{35}S]Met or 400 μCi/ml [^{3}H]Phe. Lysates were immunoprecipitated with anti-Flag-M2 antibody, resolved by SDS–PAGE (8% Tricine gel), and autoradiographed. The identified radioactive products (~34, ~12, ~8, and ~5 kDa) were excised from the gel, and the underlying proteins were microsequenced as previously described [23] on an Applied Biosystems Procise cLC Protein Sequencer.

Protease inhibitors. The protease inhibitors were used at the following final concentrations: γ-secretase inhibitor DFK-167 (50 μM; Enzyme System Products, Livermore, CA, USA), TAPI (100 μM; Sigma, Toronto, Ont., Canada), GM6001 (25 μM; Chemicon

International, Toronto, Ont., Canada), chymostatin (0.2 mM; Boehringer–Engelheim, Laval, Que., Canada), the pan-caspase inhibitor Z-VAD(OMe)-CH_2F (50 μM; Calbiochem EMD Prosciences, San Diego, CA, USA), and 1 μM of the ADAM10 inhibitors GW9020, GW5712, GW4023, and GW9901, which were generous gifts from Dr. I. Hussain (GlaxoSmithKline, UK). Phorbol 12-myristate 13-acetate (PMA) (Sigma, Toronto, Japan) and the proteasome inhibitor lactacystin (Peptide Institute, Osaka, Ont., Canada) were used at concentrations of 5 and 80 μM, respectively. Brefeldin A (BFA) was used at a final concentration of 25 μg/ml.

Results

The identification of the enzyme BACE as β-secretase [7–9] and its proof of function in vivo [24] led to a number of studies aimed at defining its biological functions and cellular trafficking [17,18,25–27]. Thus, it was realized that the cytosolic tail of BACE is phosphorylated [25] and palmitoylated [17], and that it can play an important role in defining the subcellular localization of this enzyme. Furthermore, fluorescence resonance techniques demonstrated that βAPP and BACE form a complex in early endosomes [28]. Therefore, it was important to study in more detail the cellular fate of BACE and its possible processing and degradation as a means to regulate its activity, cellular trafficking, and/or functions. Thus, it was found that BACE is first synthesized in the ER as a zymogen that is cleaved by either furin or PC5 within the TGN [17], a process that results in a modestly enhanced activity of the enzyme [20]. Removal of the transmembrane cytosolic tail of BACE resulted in a soluble form of the enzyme (sBACE) that also cleaves βAPP into C99 [17,29], but in HK293 cells it seems to favor the production of $A\beta_{40}$ [17]. This suggested that sBACE is more amyloidogenic that $BACE_F$, and that shedding of $BACE_F$ [17] may change the properties of this β-secretase. However, recent data revealed that although ADAM10 seems to be a major cell-surface BACE-sheddase, inhibition of this process by hydroxamates does not affect the level of Aβ [21].

Mature BACE cycles between the TGN, cell surface, and endosomal system several times throughout the course of its relatively long lifespan ($t_{1/2}$ 12–16 h) [30], suggesting that it may undergo endoproteolytic processing during its intracellular trafficking, a step that may regulate its substrate specificity and/or intracellular localization [30]. Thus, it was reported that $BACE_F$ is cleaved at the sequence $TEAL_{228}\downarrow A$ by an unidentified enzyme in many non-neuronal cells, resulting in fragments held together by disulfide bridges [22]. This cleavage was suggested to regulate the enzymatic activity of BACE.

C-terminal processing of BACE

In this work, the processing of BACE was studied in Neuro2a and HK293 cells transiently transfected with full length BACE ($BACE_F$) tagged at the C-terminus with a Flag epitope [17]. Following 4 h of pulse-labeling with [^{35}S]Met/Cys, the cell lysate was immunoprecipitated with either a Flag antibody or a BACE cytosolic tail (CT-) specific antibody (Ab PA1-757, not shown). The immunoprecipitated proteins were separated by SDS–PAGE under reducing (+SH) and non-reducing (−SH) conditions (Fig. 1A). The data revealed the spe-

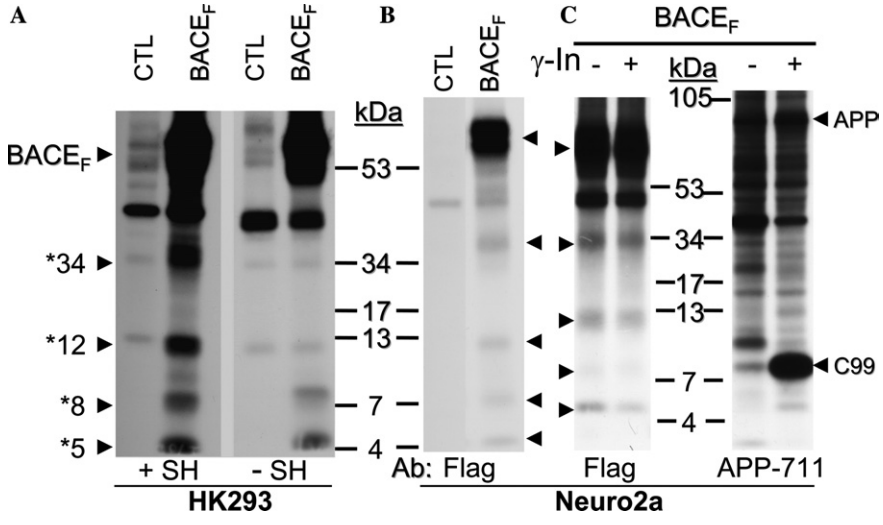

Fig. 1. Processing of $BACE_F$ in HK293 and Neuro2a cells. HK293 cells (A) or Neuro2a cells stably expressing βAPPsw (B,C) cells were transiently transfected with cDNAs of either an empty pcDNA3.1 vector (control, CTL), or a recombinant of $BACE_F$, in the presence (+) or absence (−) of the γ-secretase inhibitor (γ-In) (C). Two days post-transfections the cells were pulse-labeled for 4 h with [^{35}S]Met/Cys. Cell lysates were immunoprecipitated with either the Flag-M2 mAb or an antibody directed against aa 1–16 of Aβ (APP-711), and analyzed by SDS–PAGE on 8% Tricine gels under reducing (+SH) or non-reducing (−SH) conditions. The migration positions of $BACE_F$, and its ~34, ~12, ~8, and ~5 kDa metabolites, the βAPP fragment C99 and the molecular mass standards are emphasized.

cific formation in either cell line of relatively low levels (<25%) of at least four fragments migrating with apparent molecular masses of ~34, ~12, ~8, and ~5 kDa (Figs. 1A and B). These fragments were not background artifacts since they were not detected in the absence of BACE expression and were also observed with the BACE CT-antibody (Ab PA1-757) on untagged $BACE_F$ (not shown). Since the levels of the ~34 and ~12 kDa fragments were markedly decreased under non-reducing conditions (Fig. 1A), we concluded that they must be held together by one or more disulfide bridges. Such C-terminal processing of $BACE_F$ was not inhibited by a synthetic γ-secretase inhibitor, which indeed increased the level of C99 (γ-In, Fig. 1C) and eliminated both $A\beta_{1-40}$ and $A\beta_{11-40}$ (not shown). Thus, γ-secretase does not generate the observed BACE products. In order to define more precisely the processing sites, we produced larger amounts of these fragments radiolabeled with either [^{35}S]Met or [^{3}H]Phe. The microsequencing results are shown in Fig. 2. The ~12 kDa product is shown to occur by cleavage at $SQDD_{379}$↓CYK**F**AVSQSST GTV**M**GAVI**M** based on the presence of Phe at the sequencing position 4 and Met at positions 15 and 20. The ~34 kDa fragment revealed a Phe at residue 15 (not shown). Based on the sequence of BACE, this suggests that the ~34 kDa product is the N-terminal fragment of BACE starting at aa 46, just following the furin/PC5 processing site at $RLPR_{45}$↓ [17]. Since the ~34 and ~12 kDa products are held together by disulfide bridges [31], this would likely place the C-terminus of the ~34 kDa form at Asp_{379}. The ~8 kDa form exhibits a Phe at position ~8, suggesting cleavage at $VVFD_{407}$↓RARKRIG**F**. Finally, the ~5 kDa product shows a sequence of Met 14 and 20, and based on its size this predicts that it is the result of processing at $DMED_{442}$↓CGYNIPQTDESTL**M**TIAYV**M**.

In view of the presence of Asp at the P1 position of all the identified processing sites, it became imperative to test whether this metabolism may not be due to a caspase-type processing. However, treatment of the cells with the membrane-permeable pan-caspase inhibitor Z-VAD(OMe)-CH_2F [32] did not change the processing profile, nor did the mutation of the active site of BACE (D93A; B_{DA}) make any difference (Supplemental Fig. S1A). This suggests that the observed processing is neither due to a caspase nor is it autocatalytic, respectively. Furthermore, removal of serum also did not affect the cleavage (Supplemental Fig. S1A), a condition that would have enhanced apoptosis and caspase activation. Since ADAM10 has been reported to cleave BACE and shed it into the medium [21], we tested a number of ADAM10-selective hydroxamate inhibitors at their highest effective dose of 1 μM (Supplemental Fig. S1B). None of these significantly changed the metabolic profile of $BACE_F$. While the general metalloprotease inhibitor GM6001, the ADAM17 inhibitor TAPI [33] increased the level of the ~5 kDa product, chymostatin had no effect (see in Supplemental Fig. S1C). Interestingly, treatment of the cells with phorbol 12-myristate 13-acetate (PMA) decreased the processing to the ~5 kDa form (Supplemental Fig. S1C). This is in the opposite direction than the usual PMA-induced increased cleavage by sheddases of the ADAM family [34,35]. Thus, we can conclude that the observed cleavages are due to one or more uncharacterized enzyme(s).

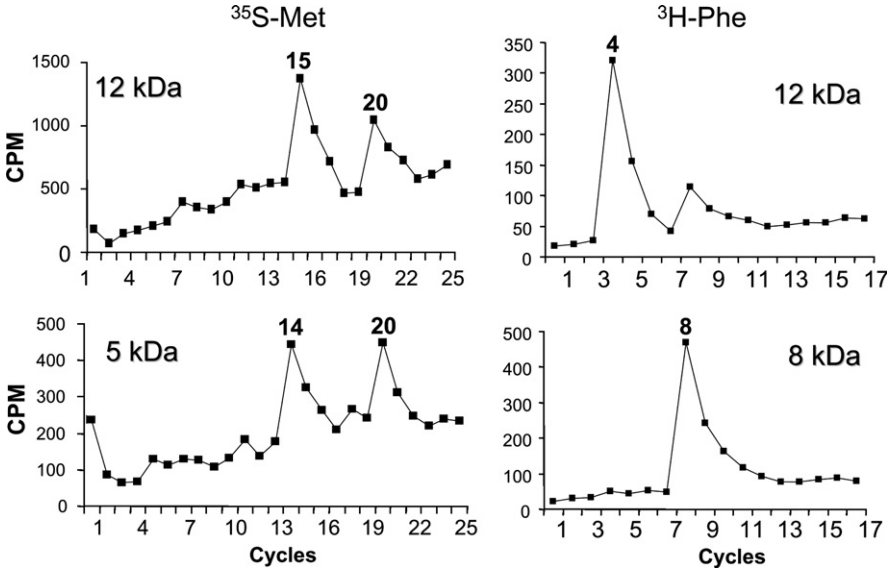

Fig. 2. Microsequence of the BACE metabolites. The results of the sequencing of the ~12, ~8, and ~5 kDa products labeled with either [^{35}S]Met or [^{3}H]Phe are shown. The deduced sequence positions are emphasized on top of each peak.

Mutagenesis of the predicted processing sites of $BACE_F$ and βAPPsw processing

Site-directed mutagenesis was used in order to test the importance of the cleavage of $BACE_F$ at the identified sites on βAPP processing. Thus, we systematically mutagenized the processing sites at Asp 379, 407, and 442. It was previously shown that proBACE localizes to the ER, whereas the zymogen processed BACE is in the TGN [17]. As shown in Fig. 3A, both forms can be readily distinguished on SDS–PAGE, and with the wild type sequence the Golgi form predominates. This is further demonstrated by the endoH sensitivity of the lower ER form and the resistance to endoH of the upper, Golgi form (not shown). The D379A and D379E mutants significantly blocked the formation of the ~12 kDa product, without significantly changing the ratio of Golgi to ER forms of $BACE_F$ (Fig. 3B). This emphasizes the importance of Asp_{379} for the generation of the ~12 kDa fragment. We note that the D379E mutant generates ~2-fold more C99 from βAPPsw than either wild type (WT) $BACE_F$ or its D379A mutant. Interestingly, the D379L mutant although resulting in lower levels of ~12 kDa product seems to also slow down the exit of $proBACE_F$ from the ER, as evidenced by increased immunoreactive levels of $BACE_F$ in this compartment. The importance of Asp_{442} in the generation of the ~8 and ~5 kDa products, respectively, is less certain since the D442A does not seem to significantly alter the generation of these products (Fig. 3B). However, we note that the mutants D407E and especially D407A do not exit efficiently from the ER, as evidenced by their higher ER/Golgi ratio, and result in lower levels of the ~8 and ~5 kDa forms, and much reduced ability to generate C99 from βAPPsw (Fig. 3C). Furthermore, the D407A mutant seems to be stuck in the ER and exhibits very low levels of its ~5 kDa metabolite. This suggests that the generation of the ~5 kDa fragment occurs in a subcellular compartment downstream from the ER.

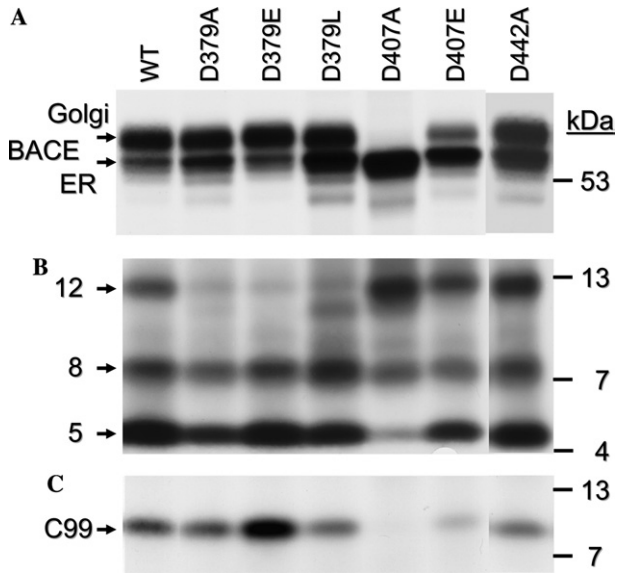

Fig. 3. Effect of inhibitors and treatments on the metabolism of $BACE_F$. HK293 cells were transiently transfected with cDNAs of either an empty pcDNA3.1 vector (CTL), or a recombinant of $BACE_F$ or its active site mutant D93A [17]. Two days post-transfection the cells were pulse-labeled for 4 h with [^{35}S]Met/Cys in the presence (+) or absence (−) of inhibitors or serum (Ser). Cell lysates were immunoprecipitated with the Flag-M2 mAb and analyzed by SDS–PAGE on 8% Tricine gels. The migration positions of $BACE_F$, and its ~12, ~8, and ~5 kDa products are shown. The inhibitors used included (A) the pan-caspase compound Z-VAD(OMe)-CH_2F (Casp), (B) the ADAM10 inhibitors GW9020, GW5712, GW4023, and GW9901, and (C) the metalloprotease inhibitor GM6001, the ADAM17 inhibitor TAPI, and chymostatin. In this experiment the cells were also pretreated (+) or not (−) with PMA.

The ~12 kDa product can be formed within the ER itself, as evidenced by the D407A mutant which is mostly in the ER (Supplemental Fig. S2). However, the formation of the ~8 and ~5 kDa products is significantly reduced in this mutant, again supporting the view that the ~12 kDa fragment has different enzymatic requirements than for the other two products. In order to further probe the compartment where the ~8 and ~5 kDa peptides are generated, we incubated HK293 cells with brefeldin A, a fungal metabolite that results in the fusion of the ER with all the compartments of the Golgi, except for the TGN [36]. Under these conditions all three metabolites are still formed (Supplemental Fig. S2). This suggests that the cognate catabolic enzyme(s) resulting in the formation of the ~8 and ~5 kDa products are recruited by the BFA treatment back to the ER from a Golgi compartment, possibly the ER/IC. In order to eliminate the proteasome as the source of the degradation enzymes, we incubated the cells with the specific proteasome inhibitor lactacystin, a *Streptomyces* metabolite that targets the 20S proteasome [37]. The data show that this inhibitor increases the level of the ~12 kDa product without significant effect on the ~8 and ~5 kDa peptides. This suggests that while the proteasome is not responsible for the generation of the ~12 kDa product, it affects its turnover.

The production of Aβ from βAPPsw by BACE and its mutants was investigated in both Neuro2a (Fig. 4A) and HK293 cells (Fig. 4B). We first noted that the efficacy of the generation of $Aβ_{40}$ and $Aβ_{11–40}$ by overexpressed $BACE_F$ is cell type dependant. Thus, while $Aβ_{40}$ is the major product secreted from Neuro2a cells, $Aβ_{11–40}$ is the major form secreted from HK293 cells. In both cell lines, of all the BACE mutants analyzed, only soluble BACE (sBACE), BACE-Δpro ($BACE_{Δp}$) and the D407A mutant result in ~1.5- to 2-fold increased levels of $Aβ_{40}$. All the other mutants did not affect the level of either $Aβ_{40}$ or $Aβ_{11–40}$. Thus, it seems that whenever $BACE_F$ is retarded in the ER (D407A, $BACE_{Δp}$), $Aβ_{40}$ is prominent, which fits with the

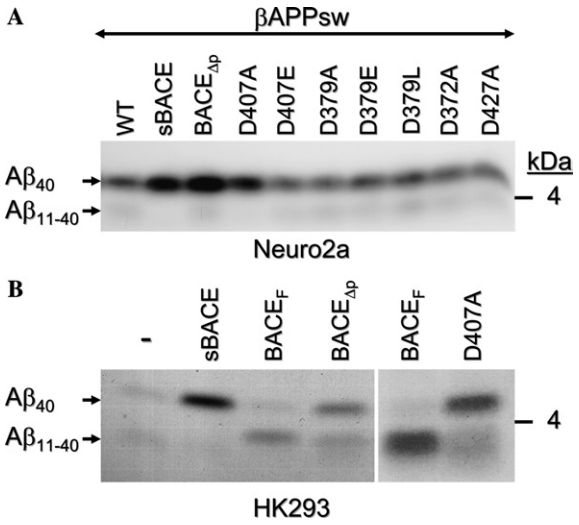

Fig. 4. Processing of βAPPsw in Neuro2a and HK293 cells by $BACE_F$ and its cleavage site mutants. (A) Neuro2a cells stably expressing βAPPsw were transfected with wild type (WT) $BACE_F$ or its cleavage site mutants, as well as its soluble form (sBACE) or a form lacking the prosegment ($BACE_{\Delta p}$). (B) HK293 cells were co-transfected with βAPPsw and either WT, or mutant $BACE_F$, its cleavage site mutants, sBACE, $BACE_{\Delta p}$, or an empty vector control. Two days post-transfections the cells were pulse-labeled for 4 h with [^{35}S]Met/Cys. The cell lysates were immunoprecipitated with an antibody directed against $A\beta_{40}$ (Ab A8326) and analyzed by SDS–PAGE on 14% Tricine gels. The migration positions of $A\beta_{40}$ and $A\beta_{11-40}$ are shown.

observation that $A\beta_{40}$ is mostly produced in the ER and $A\beta_{11-40}$ in the TGN [38]. In this compartment, we expect BACE be mostly in its zymogen form, i.e., proBACE, which is still enzymatically active [20]. Interestingly, soluble BACE (sBACE) also increases the level of $A\beta_{40}$ [17] (see Fig. 4). Accordingly, the activity involved in the upregulated production of $A\beta_{40}$ by soluble sBACE may also be the ER-localized zymogen form.

Discussion

In this study, we present evidence that $BACE_F$ is cleaved intracellularly at three other sites, resulting in ~12, ~8, and ~5 kDa C-terminal fragments (Fig. 1). All these fragments are generated by cleavages post-Asp↓, namely at SQD$\underline{\mathbf{D}}_{379}$↓C, VVF$\underline{\mathbf{D}}_{407}$↓R, and DME$\underline{\mathbf{D}}_{442}$↓CG, respectively (Fig. 2). It is interesting to note that the three fragments reported in this study were observed in neuronal (Neuro2a) and non-neuronal (HK293) cells (Fig. 1), clearly differentiating them from the TEAL$_{228}$↓A cleavage reported in an earlier study, which does not seem to occur in neuronal cells [22]. We further showed that whereas the P1 Asp_{379} is critical for the generation of the ~12 kDa fragment, Asp_{407} and Asp_{442} are not (Fig. 3). This suggests that at least two different enzymatic processes are responsible for these cleavages. In agreement, while the ~12 kDa product can be formed in the ER, the ~5 and ~8 kDa peptides are produced later on, possibly in the ER/IC (Supplemental Fig. S2). Furthermore, these cleavages are not autocatalytic and the cognate enzymes are not likely to be metalloproteases, caspases (Supplemental Figs. S1A–C), the reported BACE-sheddase ADAM10 (Supplemental Fig. S1B), nor the proteasome (Supplemental Fig. S2). Interestingly, at pH 7.4 Asp_{407} and Asp_{442} reside on either a β-strand or an α-helix extending from aa 439 to 444, respectively [39]. Thus, in view of these constrained structures at neutral pH, it is not surprising that D407A (and less so D407E) would significantly affect the secondary structure of $BACE_F$, resulting in its retention in ER. Furthermore, cleavages at Asp_{407} and Asp_{442} seem to mostly occur following exit of $BACE_F$ from the ER and sorting towards more acidic compartments, possibly allowing the exposure of such residues.

It was very recently reported that part of the newly synthesized overexpressed and endogenous BACE is proteolytically degraded by the proteasome [40]. Since the processing reported here is <25% we had to use a transient overexpression system to identify the processing products. However, similar qualitative data were also obtained upon analysis of endogenous BACE in Neuro2A cells (not shown). Our data suggest that in addition to the proteasome [40], BACE is also degraded by other enzymes residing the ER and/or ER/IC compartments. Nevertheless, we tested various cytosolic tail mutants [17] for their ability to affect the metabolism of BACE. Neither of the C478A, C482/C485A nor the triple C478/482/485A mutants affected the production of the ~12, ~8, and ~5 kDa products (not shown). Thus, Cys-palmitoylation of the cytosolic tail of BACE [17] does not affect its early metabolism in the ER and ER/IC. Furthermore, the presence of single Lys_{501} residue suggested that the observed ubiquitination of BACE [40] may occur there. However, the K501A mutant did not affect the production of the processing products reported here (not shown).

It was surprising that D379E, but not the D379A mutant, resulted in ~2-fold enhanced C99 production in HK293 cells without appreciably affecting the ER/Golgi ratio of BACE (Fig. 3C). Based on the crystal structure of BACE [39], at neutral pH Asp_{379} sits at the end of a surface-exposed loop and is followed by Cys_{380} that is disulfide linked to Cys_{330} [31]. It is possible that the bulkier Glu_{379} (as opposed to Asp_{379}) would affect the loop in such a way as to facilitate the cleavage of βAPPsw into C99. Alternatively, the D379E mutant may sort to a subcellular compartment where C99 is not as accessible to γ-secretase. This may provide a useful mutant to test the activities of various BACE inhibitors.

Since all cleavages reported herein occur post Asp↓, and that one of the proteasome activities is specific for acidic residues [41], it was plausible that they may be generated by the proteasome. Interestingly, while the

level of the ~12 kDa fragment was increased by proteasome inhibitor lactacystin, those of the ~8 and ~5 kDa were not much affected by this drug (Supplemental Fig. S2). Although this would not favor a direct action of the cytosolic/nuclear proteasome, it suggests that the ~12 kDa product is subjected to proteasomal regulation.

An informative result emanating from this study was that the ER-localized mutant proBACE D407A is more active in generating $A\beta_{40}$ from βAPPsw in either Neuro2a or HK293 cells (Fig. 4), similar to the originally observed activity of ER-enriched proBACE-Δpro in HK293 cells [17]. Intriguingly, the D407A mutant does not lead to the accumulation of C99 (Fig. 3C), but rather causes an increase in $A\beta_{40}$ formation (Fig. 4). Since the production of Aβ requires both the action of BACE and γ-secretase, this suggests that in the ER, the resident γ-secretase must rapidly transform the C99 into $A\beta_{40}$. This result supports the notion that $A\beta_{40}$ is mostly produced in the ER, whereas $A\beta_{11\text{-}40}$ occurs later in the secretory pathway, possibly in the TGN [38]. Along this line of thought, the fact that sBACE also enhances the production of $A\beta_{40}$ in both cell lines (Fig. 4) would suggest that removal of the transmembrane-cytosolic tail of $BACE_F$ results in a zymogen that could be more active in the ER than the parent enzyme. It is not clear whether the observed enhanced production of secretable $A\beta_{40}$ by the ER-enriched D407A mutant proceeds by a presenilin-dependent or presenilin-independent pathway [11]. Thus, the ER-retention properties of the D407A BACE mutant may provide a valuable tool to probe the activity of γ-secretase in this compartment and possibly the localization of βAPP as well.

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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.2004.10.019.

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