

Apical Sorting of β -Secretase Limits Amyloid β -Peptide Production*

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Polarized cells such as neurons and endothelial cells appear to be involved in two invariant pathological features of Alzheimer's disease pathology, namely the formation of senile plaques and cerebral amyloid angiopathy. This implicates polarized sorting mechanisms in the production and accumulation of amyloid β -peptide ($A\beta$). We have now studied polarized sorting of β -secretase (BACE) in Madin-Darby canine kidney (MDCK) cells. The majority of BACE is sorted to the apical surface of MDCK cells where very little β -amyloid precursor protein (β APP) is observed, because β APP undergoes basolateral sorting. Consistent with the usage of similar mechanisms for polarized sorting, BACE was also found to be targeted to axons of hippocampal neurons. The remaining basolaterally sorted BACE competes with the highly polarized basolateral α -secretase activity. Therefore, substantial amounts of BACE are targeted away from β APP to a non-amyloidogenic compartment, a cellular mechanism that limits $A\beta$ generation. In addition, no α -secretase activity was observed on the apical side whereas γ -secretase activity is observed on the basolateral and the apical side. Consistent with this finding, substantial amounts of $A\beta$ can be produced apically upon missorting of β APP to the apical surface. These data demonstrate that $A\beta$ production is limited in polarized cells by differential targeting of BACE and its substrate β APP. Moreover, our findings suggest that β APP may not be a major physiological substrate of BACE.

Proteolytic enzymes called secretases play a pivotal role in the generation of amyloid β -peptide ($A\beta^1$), the major component of the Alzheimer's disease (AD)-defining senile plaques and microvascular deposits (1). β -Secretase (BACE), a membrane-bound aspartyl protease (2–6), is responsible for the cleavage at the N terminus of the $A\beta$ domain at Asp-1 and

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¹ The abbreviations used are: $A\beta$, amyloid β -peptide; AD, Alzheimer's disease; β APP, β -amyloid precursor protein; BACE, β -site APP cleaving enzyme; MDCK, Madin-Darby canine kidney; CTF, C-terminal fragment; APPs α and APPs β , APP-soluble α cut and β cut; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MES, 2-N-morpholinoethanesulfonic acid; wt, wild type; PS, presenilin; NHS-LC, sulfo-succinimidyl-6-(biotinamido)hexanoate.

Glu-11. This heterogenous cleavage results in the generation of membrane-bound β APP C-terminal fragments (β APP CTFs), which are the immediate precursors for $A\beta$ generation (1), and in the secretion of the truncated ectodomain of β APP (7). γ -Secretase activity, which critically requires the presenilins PS1/PS2 (8–15), mediates the cleavage at the C terminus of the $A\beta$ domain. This final proteolytic event results in the secretion of $A\beta$ into biological fluids (1) and the liberation of the C-terminal domain of β APP into the cytoplasm (16). A third secretase, the α -secretase, prevents $A\beta$ generation by cleaving in the middle of the $A\beta$ -domain. The α -secretase activity is associated with three members (ADAM 9, 10, and 17) of the ADAM (a disintegrin and metalloprotease) family (17–19) and generates the secreted ectodomain of β APP (APPs α). The remaining C-terminal stub is processed by γ -secretase, which results in the secretion of p3 (1).

Polarized cells are intimately associated with two invariant pathological features of AD pathology, namely the formation of senile plaques and the cerebral amyloid angiopathy. This suggests that polarized sorting mechanisms may be involved in the production and accumulation of $A\beta$. As a model system to study polarized sorting of β APP and the three secretase activities, we used the well characterized Madin-Darby canine kidney (MDCK) cells, because they allow the generation of stably transfected cell lines with moderate expression levels and appear to use similar mechanisms for polarized sorting as neuronal cells (20). Moreover, experiments on polarized sorting of β APP in this cell line provided first evidence for the cellular mechanism underlying the enhanced $A\beta$ production caused by the Swedish mutation (21, 22), thus demonstrating the relevance of MDCK cells for studies on β APP processing. β APP is known to undergo polarized sorting in MDCK cells (21–24) as well as in neurons (25, 26). In MDCK cells the β APP holoprotein and its proteolytic products are predominantly targeted to the basolateral side. A targeting signal containing a tyrosine within the cytoplasmic domain of β APP is required for polarized sorting of membrane-associated β APP (24). Deletion of the cytoplasmic sorting signal of β APP leads to an equal surface distribution of membrane-associated β APP, whereas APPs α continues to be secreted predominantly from the basolateral surface (24). Moreover, expressing the familial AD-associated Swedish mutation, which is known to enhance BACE-mediated β APP processing (27, 28), reveals apical secretion of Swedish-APPs β (21, 22).

Understanding the cellular sorting pathways associated with proteolytic processing of β APP and, specifically, the mechanisms that allow the secretase activities to come into contact with their substrate β APP will help reveal and prevent amyloidogenesis. We therefore analyzed the sorting of BACE and its enzymatic activity in terms of $A\beta$ production in polarized

cells. Parallel investigation of the α - and γ -secretase activity revealed differential sorting of the two amyloidogenic secretases and their substrate β APP. This sorting mechanism apparently limits $A\beta$ production, by allowing efficient competition of the exclusive basolateral α -secretase activity with the low amounts of basolaterally sorted BACE.

EXPERIMENTAL PROCEDURES

cDNA Constructs—cDNA constructs encoding BACE were amplified by PCR and subcloned into the *EcoRI/XhoI* site of the pcDNA3.1/zeo(+) expression vector (Invitrogen) as described previously (29).

Tissue Culture—MDCK cells (strain II) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. All stable transfections of MDCK cells were performed with FuGENE (Roche Diagnostics) as described by the manufacturer. Cell lines expressing BACE or presenilin constructs from pcDNA3.1/Zeo were selected with $400 \mu\text{g ml}^{-1}$ zeocin. Cell lines with moderate expression levels and normal morphology were selected for the study. To obtain polarized monolayers for surface biotinylation or metabolic labeling, cells were plated at confluence on 24-mm polycarbonate Transwell filter (Corning Costar Corp.). For immunocytochemistry cells were plated on a 12-mm diameter filter. Media were changed each day, and cells were used for the corresponding experiment after 5–6 days. To increase expression levels cells were treated overnight with 10 mM butyrate. Similar results were obtained with untreated and treated cells.

Primary cultures of hippocampal neurons were prepared and cultured according to standard conditions.

Antibodies—The polyclonal antibodies 7523 and GM190 directed to the N terminus (amino acids 46–60) and the pro-peptide (amino acids 22–45) of BACE were described previously (29). The polyclonal antibody 3027 against the large loop of PS1 and the monoclonal antibody PS1N against the N terminus of PS1 were described previously (30).

Immunocytochemistry and Confocal Imaging—Cells grown on filters were washed with PBS containing 1 mM CaCl_2 , 0.5 mM MgCl_2 (PBS^+). Antibody 7523 (1:100 in 0.2% BSA/ PBS^+) was added to the apical or basolateral surface for 30 min at 4 °C. After extensive washing in PBS^+ , cells were fixed in 4% paraformaldehyde/ PBS^+ for 30 min at 4 °C, quenched for 10 min in 50 mM $\text{NH}_4\text{Cl}/\text{PBS}^+$, and then incubated with the fluorochrome-conjugated secondary antibody (Alexa 488, Molecular Probes, 1:250) for 45 min at 37 °C. After extensive washing, filters were cut out and mounted in Moviol. Confocal images were obtained with a Leica TCS SP confocal laser scanning microscope (Leica Microsystems) equipped with an oil immersion plan-apochromatic 100/1.4 numerical aperture objective lens. Fluorochromes were visualized using an argon laser with excitation wavelengths of 488 nm, emission filter 490–530 nm (for Alexa 488). Images were recorded with 512- \times 512-pixel resolution and processed with Photoshop version 5.5 (Adobe Systems, Inc.). Hippocampal neurons were transfected with an endotoxin-free BACE cDNA using Effectene (Qiagen). 36 h after transfection, neurons were fixed and stained with the affinity-purified antibody 7523 and costained with a monoclonal anti-MAP-2 antibody.

Metabolic Labeling and Immunoprecipitation—For pulse-chase experiments, MDCK cells grown on Transwell filters were starved for 45 min in methionine- and serum-free minimal essential medium and subsequently metabolically labeled with 300 μCi of [^{35}S]methionine/[^{35}S]cysteine (Promix, Amersham Biosciences, Inc.) in methionine- and serum-free minimal essential medium for 10 min and chased in the presence of excess amounts of unlabeled methionine for the indicated time points. Cells were lysed in buffer containing 1% Nonidet P-40 on ice for 30 min and scraped from the filter, and lysates were clarified by centrifugation for 20 min at $14,000 \times g$. Immunoprecipitation and separation by SDS-PAGE was performed as described previously (29). For the immunoprecipitation of APPs and $A\beta$, cells were metabolically labeled for 4 h using 300 μCi of Promix in methionine-free media containing 5% dialyzed serum. Media collected from the apical and basolateral chamber were immunoprecipitated. Quantitation was performed by phosphorimaging (PhosphorImager, Molecular Dynamics).

Surface Biotinylation—MDCK cells were grown on filters for 5 days. The monolayer was washed three times with cold PBS^+ , followed by incubation with sulfo-NHS-LC-biotin (1 mg/ml, Pierce) either apically or basolaterally for 1 h at 4 °C. 1% BSA in PBS^+ was added to the opposite chamber. The reaction was stopped by washing with PBS^+ and quenched with 10 mM glycine, 1% BSA in PBS^+ . Cell lysates were prepared and precipitated with streptavidin-Sepharose (Amersham Biosciences, Inc.). The precipitated, biotinylated proteins were separated on SDS gels and transferred to polyvinylidene difluoride mem-

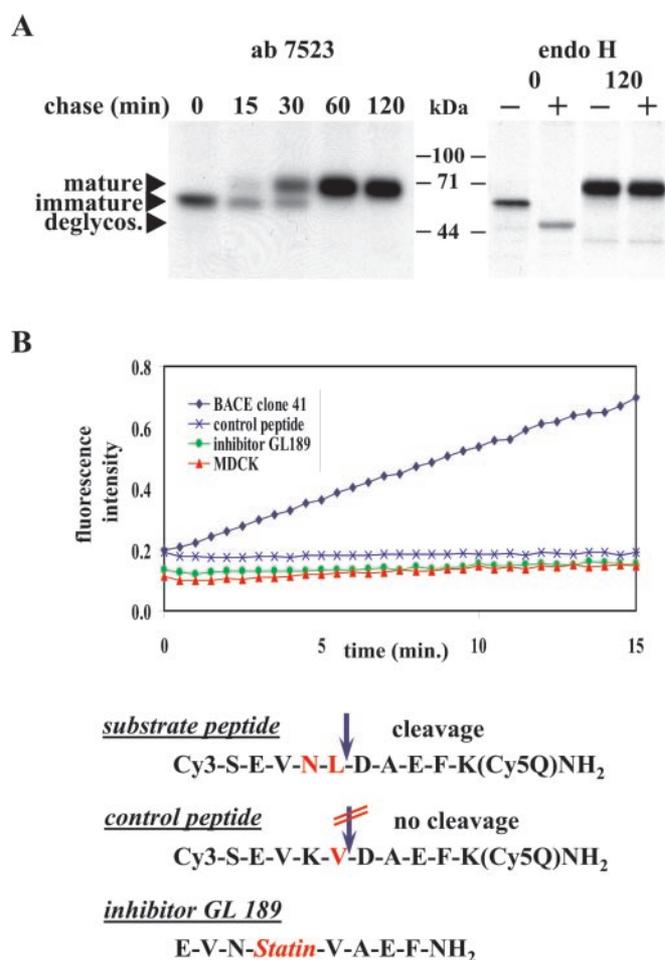


FIG. 1. Expression and maturation of functionally active BACE in MDCK cells. *A*, maturation of BACE in MDCK cells. *Left panel*, MDCK cells stably expressing BACE were pulse-labeled with [^{35}S]methionine/cysteine and chased for the indicated time points in the presence of excess amounts of unlabeled methionine. Cell lysates were immunoprecipitated with antibody 7523. Note that antibody 7523 preferentially detects mature BACE (29). *Right panel*, *N*-glycosylation and oligosaccharide modification of BACE. MDCK cells stably expressing BACE were pulse-labeled with [^{35}S]methionine and chased in the presence of excess amounts of unlabeled methionine for 2 h. Cell lysates were immunoprecipitated with antibody 7523 and treated with (+) and without (–) endoglycosidase H. Note that the mature form of BACE is endoglycosidase H-resistant. *B*, membranes from BACE-transfected (\blacklozenge) or untransfected MDCK cells (\blacktriangle) were solubilized and analyzed *in vitro* for proteolytic activity. Incubation of the fluorogenic peptide containing the Swedish mutation with solubilized membrane fractions from BACE-transfected (\blacklozenge) MDCK cells allowed a significant time-dependent proteolytic reaction, whereas the non-cleavable substrate peptide with the Met to Val mutation at the P1 side is not proteolyzed (\times). Moreover, incubation with the BACE-specific inhibitor GL189 ($5 \mu\text{M}$) blocked the proteolytic activity of solubilized membrane fractions from BACE-transfected cells (\bullet). Untransfected MDCK cells show very little BACE activity (\blacktriangle), which appears to be below the detection limit of the *in vitro* assay. Substrate peptides and the BACE-specific inhibitor GL189 are shown below. The NL mutation (Swedish mutant substrate) as well as the V mutation (non-cleavable control substrate) are shown in red.

branes. For detection of BACE, PS1, or β APP, the indicated antibody was used. Specifically bound antibodies were visualized by a horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence technique (Amersham Biosciences, Inc.) or an ^{125}I -conjugated secondary antibody.

Enzymatic Deglycosylation—These experiments were done as described previously (29).

Fluorometric BACE Activity Assay—A quenched fluorescence substrate Cy3-SEVNLDAEFK(Cy5Q)- NH_2 containing the Swedish β APP mutation (in boldface) and a non-cleavable substrate Cy3-SEVKVDAEFK(Cy5Q)- NH_2 , containing an amino acid exchange, M \rightarrow

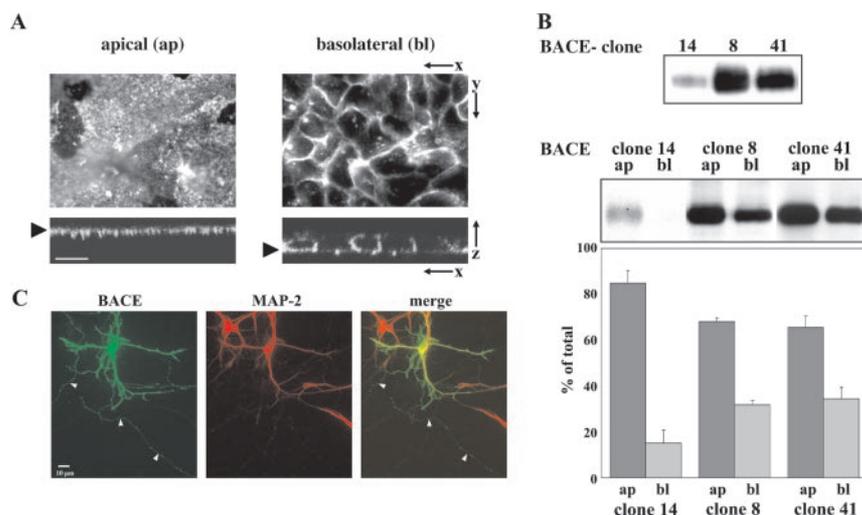


FIG. 2. Sorting of BACE in polarized MDCK cells and rat hippocampal neurons. *A*, immunocytochemical detection of BACE. Monolayers of MDCK cells stably expressing BACE were incubated with antibody 7523 on the basolateral or apical side. Bound antibodies were detected with a secondary fluorescent antibody. Note the detection of BACE on either side of the monolayer. *Lower panel*, vertical cross-sections (z axis). Basolateral and apical BACE are indicated by the arrowheads. *Bar*, 10 μ m. *B*, expression and cell surface distribution of BACE. Three independent cell lines expressing low or high levels of BACE were analyzed (detected by immunoblotting with antibody 7523; *upper panel*). *Lower panel*, cell surface biotinylation of BACE. Biotinylated surface proteins were isolated using streptavidin-coated Sepharose. Surface-biotinylated BACE molecules were detected by immunoblotting using antibody 7523 and quantitated. Data shown are average \pm S.D. of three independent experiments. *C*, axonal targeting of BACE in hippocampal neurons. 36 h after transfection of fully differentiated neurons with the human BACE cDNAs, neurons were fixed, permeabilized, and costained with the affinity-purified antibody 7523 (BACE) and a monoclonal anti-MAP-2 antibody (MAP-2) to label the somato-dendritic compartment. The merged image (*merge*) demonstrates axonal (*white arrowheads*) and somato-dendritic sorting of human BACE.

V (in boldface) were synthesized by Amersham Biosciences, Inc., UK. Membranes from BACE-transfected and non-transfected MDCK cells were prepared and extracted with 1% Triton X-100, 20 mM MES, pH 6.0, including a protease inhibitor mix (Sigma Chemical Co.). The assay was carried out in a volume of 200 μ l containing 400 nM peptide substrate or the non-cleavable control peptide, 2 μ l of the membrane preparation, and 20 mM sodium acetate, pH 4.4. Fluorescence was measured continuously over a period of 15 min, at room temperature (Fluoroskan Ascent, Labsystems, Finland, excitation 530 nm, emission 590 nm). The β -secretase inhibitor (GL189) H-EVNstatineVAEF-NH₂ was synthesized by K. Maskos and W. Bode.

Radiosequencing— $A\beta$ peptides were radiosequenced as described previously (31).

RESULTS

Maturation and Enzymatic Activity of BACE in MDCK Cells—BACE mediates the initial amyloidogenic cleavage, which produces the direct precursor for $A\beta$ production. Due to the pivotal importance of this cleavage, we first investigated BACE maturation and its proteolytic activity in MDCK cells. Because endogenous levels of BACE are too low to allow its direct biochemical and cellular analysis (2), we stably transfected MDCK cells as well as a previously described MDCK cell line (23) overexpressing human wt β APP with a BACE cDNA expression construct. In addition to overexpressed BACE, we also monitored the distribution of endogenous BACE activity by analyzing its primary cleavage product APPs β (see below). MDCK cells stably expressing human BACE were analyzed in pulse-chase experiments for maturation and glycosylation of BACE. After a short pulse, immature BACE is detected by immunoprecipitation of cell lysates with antibody 7523 (29) (Fig. 1A). Over time, efficient maturation to a higher molecular weight variant was observed (Fig. 1A), which lacks the pro-peptide (data not shown). The fully glycosylated high molecular weight BACE variant reaches a post-Golgi compartment as demonstrated by the selective resistance of this variant against endoglycosidase H (Fig. 1A). We next investigated whether overexpressed BACE is catalytically active in MDCK cells. Membranes were prepared from BACE-transfected and untransfected MDCK cell lines. Solubilized membrane fractions

were incubated with a fluorogenic β APP-derived peptide extending from P5 to P5' containing the Swedish mutation (Fig. 1B). This peptide contains one of the two known cleavage sites of BACE (see below). Compared with membrane extracts from untransfected cells, a high proteolytic activity is present in extracts derived from a BACE-transfected cell line (Fig. 1B). When solubilized membranes of BACE-transfected cells were incubated with the BACE inhibitor GL189 (Fig. 1B), a complete inhibition of the proteolytic activity was observed (Fig. 1B). Finally, we used a peptide, which contained a Met to Val substitution at the P1 site (Fig. 1B). This mutation was previously shown to inhibit endoproteolytic cleavage by BACE (2, 32). Consistent with these observations, the mutant peptide was not proteolyzed in the *in vitro* assay containing solubilized membrane fractions from BACE-transfected cells (Fig. 1B). These results thus demonstrate that fully mature biologically active BACE with its expected biochemical features is expressed in MDCK cells. In addition to the cleavage at the critical Asp-1 of the $A\beta$ domain, we also detected significant cleavage activity at Glu-11 (see below), which is consistent with previous reports (2, 33–36) and represents another major *in vivo* cleavage site of BACE.

Surface Distribution of BACE—To determine the surface distribution of BACE, the MDCK cell lines described above were grown on Transwell filters to form polarized monolayers. These were incubated with antibody 7523 to the ectodomain of BACE on the apical or basolateral surface. Laser confocal microscopy revealed BACE expression on each surface (Fig. 2A). This was further confirmed by analyzing vertical cross-section (z axis) of polarized monolayers (Fig. 2A, *lower panel*).

Cell surface targeting of BACE was analyzed by direct biotinylation of mature BACE at the plasma membrane. Three individual MDCK cell lines, which express different levels of BACE, were selected (Fig. 2B). In addition to two high expressing lines (*lines 8 and 41*) a low expressing line (*line 14*) was chosen to avoid saturation of polarized sorting. Polarized monolayers were chilled to 4 $^{\circ}$ C and labeled with sulfo-NHS-LC-biotin on the basolateral or the apical side. After lysis, biotiny-

lated surface proteins were isolated using streptavidin-coated Sepharose. Surface-biotinylated BACE molecules were detected by immunoblotting using antibody 7523. This revealed that the mature BACE variant is exclusively targeted to both cell surfaces (Fig. 2B). No pro-peptide-containing immature BACE was detected on the cell surface (data not shown). Quantitation of multiple experiments with three independent individual cell lines revealed that ~ 70 – 80% of BACE are targeted to the apical surface, whereas only about 20–30% were found on the basolateral surface (Fig. 2B) where most of the β APP substrate is located (23). The low expressing line showed the most efficient apical targeting of BACE (Fig. 2B) indicating that apical sorting of BACE can be affected by very high expression levels.

The sorting of BACE to the apical compartment together with about 20–30% basolateral targeting suggests that BACE is expressed in neurons in axons as well as in the somato-dendritic compartment (20). To prove this, fully differentiated rat hippocampal neurons were transfected with a BACE cDNA. Cells were costained with antibody 7523 to BACE and with an anti-MAP2 antibody to specifically identify somato-dendritic compartments. As shown in Fig. 2C, BACE is indeed targeted to both axons and somato-dendritic compartments.

Basolaterally Sorted BACE and α -Secretase Compete for β APP—We next investigated the polarized distribution of α - and β -secretase products in MDCK cell lines, which overexpress either human β APP alone or together with BACE. Aliquots of conditioned media from metabolically labeled cells were immunoprecipitated with the neo-epitope-specific antibody 1736 (Fig. 3A) (37), which specifically detects α -secretase-generated soluble APPs α . As reported previously (24), cells expressing only β APP secreted APPs α basolaterally (Fig. 3B). In contrast, the additional expression of BACE resulted in very low levels of APPs α secretion (Fig. 3B). In fact, basolateral secretion of APPs α was selectively reduced (Fig. 3B). This suggests that overexpressed BACE is competing with endogenous α -secretase within the basolateral targeting pathway, thus producing APPs β (which is not detected by antibody 1736) to the expense of APPs α .

To determine the amounts of APPs β produced by BACE, we immunoprecipitated secreted APPs species with antibody 5313 (38), which allows detection of both APPs α and APPs β (Fig. 3A), and compared the immunoprecipitated species with APPs α isolated with antibody 1736 (Fig. 3A). In clear contrast to antibody 1736 (Fig. 3B), antibody 5313 detected robust levels of secreted APPs in both compartments of MDCK cells overexpressing BACE and β APP (Fig. 3C). These migrated to a slightly lower molecular weight consistent with BACE-generated truncated APPs β (27, 28). Moreover, quantitation (by calculating the difference between total APPs precipitated by antibody 5313 and APPs α isolated by antibody 1736) revealed equal secretion of APPs β into the apical and basolateral compartments of double-transfected cells (Fig. 3D). This result demonstrates that APPs β can be secreted at both surfaces (Fig. 3, C and D), whereas APPs α is almost exclusively secreted from the basolateral side (Fig. 3B). This is supported by the finding that APPs β is also secreted into the apical and basolateral compartment by cells not transfected with BACE (Fig. 3, C and D). However, the significant increase of total APPs β secretion into the basolateral and apical compartments upon BACE overexpression indicates cleavage of β APP before it reaches the sorting compartment within the *trans*-Golgi (see “Discussion”).

Apically Missorted β APP Is Efficiently Proteolyzed by BACE and γ -Secretase—The above described results suggest a high endogenous BACE activity on the apical side, where it can produce APPs β in equal amounts as on the basolateral side,

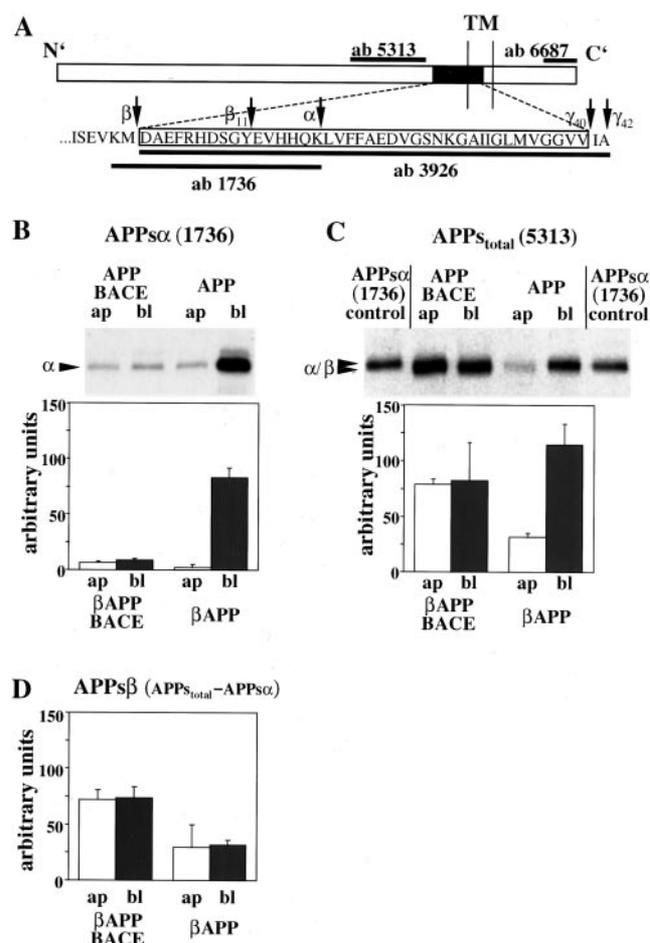


FIG. 3. Polarized secretion of APPs α and APPs β . A, schematic representation of β APP. The sites of secretase cleavages (arrows) and epitopes used for immunization (bars) are indicated. B, polarized secretion of APPs α . Conditioned media of MDCK cells stably transfected with β APP and BACE, or β APP alone were immunoprecipitated with antibody 1736 to exclusively identify APPs α . Lower panel, quantitation of the amounts of APPs α secreted on either side of the polarized monolayer. Data shown are average \pm S.D. of three independent experiments. C, polarized secretion of total APPs (APPs α and APPs β). Conditioned media of MDCK cells transfected with β APP and BACE, or β APP alone were immunoprecipitated with antibody 5313 to identify APPs α and APPs β . Antibody 5313 detects higher levels of APPs within the basolateral than in the apical compartment of cells expressing only β APP. This is due to the fact that these cells also secrete high levels of APPs α (compare immunoprecipitations with antibody 1736 in B). Lower panel, quantitation of the amounts of total APPs secreted on either side of the polarized monolayer. Data shown are average \pm S.D. of three independent experiments. D, quantitation of the amounts of APPs β secreted on either side of the polarized monolayer. The amounts of APPs β were determined by calculating the difference of total APPs and APPs α . Data shown are average \pm S.D. of three independent experiments. Note that both antibodies have similar affinities to APPs (data not shown).

although only about 10% of the β APP holoprotein is targeted to the apical side. This indicates a very efficient BACE-mediated processing of apically sorted β APP probably due to the lack of apical α -secretase activity. To prove this, we expressed a C-terminally truncated β APP derivative (β APP Δ C), which we have shown previously to be targeted to both surfaces due to the loss of the cytoplasmic sorting signal (24). Aliquots of conditioned media from metabolically labeled MDCK cells expressing β APP Δ C were immunoprecipitated with neo-epitope-specific antibody 1736 or with antibody 5313. As shown before (24), APPs α is almost exclusively secreted into the basolateral compartment, independently of the sorting of the β APP holoprotein (Fig. 4). In contrast, a significant increase of APPs β secretion

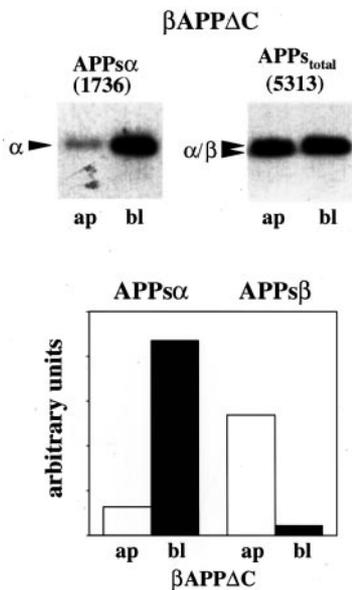


FIG. 4. Missorting of β APP differentially affects processing by α - and β -secretase. MDCK cells overexpressing β APP Δ C were metabolically labeled and conditioned media were immunoprecipitated with antibody 1736 or antibody 5313. APPs α and APPs β secretion was quantitated as described above.

into the apical compartment is observed (Fig. 4). Therefore, apically sorted full-length β APP is almost exclusively cleaved by BACE, whereas basolateral β APP appears to be processed by α - and β -secretase in a competitive manner. It is important to note that the distribution of endogenous BACE activity in the presence of mistargeted β APP reflects the distribution of cell-surface biotinylated BACE (compare Fig. 4 with Fig. 2) thus indicating apical sorting of endogenous BACE similar to that of overexpressed BACE. To further prove this, we next compared polarized $A\beta$ and p3 secretion in MDCK cells overexpressing β APP/BACE or β APP alone. Conditioned media from the apical and basolateral compartment were immunoprecipitated with antibody 3926 (39) (Fig. 5A). In cells expressing β APP alone, $A\beta$ and p3 were almost exclusively secreted into the basolateral compartment (Fig. 5A). In cells expressing both β APP and BACE, no p3 was detected (Fig. 5A), consistent with the competitive reduction of α -secretase processing, described above (Fig. 3B). Instead, high levels of an $A\beta$ species migrating between native $A\beta$ and p3 were observed in the double-transfected cells (Fig. 5A). Radiosequencing of this peptide revealed that its N terminus begins predominantly at Glu-11 (Fig. 5B). Cleavage at Glu-11 is a typical feature of the β -secretase activity and is observed in cells expressing endogenous (see below) (31) and overexpressed BACE (2, 33–36). The truncated $A\beta$ species is secreted only basolaterally, although the corresponding APPs β is equally secreted into the apical and basolateral compartment (see Fig. 3D). The reason might be that the precursor of $A\beta$, the BACE-generated β APP CTF, still contains the sorting signal for basolateral transport (see Fig. 5D for further evidence). We next investigated if a similar truncated $A\beta$ species could be produced on the apical side, where high levels of BACE, but little, if any, α -secretase activity was observed. To do so, β APP Δ C was expressed, which allows the trafficking of significant amounts of the β APP holoprotein to the apical side (24). In contrast to the basolateral secretion of $A\beta$ /p3 in wt β APP-expressing cells, overexpression of the C-terminally truncated β APP derivative revealed $A\beta$ secretion from both surfaces (Fig. 5C). Indeed, high levels of a truncated $A\beta$ species were observed on the apical side. Due to the preferentially apical distribution of BACE and the compe-

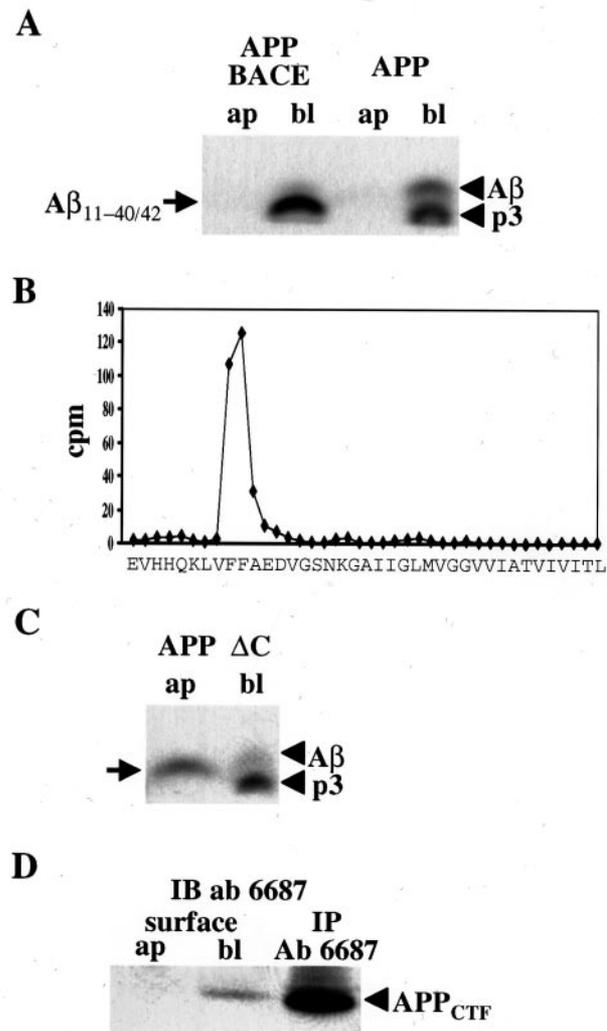


FIG. 5. Apical BACE and γ -secretase activity. **A**, polarized distribution of $A\beta$ and p3 in cells expressing β APP alone or β APP and BACE. Conditioned media was immunoprecipitated with antibody 3926 (39). Note the enhanced production of a truncated $A\beta$ species in cells double-transfected with β APP and BACE (arrow). **B**, radiosequencing of $A\beta$ secreted from BACE-expressing cells. Cells were metabolically labeled with [3 H]Phe, and $A\beta$ was isolated from conditioned media by immunoprecipitation with antibody 3926. The peak of radioactivity in cycles 9 and 10 demonstrates that the N terminus of this $A\beta$ species begins at Glu-11. **C**, polarized distribution of $A\beta$ and p3 in cells expressing β APP Δ C. Conditioned media were immunoprecipitated with antibody 3926. Note that $A\beta$ peptides are secreted on both sides, whereas p3 is exclusively observed within the basolateral compartment. Consistent with previous findings, increased levels of p3 are found upon deletion of the β APP cytoplasmic domain (24). A truncated $A\beta$ species (most likely starting at Glu-11; see above) is found predominantly on the apical side (arrow). **D**, detection of β APP CTFs on the basolateral cell surface. Biotinylated surface proteins were isolated using streptavidin-coated Sepharose. Surface-biotinylated β APP CTFs were detected by immunoblotting using antibody 6687. As shown by the direct immunoprecipitation with antibody 6687, the α -secretase-generated β APP CTF is very abundant in MDCK cells, whereas only very small amounts of β -secretase generated β APP CTFs were observed.

titution of BACE with α -secretase on the opposite side, smaller amounts of $A\beta$ were detected basolaterally (Fig. 5C). Moreover, consistent with the above described basolateral activity of α -secretase (Figs. 3B and 4) p3 was exclusively observed in the basolateral compartment (Fig. 5C). Efficient $A\beta$ production on the apical site again supports the notion that endogenous BACE undergoes predominantly apical targeting like the overexpressed enzyme. This also demonstrates that the Glu-11

sorting in MDCK cells (41, 42). Consequently, the strongly increased APPs β undergoes unpolarized secretion. In contrast, the membrane-bound cleavage products of BACE, the β APP CTFs, which are the immediate precursors for $A\beta$ generation, still contain their cytoplasmic sorting signal and are consequently targeted to the basolateral side where they can be further processed to $A\beta$ (see above).

Finally, our data suggest that, at least in MDCK cells, β APP is not the major physiological substrate of BACE, because both are targeted in the opposite direction. This is supported by the observation that the β -secretase activity in mice is unable to efficiently cleave endogenous β APP whereas human β APP, which has a slightly different amino acid sequence in its $A\beta$ domain, is cleaved normally (43). Therefore, β APP cannot be the major BACE substrate in mice as well. Indeed, while this report was under review, a novel BACE substrate was identified (44).

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