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Polarized secretion of β-amyloid precursor protein and amyloid β-peptide in MDCK cells

(Alzheimer disease/polarized trafficking/proteolytic processing)

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ABSTRACT **The β-amyloid precursor protein (βAPP) is a widely expressed integral membrane protein that is proteolytically processed to yield several secreted derivatives, including soluble APP (APP_s), the 4-kDa amyloid β-peptide (Aβ), and a related 3-kDa peptide (p3). To understand βAPP trafficking and processing, we analyzed the sorting of βAPP in Madin–Darby canine kidney (MDCK) cells, an epithelial cell known to possess physiologically distinct apical and basolateral plasma membranes. Processing of βAPP resulted in highly polarized secretion of APP_s. More than 90% of APP_s was detected in the basolateral compartment, and less than 10% was found in the apical compartment. This was associated with a preferential localization of βAPP on the basolateral cell surface. Activation of protein kinase C, which is known to enhance the secretion of APP_s, did not change the polarity of APP_s release but significantly increased the amount secreted. Aβ and p3 peptides were also secreted predominantly basolaterally. In addition, MDCK cells secreted a truncated form of Aβ beginning at Arg-5. These data show that the proteolytic processing products of βAPP undergo polarized secretion. Moreover, the results suggest that the amyloidogenic Aβ peptide is generated following the polarized sorting of βAPP. The polarized basolateral secretion of Aβ in these epithelial cells provides a potential mechanism for the accumulation of Aβ in the abluminal basement membrane of brain microvessels during Alzheimer disease.**

In Alzheimer disease (AD) and to a lesser extent during normal aging, amyloid deposits accumulate as spherical plaques in brain tissue and within the basement membranes of cerebral and meningeal blood vessels (1). These amyloid deposits are composed of the 39- to 43-aa amyloid β-peptide (Aβ) (2). Aβ is derived by proteolytic cleavages from the β-amyloid precursor protein (βAPP) (3), a widely expressed membrane-spanning glycoprotein (4). During normal processing, βAPP is cleaved close to the membrane, releasing most of its N-terminal ectodomain as a large soluble molecule (APP_s) (4). This cleavage is mediated by an as yet unidentified enzyme designated "secretase." The secretory cleavage can occur on the cell surface, as shown by cell surface biotinylation (5) and surface iodination experiments (6). However, these experiments do not exclude the possibility that cleavage can also occur intracellularly, as has recently been shown in PC12 cells (7). Secretase cleavage occurs preferentially within the Aβ sequence, thus precluding Aβ formation (8). Longer forms of APP_s, perhaps containing the entire Aβ region, also seem to be secreted from some cells (9).

Besides the secretory pathway, βAPP is also processed and degraded within an endosomal/lysosomal pathway. Full-length βAPP can be reinternalized from the cell surface and targeted to endosomes and lysosomes (5). Indeed, the holoprotein has been identified within clathrin-coated vesicles (10). C-terminal fragments containing the entire Aβ sequence have been detected within late endosomes/lysosomes upon treatment of cultured cells with lysosomal protease inhibitors (5, 11). Similar fragments can also be identified within the human brain and its vasculature (12, 13). These C-terminal fragments have been postulated to serve as the precursors for Aβ (11, 12).

Recently, we (14) and others (15, 16) found that Aβ is a product of normal cellular metabolism, in that Aβ and a variety of closely related low molecular weight peptides (14) are continuously secreted into the medium. These findings clearly show that aberrant or pathological processing of βAPP is not necessary to produce Aβ. Aβ is similarly found in the cerebrospinal fluid of both AD patients and controls (15, 16), which indicates that Aβ is produced normally *in vivo*. Little is known about the mechanism of Aβ production. It appears that an acidic environment is necessary to generate Aβ (16, 17), although the peptide has not been detected in isolated lysosomes (17) or cell lysates (14, 16, 17).

To understand the mechanism of Aβ generation, a detailed knowledge of βAPP trafficking and processing and the sorting of its proteolytic fragments is necessary. Aβ accumulates preferentially in the abluminal basement membrane of brain microvessels in AD, just basal to the endothelial cells (18). Moreover, parenchymal plaques of Aβ in AD cortex are tightly associated with certain vascular basement membrane components, including laminin and heparan sulfate proteoglycan (19, 20). We have therefore started to analyze the sorting of βAPP and its proteolytic fragments in Madin–Darby canine kidney (MDCK) cells. These epithelial cells provide a well-characterized model system for the study of polarized protein sorting (21, 22). In MDCK cells, sorting takes place within the trans-Golgi network (21, 23–27), where proteins are thought to be sorted into distinct transport vesicles for direct delivery to the apical or basolateral surface. In the case of membrane-spanning proteins, it has been shown that basolateral sorting of these proteins is dependent on cytoplasmic signal sequences (21, 25–28). Soluble proteins can also undergo polarized secretion, a process that might be regulated by signals distinct from those present in membrane-bound proteins (29–32). In this regard, βAPP provides a particularly interesting model system for studying polarized protein trafficking, because it is initially made as a transmembrane precursor, which is then processed into a variety of secreted proteolytic products. Moreover, the analysis of vectorial transport of βAPP in MDCK cells may also help to identify the cellular locus of Aβ generation.

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer disease; APP_s, soluble βAPP; βAPP, β-amyloid precursor protein; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C.

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MATERIALS AND METHODS

Tissue Culture. MDCK cells were cultured as described (33). For analysis of polarized secretion, MDCK cells were plated on polycarbonate filters in Transwell chambers (Costar). We used 24-mm diameter filters containing 0.4-μm pores. Identical results were obtained using filters with 3.0-μm pores. For metabolic labeling, MDCK cells were plated onto the filters and labeled in methionine-free medium with 40 μCi of [^{35}S]methionine (1195 Ci/mmol; 1 Ci = 37 GBq) per ml. For analysis of the polarized secretion of APP_s, cells were labeled for 3 hr using methionine-free medium containing 10% (vol/vol) dialyzed fetal bovine serum. For the detection of Aβ and other low molecular weight peptides, we labeled the cells for 12 hr using methionine-free medium with undialyzed fetal bovine serum (14). [^{3}H]Inulin was used as a tracer to confirm that the cells formed tight monolayers (34). During the 3-hr labeling period, we found 0.5–0.8% of the inulin in the opposite chamber, and during the 12-hr labeling period, we detected $\leq$2% within the opposite chamber, demonstrating that passive diffusion is negligible. The polarized phenotype of MDCK cells was proven using [^{35}S]methionine uptake as a basolateral marker (33) and detection of the p40, p35, and p30 proteins as apical markers (29–32).

Antibodies, Immunoprecipitation, and Gel Electrophoresis. We used antibody R1736 (14) to residues 595–611 of βAPP_{695} for immunoprecipitation of APP_s. This antibody recognizes an epitope that is specific to βAPP and does not exist in the recently described amyloid precursor-like proteins I and II (35, 36). Aβ, p3, and related low molecular weight peptides were immunoprecipitated with R1280 (14). Media from the basolateral and the apical chambers were brought to identical volumes, and equal amounts of antibodies were added. The immunoprecipitated derivatives were quantitated by cutting out the precipitated proteins from the dried gel and counting the slices in 5 ml of scintillation fluid (Econofluor; NEN). Independent experiments were carried out 3–10 times. All results are expressed as the average ± SEM.

Phorbol Ester Treatment. For the induction of APP_s secretion, MDCK cells grown on polycarbonate filters in Transwell chambers were pulse labeled for 2 h and chased in the presence or absence of 1 μM phorbol 12,13-dibutyrate (PDBu) for 2 hr in medium containing excess unlabeled methionine. Independent experiments were carried out three times. All results are expressed as the average ± SEM.

Transfection and Isolation of Single-Cell Clones. MDCK cells were cotransfected by electroporation with a cDNA construct encoding βAPP_{695} (CMV 695) (17) and the pRc/CMV vector (Invitrogen) encoding neomycin resistance. Thirty micrograms of CMV 695 and 0.6 μg of pRc/CMV were mixed with 5×10^6 cells in serum-free medium and pulsed once at 1000 mV (capacitance, 25 μF) at room temperature. Stably transfected cell clones were selected with G418 (400 μg/ml). Single-cell clones were identified using a filter screening method (37); the filters were immunoblotted with antibody R1736 to detect secreted APP_s. High-expressing single-cell clones were isolated using cloning cylinders. Two independent clones were analyzed for polarized secretion.

Cell Surface Biotinylation. MDCK cells plated on polycarbonate filters were biotinylated apically and basolaterally at 4°C as described (5). After washing, cells were warmed to 37°C for 1 hr. Media from the apical and basolateral labeled chambers were then immunoprecipitated with R1736. Biotinylated APP_s was electrophoresed and detected as described (5).

Determination of the Cell Surface Distribution of βAPP. A monoclonal antibody, 5A3, which recognizes the midregion of the βAPP ectodomain, was used to quantitate the amount of cell surface βAPP. Fab fragments were produced after pepsin digestion and radioiodinated with Iodogen (Pierce), following the manufacturer's directions, to a specific activity of 3–5 μCi/μg of protein. βAPP_{695}-transfected MDCK cells grown on polycarbonate filters were used for the binding assay. The cells were chilled on ice and then washed twice with binding medium consisting of RPMI 1640 supplemented with 0.2% (wt/vol) bovine serum albumin. The cells were then incubated for 1 hr at 4°C with the labeled antibody (at 10 nM per well) in either the apical or basolateral chamber. Cells were washed five times over 30 min and lysed with 0.2 M NaOH, and then radioactivity (cpm) was determined. In all experiments, cpm from the untransfected MDCK cells were subtracted from the cpm obtained from the transfected cells. All samples were performed in triplicate, and results are expressed as the average ± SEM.

Radiosequencing. Radiosequencing of gel-purified p3 and Aβ was carried out as described (14).

RESULTS

To determine whether cells that exhibit a polarized cell surface secrete the various proteolytic fragments of βAPP in a polarized manner, MDCK cells were grown in Transwell chambers and metabolically labeled with [^{35}S]methionine. APP_s was then immunoprecipitated from the apical or the basolateral chamber. APP_s was predominantly secreted into the basolateral compartment (Fig. 1*A*). Quantitation showed that $\approx$90% of the secreted APP_s molecules were detected in the basolateral compartment, with $\approx$10% found in the apical compartment (Fig. 1*B*).

To prove that the MDCK cells used for the experiments exhibited the expected polarized phenotype, we analyzed apical and basolateral markers. As an apical marker, we used

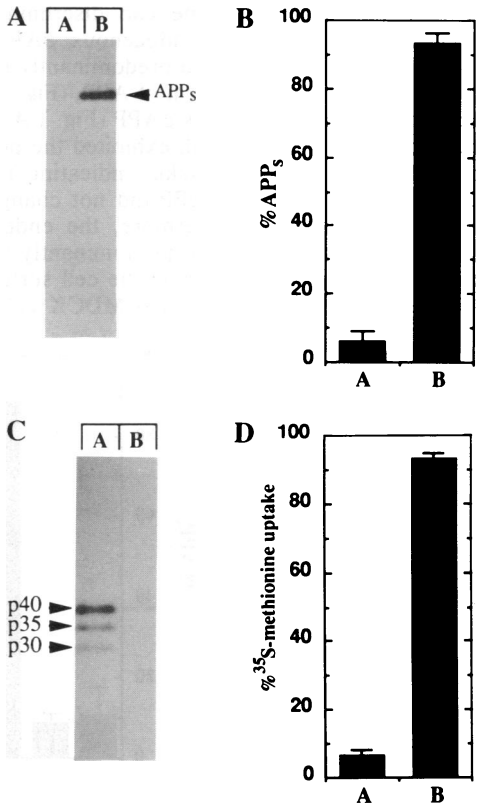

FIG. 1. (*A*) Polarized secretion of APP_s into the basolateral compartment. MDCK cells were metabolically labeled with [^{35}S]methionine, and APP_s was immunoprecipitated from both chambers. (*B*) Quantitation of the polarized secretion of APP_s. (*C*) Three apical marker proteins (p40, p35, and p30) are secreted predominantly into the apical compartment. (*D*) [^{35}S]Methionine is taken up predominantly at the basolateral cell surface. A, apical; B, basolateral.

the detection of three low molecular weight proteins (p40, p35, and p30) that have previously been shown to be predominantly secreted into the apical compartment of MDCK cells (29–32). As expected, these proteins were almost exclusively detected in the apical compartment in our cells (Fig. 1*C*). As a known basolateral marker, we used a methionine uptake assay (33). Labeling our MDCK cells on either the basolateral or apical side resulted in methionine uptake predominantly from the basolateral compartment (Fig. 1*D*). These data show that the MDCK cells used for the experiments exhibit the expected polarized phenotype.

Activation of protein kinase C (PKC) by phorbol esters (38) or certain first messengers (39) results in a significant increase in the secretion of APP_s. To establish whether PKC activation in MDCK cells influences the sorting of APP_s, the cells were pulse labeled with [^{35}S]methionine and chased in the presence or the absence of phorbol ester. PKC activation resulted in an increased secretion of APP_s in MDCK cells, as reported for other cell types (Fig. 2*A*). However, APP_s was still secreted predominantly into the basolateral compartment (Fig. 2). This result shows that activation of APP_s secretion does not change its basolateral sorting.

The data so far clearly demonstrate polarized secretion of APP_s. However, because APP_s is proteolytically derived from a transmembrane precursor, it is possible that the cleavage event rather than βAPP localization is polarized. To determine whether such an indirect mechanism of polarized secretion occurs or whether βAPP itself is sorted in a polarized manner, we determined the cell surface distribution of βAPP. To achieve sufficiently high expression of βAPP for this experiment, we stably transfected MDCK cells with a cDNA clone encoding βAPP_{695} (17). This shorter isoform of βAPP has the advantage that one can discriminate the transfected βAPP protein from endogenous $\beta APP_{751/770}$. Isolated single-cell clones showed a predominantly basolateral secretion of the transfected form of APP_s (Fig. 3 *A* and *B*), as shown above for endogenous βAPP (Fig. 1 *A* and *B*). These stably transfected clones still exhibited the predominantly basolateral methionine uptake, indicating that the transfection and expression of βAPP did not change their polarity (data not shown). Furthermore, the endogenous $\beta APP_{751/770}$ molecules still undergo predominantly basolateral secretion (Fig. 3*A*). To determine the cell surface distribution of βAPP, βAPP_{695}-transfected MDCK cells were plated on polycarbonate filters and incubated with a radioiodinated Fab fragment of monoclonal antibody 5A3 added to either chamber. As shown in Fig. 3*C*, βAPP was localized predominantly to the basolateral surface of MDCK cells. Furthermore, cell surface biotinylation experiments showed that βAPP can be cleaved from both cell surfaces. βAPP_{695}-transfected MDCK cells were biotinylated at 4°C on either side. After 1 hr at 37°C, APP_s was immunoprecipitated from the apical or basolateral biotinylated side. Surface-biotinylated APP_s was detected in both compartments (Fig. 3*D*). However, due to the differential distribution of βAPP on the cell surface, we detected most of the cell-surface derived APP_s after biotinylation in the basolateral chamber. These results argue against the indirect sorting mechanism of APP_s and show that βAPP itself exhibits a polarized distribution resulting in the preferential basolateral secretion of APP_s.

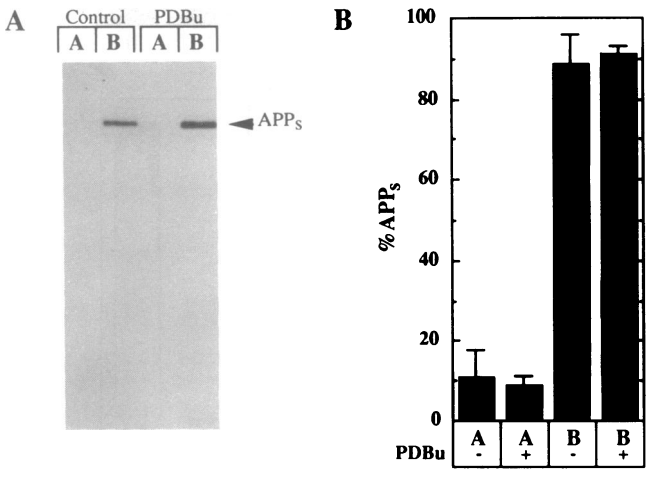

FIG. 2. PKC-mediated upregulation of APP_s secretion does not alter polarized sorting of βAPP. (*A*) MDCK cells were pulse labeled and then chased with or without the addition of PDBu. APP_s was immunoprecipitated from the apical and basolateral compartments. (*B*) Quantitation of the polarized secretion of APP_s during a pulse chase with (+) or without (−) the addition of PDBu. A, apical; B, basolateral.

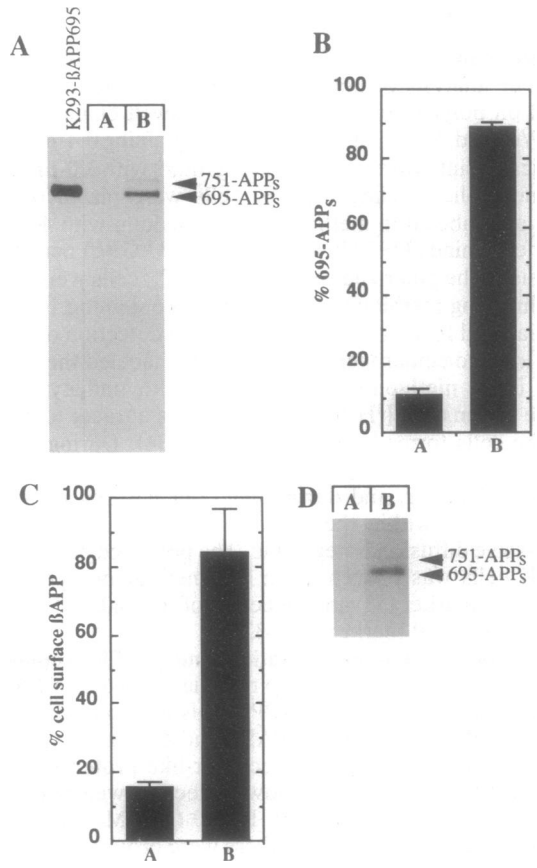

FIG. 3. (*A*) Polarized secretion and differential surface distribution of βAPP after stable transfection of βAPP_{695}. MDCK cells stably transfected with βAPP_{695} secrete APP_s predominantly into the basolateral compartment. As a control, an immunoprecipitate of conditioned medium from kidney 293 cells stably transfected with βAPP_{695} was run in parallel. The second faint APP_s band is derived from endogenously expressed $\beta APP_{751/770}$. (*B*) Quantitation of the polarized secretion of transfected 695-APP_s. (*C*) βAPP is predominantly localized on the basolateral cell surface, as determined by the binding of a radiolabeled Fab fragment of the monoclonal antibody 5A3. (*D*) Differentially distributed cell surface βAPP is a substrate for the secretory cleavage. βAPP_{695}-transfected MDCK cells were surface biotinylated in either chamber on ice. Cells were then washed and warmed to 37°C for 1 hr. Biotinylated APP_s was immunoprecipitated from the respectively labeled chambers and detected as described (5). A, apical; B, basolateral.

Previously, we (14) and others (15, 16) showed that Aβ is secreted by cultured cells under normal metabolic conditions. In addition to Aβ, we also detected a variety of alternative low molecular weight cleavage products, including a 3-kDa peptide (p3) that appeared to be derived from the

10-kDa C-terminal fragment of βAPP created by the secretase cleavage (14, 17). MDCK cells with or without transfected βAPP_{695} were plated on Transwell polycarbonate filters and metabolically labeled for 12 hr with [^{35}S]methionine to determine whether the sorting of Aβ and p3 follows that of APP_s. Like APP_s, Aβ and p3 were secreted predominantly basolaterally in both untransfected and βAPP_{695}-transfected cells (Fig. 4 *A* and *B*). Surprisingly, we found that the Aβ peptides in the 4-kDa range migrated as a closely spaced doublet (Fig. 4*A*), in contrast to the usual singlet observed in other cell types examined to date (14). This phenomenon was observed in both untransfected and transfected cells. [^{3}H]Phenylalanine-labeled 4- and 3-kDa peptides were isolated from medium of βAPP_{695}-transfected MDCK cells and subjected to radiosequencing (14). The 4-kDa material revealed a predominant double peak of radioactivity occurring at cycles 15 and 16, four cycles earlier than would be expected for the phenylalanine at residues 19 and 20 in Aβ starting at Asp-1 (Fig. 4*C*). This position for the two adjacent phenylalanine residues corresponds to a peptide beginning at Arg-5 (Fig. 4*D*). This peptide represented ≈80% of the total cpm recovered in the sequencing run on the 4-kDa material. This Arg-5 fragment has not been detected in other cell types expressing wild-type βAPP (14–16). Small signals were also obtained at cycles 4, 19, and 20 and also 7, 22, and 23, corresponding to peptides beginning at Asp-1 (Aβ) and Val (−3), respectively (Fig. 4 *C* and *D*), as observed previously (14). Approximately 10% of the total cpm were contributed by each of these two peptides. Sequence analysis of p3 produced a predominant doublet of radioactivity at cycles 3 and 4 (data not shown), indicating a peptide beginning at Leu-17 (Fig. 4*D*) (14, 17).

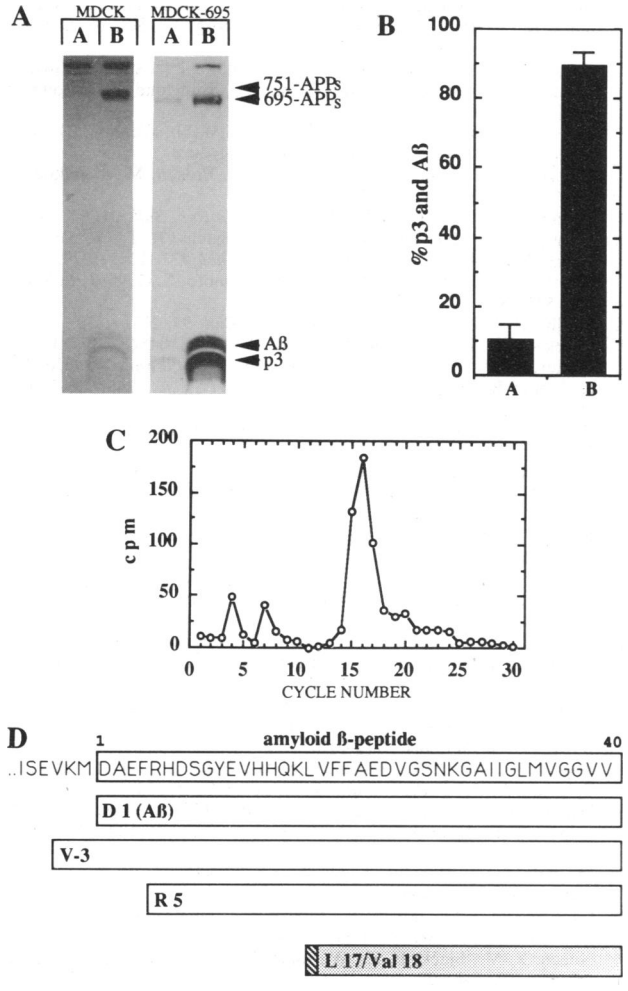

FIG. 4. (*A*) Polarized secretion of Aβ, Aβ-like low molecular weight peptides, and p3 in MDCK cells (*Left*) and βAPP_{695}-transfected MDCK cells (*Right*). Note the tightly spaced double band at ≈4 kDa. Longer exposure times revealed the same doublet in the untransfected MDCK cells. Antibody R1280 immunoprecipitates only a minor, variable quantity of APP_s (14, 17). (*B*) Quantitation of the polarized secretion of Aβ and p3. A, apical; B, basolateral. (*C*) Radiosequencing after [^{3}H]phenylalanine labeling of the double band at ≈4 kDa shown in *A* (MDCK-695). (*D*) Schematic diagram showing the identity of the different Aβ-related peptides secreted by MDCK cells as determined by radiosequencing. The shaded bar indicates the sequences of the p3 species. Note that the exact C termini of Aβ and Aβ-related peptides are currently unknown.

DISCUSSION

In this study, we demonstrate the basolateral secretion of APP_s, Aβ, and several Aβ-related low molecular weight peptides, including p3 and a species beginning at Arg-5 of Aβ.

Because βAPP is initially synthesized and transported as a membrane-bound precursor, the polarized secretion of multiple proteolytic fragments of βAPP raises interesting mechanistic questions. The signals mediating polarized sorting of βAPP might act on the full-length, membrane-bound precursor molecule. Indeed, it has been shown that signals mediating basolateral sorting of a variety of transmembrane proteins are located within their cytoplasmic domains (21, 25–28). Deletions of these sequences resulted in the inhibition of basolateral sorting of these molecules. Instead of being targeted to the basolateral plasma membrane, these truncated proteins were delivered directly to the apical plasma membrane. Matter *et al.* (28) have shown that two tyrosine-dependent targeting signals are present in the low density lipoprotein receptor. One of these signals is colinear with the NPXY motif known to be necessary for the coated pit-mediated reinternalization of cell surface proteins. Such a tyrosine-dependent signal is present within the cytoplasmic tail of βAPP (40) and a variety of other proteins (21, 25–27). Moreover, βAPP has been shown to be reinternalized from the cell surface as a full-length molecule (5) and has been identified within clathrin-coated vesicles (10). One could therefore speculate that a cytoplasmic signal, which is colinear with the NPXY sequence motif, is involved in the basolateral sorting of βAPP. However, the proteolytic processing of βAPP makes such a prediction difficult. Although cell surface full-length βAPP can give rise to the secreted form of APP_s (refs. 5 and 6 and this paper), we have found that βAPP can also be cleaved intracellularly to create APP_s in MDCK cells (C.H. and D.J.S., unpublished data), as has recently been shown in PC12 cells (7). Therefore, it is possible that APP_s itself is sorted intracellularly as a soluble derivative, making the cytoplasmic tail unnecessary for polarized trafficking. Moreover, both sorting mechanisms could act in parallel on membrane-bound precursors and on intracellularly generated APP_s. In such a case, two independent sorting signals in the cytoplasmic tail and in the ectodomain of βAPP have to be proposed.

It is known that neuronal βAPP undergoes fast axonal transport (41) and is localized to axons in mature cultured neurons (42). According to the proposal of Dotti and Simons (43), these findings would predict that βAPP might undergo apical sorting in MDCK cells. However, certain other neuronal proteins have been shown not to follow the axonal/apical dendritic/basolateral model (27). Furthermore, the site(s) of release of APP_s, Aβ, and p3 from neurons has not yet been determined, precluding such a prediction for βAPP.

In our study, βAPP as well as all of its secreted proteolytic products, including Aβ, are preferentially targeted to the basolateral compartment. This finding suggests that Aβ generation is an event that probably takes place after polarized βAPP sorting, since it is unlikely that all proteolytic fragments contain their own sorting signals. In this regard, it is interesting to note that an acidic environment has been shown

to favor Aβ generation, although the peptide has not been found within isolated lysosomes (16, 17). A compartment that fulfills these requirements could therefore be the early endosome or a late Golgi transport vesicle.

The proteases mediating the generation of Aβ and related low molecular weight fragments are currently unknown. Here, we show that epithelial cells have somewhat different proteolytic mechanisms for generating these peptides as compared to embryonic kidney 293 cells, which have previously been studied in considerable detail (14, 17). In both untransfected and βAPP_{695}-transfected MDCK cells, the majority of the 4-kDa peptides starts at Arg 5. Transfecting the same isoform of βAPP into kidney 293 cells results in a cleavage pattern creating mostly Aβ starting at Asp 1, plus minor peptides beginning at Val −3, Ile −6, or Phe 4 of Aβ (14). This difference in Aβ species indicates that βAPP might be a substrate for proteases with different tissue-specific and cell-specific expression patterns. Degradation of Aβ starting at Asp 1 into the Arg 5 species seems unlikely, since we have observed that Aβ and Aβ-related peptides are very stable and have a long half-life (ref. 17; C.H. and D.J.S., unpublished data).

The βAPP trafficking data presented here may have functional implications. Secreted APP_s has been found to have trophic effects on cultured cells (44). In epithelial cells, a molecule with these properties might be expected to be expressed on the basolateral plasma membrane, as we have indeed shown. Furthermore, it has also been shown that APP_s acts as a cell adhesion protein *in vitro* (45). Such a protein would be expected to undergo basolateral secretion, as laminin does (27). Moreover, secreted APP_s from $\beta APP_{751/770}$ containing the KPI domain (also known as protease nexin II) has been proposed to be involved in the blood coagulation cascade and wound healing (46). MDCK epithelial cells endogenously express the βAPP isoforms proposed to be involved in blood coagulation, and these molecules are secreted almost entirely into the abluminal compartment under both basal and stimulated conditions. In this regard, it is interesting to note that von Willebrand factor is also released basolaterally from endothelial cells and binds to the extracellular matrix upon induction of its secretion (47). This phenomenon has been interpreted to play an important role in wound healing in blood vessels. Therefore our data support the current concept of a biological function for βAPP in cell adhesion, growth promotion, and blood coagulation.

Finally, the basolateral secretion of Aβ that we define here might also play an important role in generating the typical amyloid pathology of AD within blood vessels of the brain. Virtually all AD cases display some, and often many, cerebral microvessels that contain filamentous Aβ deposits. These are consistently localized to the abluminal basement membrane of capillaries, arterioles, and small arteries, including arteries in the meningeal space (1, 18). Our results suggest that polarized release of Aβ and closely related peptides from the basolateral endothelial surface could provide one explanation for this preferential localization. Besides its pathological implications, the polarized trafficking of both membrane-anchored βAPP and its various secretory derivatives, including Aβ, provides an excellent system for the analysis of the sorting signals of alternative proteolytic products derived from an integral membrane precursor.

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