

Polarized Sorting of β -Amyloid Precursor Protein and Its Proteolytic Products in MDCK Cells Is Regulated by Two Independent Signals

Christian Haass, Edward H. Koo, Anja Capell, David B. Teplow, and Dennis J. Selkoe

Departments of Neurology and Pathology, Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, Massachusetts 02115

Abstract. Progressive cerebral deposition of the amyloid ($A\beta$) β -protein is an early and invariant feature of Alzheimer's disease. $A\beta$ is derived by proteolysis from the membrane-spanning β -amyloid precursor protein (β APP). β APP is processed into various secreted products, including soluble β APP (APP_s), the 4-kD $A\beta$ peptide, and a related 3-kD peptide (p3). We analyzed the mechanisms regulating the polarized basolateral sorting of β APP and its proteolytic derivatives in MDCK cells. Deletion of the last 32 amino acids (residues 664-695) of the β APP cytoplasmic tail had no influence on either the constitutive $\sim 90\%$ level of basolateral sorting of surface β APP, or the strong basolateral secretion of APP_s , $A\beta$, and p3. However, deleting the last 42 amino acids (residues 654-695) or changing tyrosine 653 to alanine altered the distribution of cell surface β APP so that $\sim 40\text{--}50\%$ of the molecules were inserted apically. In parallel, $A\beta$ was

now secreted from both surfaces. Surprisingly, this change in surface β APP had no influence on the basolateral secretion of APP_s and p3. This result suggests that most β APP molecules which give rise to APP_s in MDCK cells are cleaved intracellularly before reaching the surface. Consistent with this conclusion, we readily detected intracellular APP_s in carbonate extracts of isolated membrane vesicles. Moreover, ammonium chloride treatment resulted in the equal secretion of APP_s into both compartments, as occurs with other non-membranous, basolaterally secreted proteins, but it did not influence the polarity of cell surface β APP. These results demonstrate that in epithelial cells two independent mechanisms mediate the polarized trafficking of β APP holoprotein and its major secreted derivative (APP_s) and that $A\beta$ peptides are derived in part from β APP holoprotein targeted to the cell surface by a signal that includes tyrosine 653.

THE β -amyloid precursor protein (β APP)¹ is a type I membrane-spanning glycoprotein which is ubiquitously expressed in mammalian cells. The cellular processing of β APP has attracted great interest because progressive cerebral deposition of its ~ 40 residue amyloid β -protein ($A\beta$) fragment is an early and constant feature of Alzheimer's disease (AD). A central role for $A\beta$ in the pathogenesis of AD is supported by the discovery of missense mutations within and immediately flanking the $A\beta$ region of the β APP gene in several families with autosomal dominant AD (FAD) (for review see Mullan and Crawford, 1993). Moreover, aggregated forms of $A\beta$ have been shown to induce neurotoxicity and neuronal death in tissue culture

systems (Koh et al., 1990; Mattson et al., 1992; Pike et al., 1993; Yankner et al., 1990). β APP is encoded by a single gene on human chromosome 21 (Kang et al., 1987). Increased expression of β APP in patients with trisomy 21 (Down's syndrome) may explain the invariant development of severe $A\beta$ deposition and subsequent histopathology of AD in this disorder.

β APP provides an intriguing model for studying the processing of membrane proteins because it serves simultaneously as a cell surface receptor-like molecule and the precursor of several secreted derivatives, some of which appear to act as ligands for other receptors. The complex metabolism of β APP includes at least two principal processing pathways: (a) cleavage within the $A\beta$ region by an unidentified enzyme(s) called α -secretase (see Fig. 1 A) to release the large soluble ectodomain (APP_s) (Esch et al., 1990; Sisodia et al., 1990; Weidemann et al., 1989) and (b) reinternalization of the holoprotein from the cell surface and proteolytic processing within endosomes and lysosomes (Ferreira et al., 1993; Golde et al., 1992; Haass et al., 1992a; Koo and Squazzo, 1994). Regarding the exocytic pathway, it has been shown that surface β APP can serve as a substrate for α -secretase (Haass et al., 1992a; Roberts et al., 1994; Siso-

Please address all correspondence to Dr. D. Selkoe, Departments of Neurology and Pathology, Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115. Tel.: (617) 732-6454. Fax: (617) 732-7787.

1. *Abbreviations used in this paper:* A, apical; $A\beta$, amyloid β -protein; AD, Alzheimer's disease; B, basolateral; β APP, β -amyloid precursor protein; APP_s , soluble β -amyloid precursor protein; FAD, familial Alzheimer's disease; p3, 3 kD COOH-terminal peptide of $A\beta$; PNS, postnuclear supernatant.

dia, 1992), but intracellular generation of APP, the major secreted derivative, has also been demonstrated (De Strooper et al., 1993; Kuentzel et al., 1993; Sambamurti et al., 1992). Such processing is referred to as non-amyloidogenic because the α -secretase cleavage occurs principally between residues 16-17 of the A β region, precluding release and deposition of intact A β . In the endocytic pathway, molecules which avoid α -secretase cleavage can undergo reinternalization (Haass et al., 1992a; Koo and Squazzo, 1994), presumably via a cytoplasmic sequence motif (Asn-Pro-Thr-Tyr) known to mediate trafficking via clathrin-coated vesicles (Chen et al., 1990; Nordstedt et al., 1993; Yamazaki, T., D. J. Selkoe, E. H. Koo. 1993. *Soc. Neurosci. Abstr.* 19:396). Because only a minority of synthesized β APP molecules undergo α -secretory processing or reinternalization from the cell surface, it is likely that additional intracellular trafficking pathways exist, including from the *trans*-Golgi to endosomes/lysosomes, possibly using the clathrin-mediated pathway to exit the *trans*-Golgi network (Kuentzel et al., 1993).

Delineation of these two general processing routes did not answer the question of how the A β fragment is produced and released. The localization of the COOH terminus of A β in the hydrophobic transmembrane domain of β APP predicted that a pathological alteration of membrane structure would be required to release this fragment. However, it has been shown that intact A β is constitutively secreted into the media of cultured cells under physiologic conditions, presumably after cleavage of β APP by enzyme activities designated β - and γ -secretase (Fig. 1 A; [Busciglio et al., 1993; Haass et al., 1992b; Shoji et al., 1992; for review see Haass and Selkoe, 1993]). That such A β secretion also occurs normally *in vivo* has been proven by the identification of A β in cerebrospinal fluid and plasma (Seubert et al., 1992; Shoji et al., 1992; Suzuki, N., T. T. Cheung, X.-D. Cai, A. Odaka, L. Otvos, Jr., S. L. Gillespie, L. Ho, M. Shoji, C. Eckman, T. E. Golde, and S. G. Younkin. 1994. The familial amyloid β protein precursor (β APP717) mutations increase production of long amyloid β protein. *Neurobiol. Aging.* 15:S54).

In addition to A β , a related COOH-terminal fragment of molecular mass \sim 3 kD (p3) is also secreted into culture media. Its NH₂ terminus is at, or adjacent to, the α -secretase cleavage site, suggesting that p3 derives from the \sim 10-kD COOH-terminal fragment of β APP that remains membrane bound after α -secretase cleavage (Haass et al., 1993, 1992b). Despite their secretion at nanomolar levels, A β and p3 have not been detected intracellularly (Busciglio et al., 1993; Haass et al., 1993, 1992b; Shoji et al., 1992), except in one human neuronal cell line (Wertkin et al., 1993).

It is important to establish the mechanism of A β generation and the intracellular organelle(s) within which β APP proteolysis occurs. Agents which inhibit the trafficking of β APP through the Golgi (e.g., Brefeldin A) or alter intravesicular pH (e.g., ammonium chloride, chloroquine) block A β formation (Buxbaum et al., 1994; Haass et al., 1993; Koo and Squazzo, 1994; Shoji et al., 1992). A β cannot be detected within isolated lysosomes (Haass et al., 1993). Moreover, A β and p3 appear to be derived from distinct pathways (Dyrks et al., 1993; Haass et al., 1993; Koo and Squazzo, 1994). Taken together, the data suggest that A β is formed after β APP matures through the Golgi and is cleaved at the NH₂ terminus of the A β region in a non-lysosomal acidic compartment.

To understand the mechanism of β APP trafficking and its

relationship to A β production in greater detail, we examined β APP processing and the secretion of its various derivatives in MDCK epithelial cells (Haass et al., 1994a). This model system is of interest in view of the high expression of β APP in a number of polarized cell types *in vivo*, including neurons and endothelial cells (Shivers et al., 1988; Haass et al., 1992a). In initial studies, we showed that β APP undergoes highly polarized sorting, principally to the basolateral surface of MDCK cells, and that APP, A β , and p3 are all preferentially secreted from the basolateral surface (Haass et al., 1994a). Because the sorting signals which regulate protein trafficking in such cells have been identified only for uncleaved membrane-bound proteins (for review see Rodriguez-Boulan and Powell, 1992), β APP provides a novel model molecule which can be used to define signals for the polarized sorting of a membrane-bound precursor and its soluble proteolytic products. Moreover, the polarized trafficking and secretion of A β in epithelial cells could prove directly relevant to the preferential localization of A β deposits in the basolateral basement membrane of cerebral endothelial cells (Yamaguchi et al., 1992).

We have therefore conducted an analysis of the sorting signals in the β APP cytoplasmic tail, using β APP cDNA constructs containing a variety of deletions and substitutions. This analysis demonstrated two independent pathways mediating the polarized sorting of β APP and its derivatives. One mechanism was dependent in part on cytoplasmic signals close to the membrane, including a critical tyrosine at residue 653 of β APP₆₉₅. This trafficking route regulates the localization of both surface-bound β APP and, at least in part, secreted A β . The second mechanism appears to involve specialized signals within the ectodomain of β APP. This mechanism directs β APP into a basolateral sorting pathway, where most of the α -secretase-derived APP is produced. Our findings extend the understanding of the cellular processing of proteins that can exist in both membrane-bound and soluble forms and, in the specific case of β APP, help elucidate the mechanisms regulating β APP trafficking and A β production.

Materials and Methods

Culture and Analysis of MDCK Cell Monolayers

MDCK cells were cultured as described (Haass et al., 1994a; Hunziker and Mellman, 1989). We plated MDCK cells on carbonate filters in Transwell chambers (Costar Corp., Cambridge, MA). 24-mm-diam filters with pore sizes of 0.4 μ m and 3 μ m were used for labeling and binding experiments, respectively. MDCK cells were metabolically labeled and their apical and basolateral media immunoprecipitated as described (Haass et al., 1994a).

Pulse Chase Experiments

MDCK cells stably transfected with the β APP₆₉₅Y653A cDNA (see below) were cultured on Transwell chambers as described above. Cells were washed twice in methionine/serum free medium and incubated in that medium for 20 min. Cells were pulse labeled in methionine/serum free medium containing 140 μ Ci [³⁵S]methionine per Transwell chamber for 15 min. Cells were washed 3 \times in DMEM, 10% FBS at 4°C, and chased for 0.5, 1 h, 4 h, and 8 h at 37°C. Media were collected and APP_s was immunoprecipitated.

Antibodies, Immunoprecipitation, and Gel Electrophoresis

Immunoprecipitation and gel electrophoresis were carried out as described before (Haass et al., 1991, 1994a, 1992b). For the immunoprecipitation of

APP_s, we used antibody 1736 (Haass et al., 1992b) raised to residues 595-611 (β APP₆₉₅ numbering). The antibody was diluted 1:300. A β and p3 were immunoprecipitated with antibody 1280 to A β 1-40 (Haass et al., 1992b) at a 1:300 dilution. Immunoprecipitations were quantitated as described before (Haass et al., 1994a; Hung et al., 1993; Hung and Selkoe, 1994).

For immunoblotting, we used antibody C7 (raised to the last 20 amino acids of β APP; Podlisny et al., 1991), antibody 1736, and antibody B5 (raised to amino acids 444-592 of β APP 695; Oltersdorf et al., 1990). The epitopes of all antibodies are shown in Fig. 1 A.

Construction of Mutant β APP cDNAs and Transfection

All β APP deletion constructs were generated by the PCR using oligonucleotides designed to introduce stop codons at the corresponding position. Stop codons were introduced using the following oligonucleotides as a 3' primer:

CCTCTAGACTATCGTAGCCGTTCTGC (Δ 12 oligonucleotide)
CCTCTAGACTAGTGGCGCTCCTCTGGGG (Δ 22 oligonucleotide)
CCTCTAGACTAAACCTCCACACACC (Δ 32 oligonucleotide)
CCTCTAGACTIAGTACTGTTCTTCTT (Δ 42 oligonucleotide)
(underlined sequences = XbaI restriction site; bold letters = stop codon)

The following oligonucleotide was used as 5' primer:

GATGCAGAATTCGACAT
(underlined sequence = EcoRI restriction site).

The PCR products were digested with EcoRI and XbaI and subcloned into EcoRI-SpeI-linearized CMV β APP₆₉₅ plasmid, generating the plasmids CMV β APP₆₉₅ Δ 12, CMV β APP₆₉₅ Δ 22, CMV β APP₆₉₅ Δ 32, and CMV β APP₆₉₅ Δ 42 (Haass et al., 1993). The sequence was confirmed by sequencing both DNA strands of the subcloned PCR products.

The Y653A substitution was generated by oligonucleotide-directed mutagenesis according to standard procedures (Kunkel, 1985), using the following oligonucleotide (bold letters indicate the mutated triplet coding for Ala instead of Tyr):

ATGAATGGATGGCCTGTTCTTCTT (Y653A oligonucleotide).

The ~0.9-kb EcoRI-HindIII fragment of CMV β APP₆₉₅ was subcloned into M13mp18 and used as a single stranded template for mutagenesis (Hung and Selkoe, 1994). The mutated sequence was isolated by digestion with EcoRI-HindIII and subcloned into the EcoRI-SpeI linearized CMV β APP₆₉₅. The mutated sequence was confirmed by DNA sequencing. The β APP₆₉₅Y653A Δ 12 cDNA construct was generated by PCR using the Δ 12 oligonucleotide as a 3' primer, the 5' primer (see above), and the CMV₆₉₅Y653A cDNA as a template. The PCR product was subcloned as described and its sequence confirmed by DNA sequencing.

Stable transfection of MDCK cells and selection of single cell clones were carried out as described before (Haass et al., 1994a). The polarized phenotype of all single cell clones was confirmed as described (Haass et al., 1994a). Endogenous APP_s, which is strongly secreted basolaterally, served as an additional marker. Independent cell lines from each transfection with the different cDNA constructs were analyzed.

We did not induce the transcription of the stably transfected cDNA constructs with butyrate, because we found major changes in A β generation upon butyrate treatment.

Determination of the Cell Surface Distribution of β APP in MDCK Cells

The cell surface distribution of β APP was determined using radioiodinated Fab fragments of the monoclonal antibody 5A3, as described (Koo and Squazzo, 1994; Haass et al., 1994b). This antibody recognizes the mid-region of the β APP ectodomain.

Carbonate Extraction of Isolated Membranes

A postnuclear supernatant (PNS) was prepared as described before (Haass et al., 1992a). The PNS was pelleted to remove cytosolic proteins by spinning for 1 h at 50,000 rpm (~233,000 g max) at 4°C in a 55 Ti rotor (Beckman Instrs., Fullerton, CA). Isolated membranes were extracted in sodium carbonate as described by Fujiki et al. (1982). The homogenate was then pelleted as described above. The carbonate-releasate was concentrated on a Centricon 10 column, washed in STEN buffer (Weidemann et al., 1989) and again concentrated. The protein concentration of each fraction was de-

termined and equal amounts loaded on a 10% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose and immunoblotted as described before (Haass et al., 1989).

NH₄Cl Treatment

MDCK cells stably transfected with β APP₆₉₅ were plated on Transwell filters and metabolically labeled with [³⁵S]methionine for 3 h in the presence or absence of 10 mM NH₄Cl (Caplan et al., 1987). Media from each chamber were immunoprecipitated with antibody 1736, and the amounts of APP_s were quantitated. The media from cells kept as a control were brought to 10 mM NH₄Cl just before immunoprecipitation. [³H]Inulin was used as a tracer to ascertain the intactness of the tight junctions (Lisanti et al., 1989), and the polarized phenotype of MDCK cells during NH₄Cl treatment was determined by the methionine uptake assay (Hunziker and Mellman, 1989).

Radiosequencing and Compositional Analyses of Secreted A β Peptides

Radiosequencing and quantitation of the different A β and A β -like 4-kD peptides were performed essentially as described (Haass et al., 1994b).

Results

The Surface Distribution of β APP in MDCK Cells Is Regulated by Cytoplasmic Signals

Tyrosine-containing cytoplasmic sequence motifs have been shown to serve as basolateral sorting signals for a variety of membrane-bound proteins (for reviews see Hopkins, 1991; Rodriguez-Boulan and Powell, 1992). In MDCK cells, deletion of these signals results in a redistribution of the proteins from the basolateral to the apical surface. To define sorting signals mediating the highly polarized surface expression of β APP, we produced a set of β APP mutants containing progressively larger deletions of the cytoplasmic tail (Fig. 1 B). MDCK cells were stably transfected with cDNAs encoding wild-type or truncated β APP molecules, grown on polycarbonate filters in Transwell chambers, and then the cell surface distribution of β APP was determined by an antibody-binding assay using a monoclonal antibody to the β APP ectodomain (Haass et al., 1994a). MDCK cells expressing wild-type β APP₆₉₅ (β APP₆₉₅) or β APP₆₉₅ molecules missing the last 12 (β APP₆₉₅ Δ 12), 22 (β APP₆₉₅ Δ 22), or 32 (β APP₆₉₅ Δ 32) residues did not exhibit significant differences in the surface distribution of β APP; i.e., ~90% of labeled surface molecules were found on the basolateral membrane and ~10% were inserted apically (Fig. 2). This result is of particular interest because deleting the last 12 residues eliminates the Asn-Pro-Thr-Tyr putative reinternalization signal (Fig. 1 B), suggesting that this signal is not involved in the polarized sorting of β APP. However, deleting almost the entire cytoplasmic tail up to tyr653 (β APP₆₉₅ Δ 42; Fig. 1 B) resulted in a ~60:40 basolateral:apical distribution of β APP (Fig. 2). This result suggested that a cytoplasmic signal sequence close to the transmembrane domain influences the cell surface distribution of β APP. Since the β APP₆₉₅ Δ 42 cDNA construct has a stop codon inserted after tyrosine 653, we assumed that the deletion might interfere with a tyrosine-containing basolateral targeting motif very close to the cell membrane. We therefore constructed a point mutation exchanging tyrosine 653 to alanine, because such mutations are known to inactivate tyrosine-containing sorting signals (Matter et al., 1992). MDCK cells stably transfected with this cDNA construct exhibited an almost equal cell surface

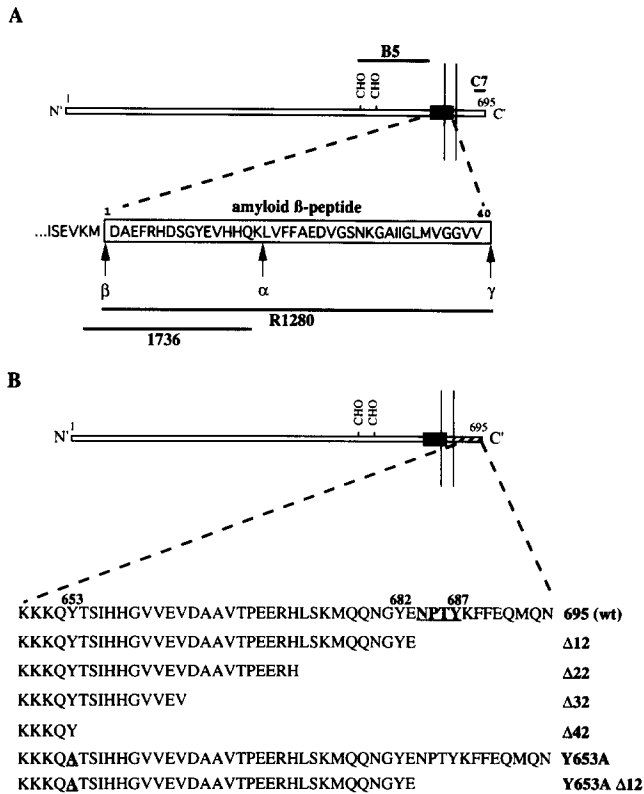


Figure 1. (A) Schematic representation of the β APP molecule. The black box indicates the A β domain and vertical lines indicate the cell membrane. The A β amino acid sequence is enlarged and shown in the white box. CHO, sites of glycosylation. Arrows indicate α -, β -, and γ -secretase cleavage sites. Bars indicate epitopes of antibodies used in this study. (B) Schematic representation of the deletion and substitution constructs used. The amino acid sequence of the cytoplasmic domain of β APP (striped box) is enlarged. The potential clathrin-coated pit reinternalization motif (NPTY) is underlined. wt, wild type.

expression of β APP (Fig. 2). To exclude the possibility that two partially redundant signals (Matter et al., 1992) within the cytoplasmic tail of β APP mediate polarized sorting, we transfected MDCK cells with a cDNA construct harboring both the tyrosine to alanine exchange at position 653 and the deletion of the last 12 amino acids (β APP₆₉₅Y653A Δ 12). Determination of the cell surface expression of β APP again revealed an almost equal expression on both surfaces (Fig. 2). Taken together, these data indicate that a sorting signal containing tyrosine 653 mediates the polarized sorting of cell surface β APP in MDCK cells.

Basolateral Secretion of APP_s Occurs Independently of the Cytoplasmic Tail

The above experiments examined only surface-bound holo- β APP. To analyze the polarized release of the major secreted product, APP_s, we metabolically labeled MDCK cells expressing the various cDNA constructs, collected the media from both chambers and detected APP_s by immunoprecipitation with antibody 1736 (Fig. 1 A), specific for α -secretase cleaved APP_s (Fig. 3 A). Quantitation of the immunoprecipitations (Fig. 3 B) showed that none of the COOH-terminal deletions or point mutations had a major influence on the

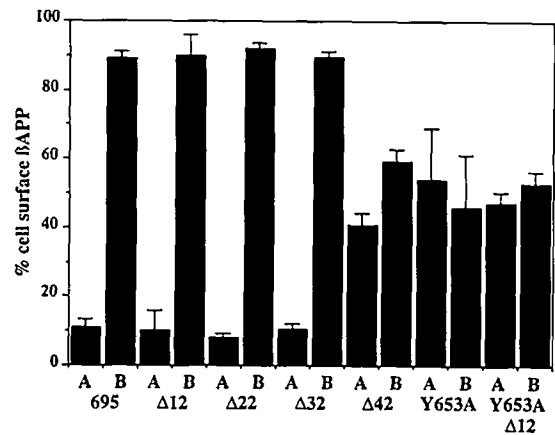


Figure 2. Cell surface distribution of β APP in MDCK cell lines expressing β APP₆₉₅, β APP₆₉₅ Δ 12, β APP₆₉₅ Δ 22, β APP₆₉₅ Δ 32, β APP₆₉₅ Δ 42, β APP₆₉₅Y653A, and β APP₆₉₅Y653A Δ 12, as determined by the binding of a radiolabeled Fab fragment of the monoclonal antibody 5A3 (Haass et al., 1994). Data are expressed as % of total surface β APP located on the apical (A) or basolateral (B) cell surface \pm standard errors of the means (SEM); $n = 3$ –12 for each construct.

basolateral secretion of APP_s. Even deleting almost the entire cytoplasmic tail (Δ 42) or expressing the β APP₆₉₅Y653A or β APP₆₉₅Y653A Δ 12 cDNAs still resulted in efficient basolateral secretion of APP_s. These data indicate that the polarized secretion of APP_s is independent of virtually the entire cytoplasmic sequence of β APP and uses sorting signals different from those mediating trafficking of the holoprotein to the cell surface (compare Fig. 2).

To exclude the possibility that the change of cell surface β APP but not APP_s polarity observed in the mutant-expressing cells is due to a change in the kinetics of APP_s secretion into the apical and basolateral compartments, we performed pulse chase experiments using the MDCK cell line stably transfected with the β APP₆₉₅Y653A cDNA. During a cold chase, APP_s molecules derived from the mutant cDNA construct and from the wild-type endogenous β APP gene were secreted with very similar kinetics (Fig. 4). APP_s molecules derived from the mutant cDNA construct are secreted at all time points overwhelmingly into the basolateral compartment. Even after an 8-h cold chase, no accumulation of APP_s in the apical compartment occurs (Fig. 4). This result makes it unlikely that this mutation interferes with the kinetics of APP_s secretion.

To confirm the hypothesis that polarized secretion of APP_s involves sorting signals in the ectodomain rather than in the transmembrane or cytoplasmic domains, we examined secretion from a truncated β APP cDNA construct (β APP695/S) that bears a stop codon at the α -secretase cleavage site (Hung and Selkoe, 1994). The resultant protein is expressed and secreted as a soluble, non-cleaved molecule. When MDCK cells were stably transfected with this construct and metabolically labeled, APP_s was recovered from both chambers (Fig. 5, A and B). However, \sim 70% of total secreted APP_s was still released into the basolateral chamber, clearly demonstrating that APP_s itself contains basolateral sorting information. Similar results were obtained when we expressed a cDNA construct containing a stop codon at the β -secretase site (i.e., after methionine₃₉₆ of

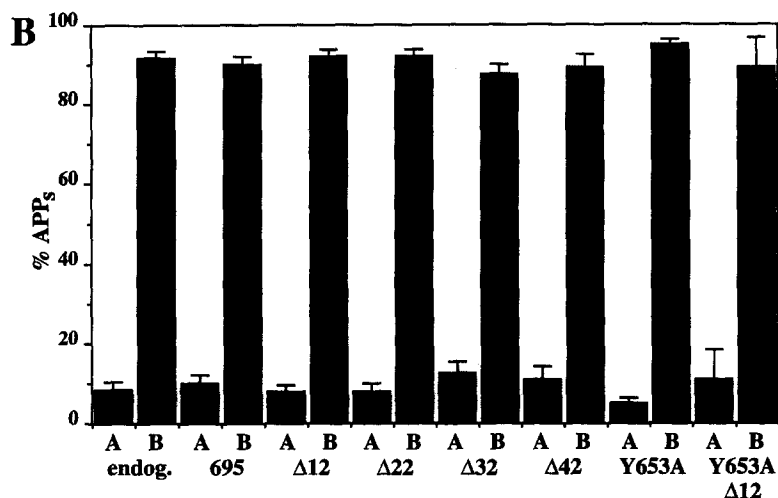
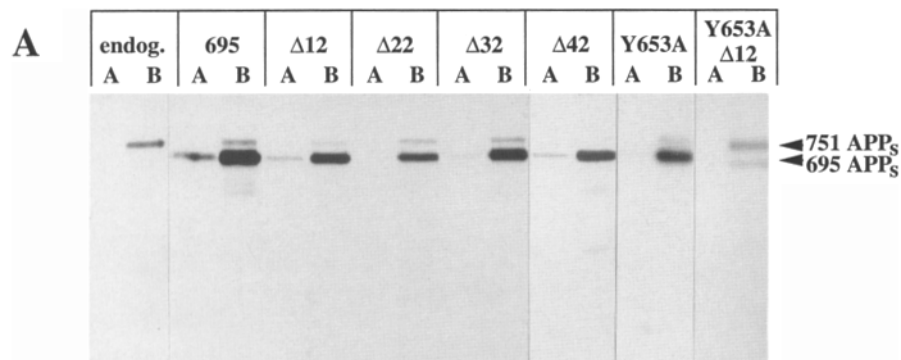


Figure 3. Polarized secretion of APP. (A) MDCK cells stably transfected with β APP₆₉₅, β APP₆₉₅Δ12, β APP₆₉₅Δ22, β APP₆₉₅Δ32, β APP₆₉₅Δ42, β APP₆₉₅Y653A, or β APP₆₉₅Y653A Δ12 were metabolically labeled with [³⁵S]methionine. APP_s was immunoprecipitated from the apical (A) and basolateral (B) chambers with antibody 1736. The upper band (751 APP_s) represents endogenous APP_s known to be secreted into the basolateral compartment (Haass et al., 1994a). The lower band corresponds to APP_s derived from the transfected β APP₆₉₅ constructs. (B) Quantitation of the immunoprecipitations shown in A. Data are expressed as % of total secreted APP_s released into the apical (A) or basolateral (B) compartment \pm standard errors of the means (SEM); n = 6–12.

β APP₆₉₅) resulting in the preferential basolateral secretion of a truncated form of APP, (data not shown). This finding excludes the possibility that a basolateral sorting signal is located between the β - and α -secretase sites.

Basolateral Secretion of APP_s Is Sensitive to Ammonium Chloride

The persistence of basolateral APP_s secretion despite changes in the cell surface distribution of β APP (Figs. 2 and 3) suggests that the majority of secreted APP_s molecules are

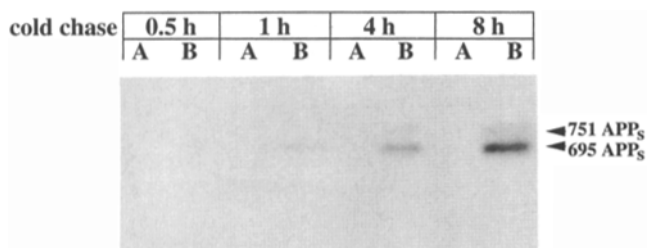


Figure 4. Pulse chase experiment with a MDCK cell line stably expressing the β APP₆₉₅Y653A cDNA. Cells were metabolically labeled for 15 min with [³⁵S]methionine and chased in the presence of excess amounts of unlabeled methionine for the indicated time points. Note that even after a 8-h cold chase period, no accumulation of APP_s occurs within the apical compartment.

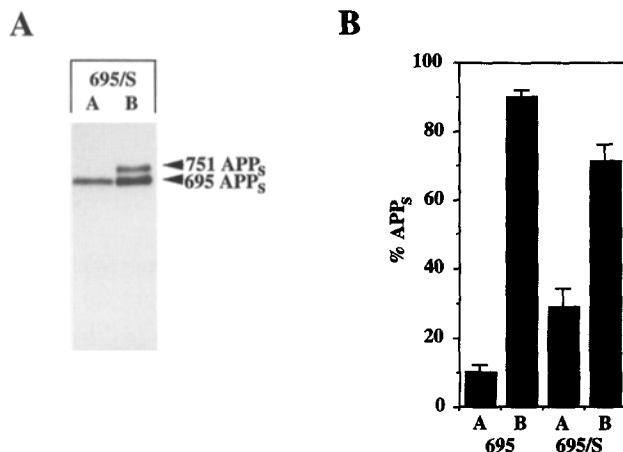


Figure 5. MDCK cells expressing the cDNA construct β APP₆₉₅-695/S secrete ~70% of their APP_s into the basolateral compartment. (A) Immunoprecipitation of conditioned media with antibody 1736. Note that the endogenous APP_s 751 still undergoes almost complete basolateral sorting. (B) Quantitation of the data shown in A. Data are expressed as described in Fig. 3 B; n = 9. A, apical; B, basolateral.

generated intracellularly before β APP reaches the surface. However, it is important to note that full-length β APP inserted at the basolateral surface also contributes to the pool of APP_s, because surface biotinylated β APP is subsequently recovered as biotinylated APP_s in the basolateral compartment (Haass, 1994a). It is known that soluble, non-membrane-bound proteins are secreted into the basolateral compartment of epithelial cells by an NH₄Cl-sensitive pathway (Caplan et al., 1987). For example, treating cells with NH₄Cl during a short labeling period equalizes the normally basolateral secretion of laminin and cathepsin D but does not change the distribution of apically secreted proteins or cell surface marker proteins such as (Na⁺+K⁺) ATPase (Caplan et al., 1987). Treating MDCK cells stably expressing β APP₆₉₅ with a low dose of NH₄Cl did, in fact, equalize secretion of APP_s (Fig. 6, A and B). This treatment did not alter the tight junctions, as shown by the exclusion of the radioactive tracer [³H]inulin, nor did it change detectably the polarized phenotype of these cells, as shown by the preferential basolateral uptake of [³⁵S]methionine (Fig. 6 B). The distribution of surface β APP was also not changed by NH₄Cl treatment, as shown by an antibody-binding assay (Fig. 6 B). The latter finding again demonstrates that in MDCK cells two independent mechanisms determine the sorting of surface β APP and of APP_s. Moreover, the selective NH₄Cl sensitivity of the polarized secretion of APP_s strongly suggests a sorting mechanism capable of acting on soluble, non-membrane-bound forms of β APP.

Detection of Intravesicular APP_s in MDCK Cells

Because the above data suggest the existence of soluble APP_s molecules within intracellular vesicles, we prepared a PNS of MDCK cells stably transfected with β APP₆₉₅. The PNS was centrifuged at 233,000 g (max) to pellet microsomal membranes. These membranes were then extracted with sodium carbonate buffer, and the released material (carbonate-supernatant) was separated from the membranes (carbonate-pellet) by a 233,000-g (max) spin. This treatment is known to release luminal contents of membrane vesicles without releasing membrane-bound molecules (Fujiki et al., 1982). Equal amounts of protein from these various fractions (PNS; PNS-pellet; carbonate-pellet; carbonate-supernatant) were analyzed by immunoblotting. Using antibody C7 (Fig. 1 A) against the cytoplasmic tail of β APP, we detected two bands corresponding to N- and N-plus O-glycosylated full-length β APP molecules (Fig. 7). These bands were enriched in the PNS-pellet as compared to unfractionated PNS and were further enriched in the carbonate-pellet. Only very minor amounts of full-length β APP were detected within the carbonate-supernatant (Fig. 7 A), consistent with the knowledge that carbonate does not extract membrane-bound proteins (Fujiki et al., 1982). To detect soluble APP_s, as well as full-length β APP, identical fractions were immunoblotted with antibody B5 to the β APP ectodomain (Fig. 1 A). Again, we observed a gradual enrichment of β APP holoproteins during the fractionation procedure (Fig. 7 A). However, the carbonate-supernatant also contained substantial amounts of a slightly smaller β APP species reacting selectively with antibody B5 but not antibody C7 (Fig. 7 A, arrowhead) and migrating at the position expected for APP_s. To further confirm that the β APP molecules detected by antibody B5

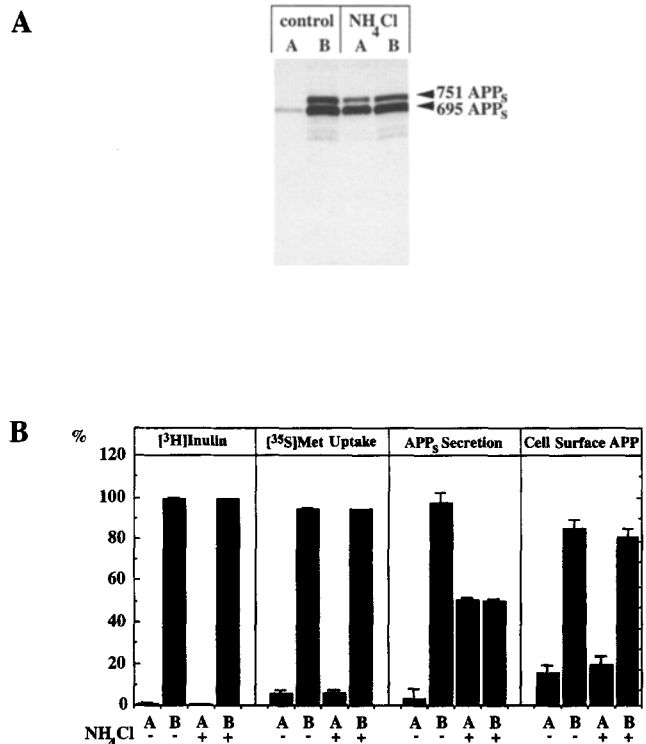


Figure 6. Polarized secretion of APP_s is NH₄Cl sensitive. (A) Addition of 10 mM NH₄Cl during a 4-h metabolic-labeling period results in equal secretion of APP_s into both compartments. APP_s was immunoprecipitated with antibody 1736. A, apical; B, basolateral. (B) Quantitation of the immunoprecipitations shown in A (APP_s Secretion; third box). NH₄Cl has no effect on the polarized distribution of cell surface β APP (Cell Surface APP) and no influence on the formation of tight junctions (³H]Inulin). NH₄Cl likewise has no effect on the polarized phenotype of MDCK cells (³⁵S]Met Uptake). Data are expressed as described in Fig. 3 B. n = 3–6. A, apical; B, basolateral.

represent authentic APP_s cleaved at the α -secretase site and do not correspond to random lysosomal degradation products of full-length β APP, the same protein fractions were immunoblotted with antibody 1736 (Fig. 7 B). This antibody was raised to the free COOH-terminal end of conventional (α -secretase cut) APP_s (Fig. 1 A) and preferentially recognizes APP_s molecules ending at lysine 16. Antibody 1736 strongly identified the APP_s released into the carbonate supernatant in a very similar manner to antibody B5, consistent with the existence of authentic intracellular APP_s cleaved at the α -secretase site (Fig. 7 B).

Because the APP_s protein in the carbonate supernatants is soluble, does not contain the C7 epitope, migrates with the appropriate M_r in SDS-PAGE, and specifically reacts with antibody 1736, it very likely represents intracellular APP_s created by α -secretase, as described previously by others (De Strooper et al., 1993; Kuentzel et al., 1993; Sambamurti et al., 1992). It is unlikely that these APP_s molecules were generated during the isolation procedure, since APP_s is already detected in the PNS of MDCK cells (Fig. 7, A and B) and in total lysates of kidney 293 cells (Fig. 7, A and B). In this regard, it is important to note that we have previously shown that no measurable reuptake of secreted

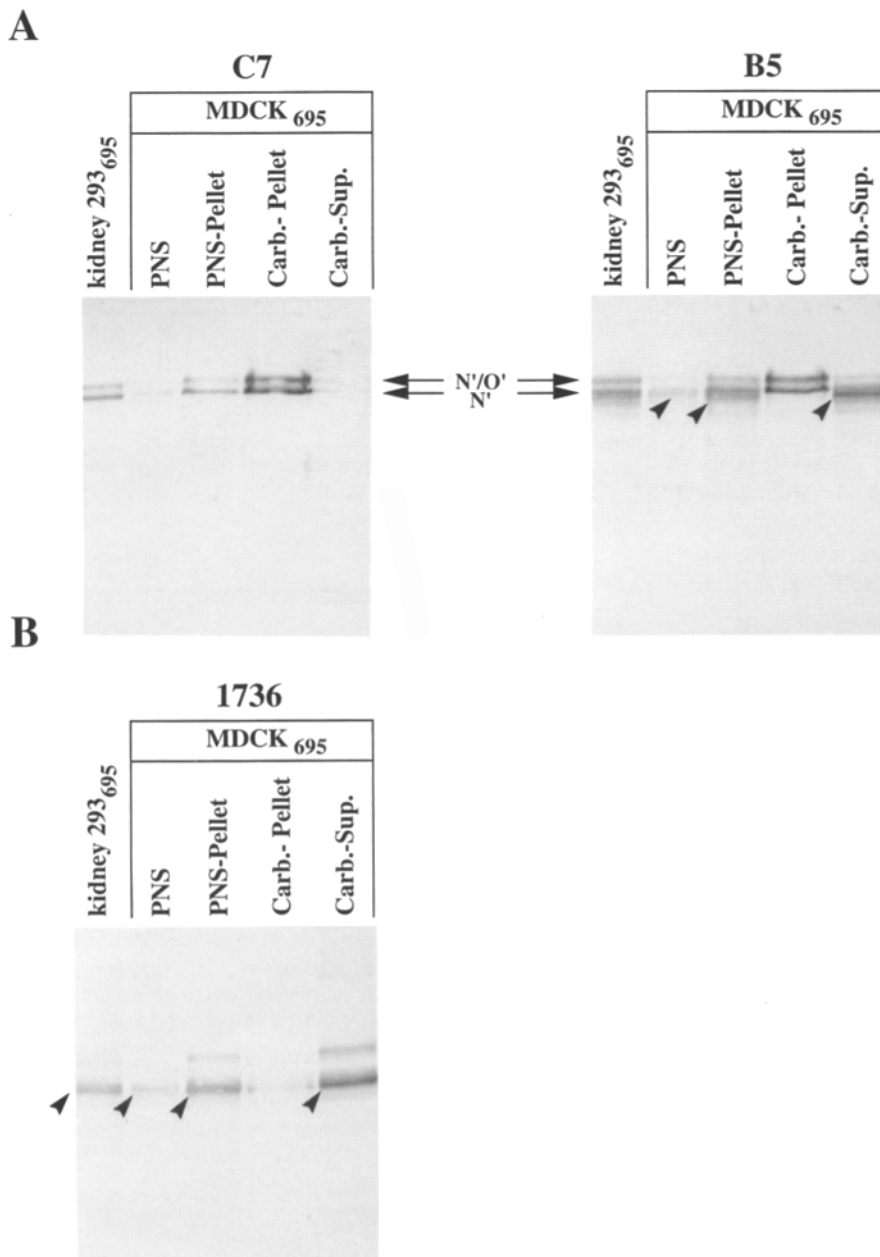


Figure 7. (A) Detection of APP, within cell lysates. Equal amounts (50 μ g) of protein derived from total PNS, PNS-pellet, carbonate-pellet (*Carb.-Pellet*) and carbonate supernatant (*Carb.-Sup.*) were immunoblotted with antibody C7 (*left panel*) and B5 (*right panel*). Antibody B5 detects high amounts of intracellular APP, within the carbonate supernatant. Proteins were separated on 10% SDS-polyacrylamide gels. (B) Identical protein fractions as described in A were immunoblotted with antibody 1736, which selectively recognizes α -secretase generated APP, and exhibits very low affinity for full-length β APP. Proteins were separated on 7% SDS-polyacrylamide gels. Arrowheads indicate the intravesicular APP_s. Arrows indicate membrane-bound immature (N'-glycosylated) and mature (N'/O' glycosylated) β APP. The upper band identified by antibody 1736 represents endogenous 751 APP_s.

APP, by cultured cells occurs under these culture conditions (Haass et al., 1992a). The present results, therefore, strongly support the hypothesis that APP_s can be generated within intracellular vesicles. Taken together, our observations suggest that intracellularly cleaved APP_s and full-length β APP are sorted for secretion and cell surface insertion, respectively, through two independent pathways.

Effects of Cytoplasmic Deletions on Polarized Secretion of A β

Having obtained evidence of distinct mechanisms for the polarized sorting of surface β APP and APP_s, we next analyzed the polarity of A β and p3 peptides in MDCK cell lines expressing the mutant β APP molecules described above. Deleting the last 12 amino acids of the cytoplasmic tail of

β APP (Fig. 1 B) had no effect on the release of A β or p3, indicating that the Asn-Pro-Thr-Tyr clathrin-binding domain by itself does not regulate the polarized secretion of these two peptides (Fig. 8). Similar results were observed when the last 22 or 32 amino acids of the cytoplasmic tail were removed (Fig. 8). However, deleting the cytoplasmic tail up to amino acid 654 (β APP₆₉₅ Δ 42; Fig. 1 B) resulted in an increased secretion of A β into the apical compartment. Interestingly, the polarized basolateral secretion of p3 was less affected by this deletion (Fig. 8). A similar result was obtained by analyzing the distribution of A β and p3 secreted from cells expressing the β APP₆₉₅Y653A cDNA (Fig. 8). We conclude from these data that a tyrosine-containing sequence motif close to the transmembrane domain of β APP mediates in part the polarity of A β secretion. These results correlate with the expression of the mutant β APP molecules

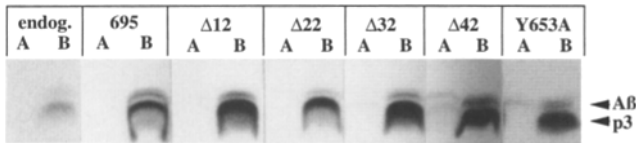


Figure 8. Polarized secretion of A β and p3. A β and p3 derived from cells expressing β APP₆₉₅, β APP₆₉₅ Δ 12, β APP₆₉₅ Δ 22, β APP₆₉₅ Δ 32, β APP₆₉₅ Δ 42, and β APP₆₉₅Y653A during a 12-h metabolic-labeling period are predominantly detected in the basolateral compartment. Although cells expressing β APP₆₉₅ Δ 42 and β APP₆₉₅Y653A still target A β into the basolateral compartment, A β can also be readily detected within the apical compartment. Note the relative increase of A β as compared to p3 in the apical vs the basolateral compartment in these last two mutants. A, apical; B, basolateral.

on the cell surface, in that a change in β APP surface polarity occurs only after deleting the last 42 amino acids or mutating tyrosine 653 (see Fig. 2). However, it should be noted that the increased secretion of A β into the apical compartment is not as marked as the observed redistribution of surface β APP.

Closer analysis of the electrophoretic gels shown in Fig. 8 revealed that all four cytoplasmic deletions resulted in a specific decrease in the upper band of a tightly spaced doublet corresponding to the 4-kD A β species. We showed previously that this characteristic 4-kD A β doublet produced by MDCK cells is composed of peptides beginning predominantly at A β positions Arg 5 (~80%), Val -3 (~10%) and Asp 1 (~10%) (Haass et al., 1994a). To establish the identities of the 4-kD peptides produced in MDCK cells from COOH terminally deleted β APP, we radiosequenced the peptides in the doublet species derived from the β APP₆₉₅ Δ 42 transfected cell line. We found an elimination of the normal radioactive phenylalanine peaks at sequencing cycles 4, 19, and 20, indicating an absence of A β species beginning at Asp 1. In this regard, it is important to note that A β purified from amyloid deposits in AD brain begins predominantly at Asp 1 (Roher et al., 1993). Moreover, it should be noted that alternative A β peptides could be generated within different subcellular compartments than typical A β starting at apartate 1. Therefore, the results in Fig. 8 may not reflect the sorting of typical A β but rather that of alternative A β -like peptides.

Discussion

β APP is an attractive model protein for identifying sorting signals because, unlike transmembrane proteins studied to date (Rodriguez-Boulant and Powell, 1992), it is proteolytically processed into a variety of secreted derivatives in addition to existing as an uncleaved, membrane-bound surface protein. One of the derivatives is the A β peptide, which appears to play a central role in the pathogenesis of AD (Selkoe, 1994). Little is known about the cellular mechanisms regulating the production of A β . Analyzing the sorting of wild-type and mutant β APP in MDCK cells should help define amyloidogenic and non-amyloidogenic processing pathways, and our results demonstrate that this is indeed the case. Here, we report the surprising finding that the sorting of β APP in MDCK cells involving two populations of β APP molecules and two independent mechanisms (Fig. 9). Sur-

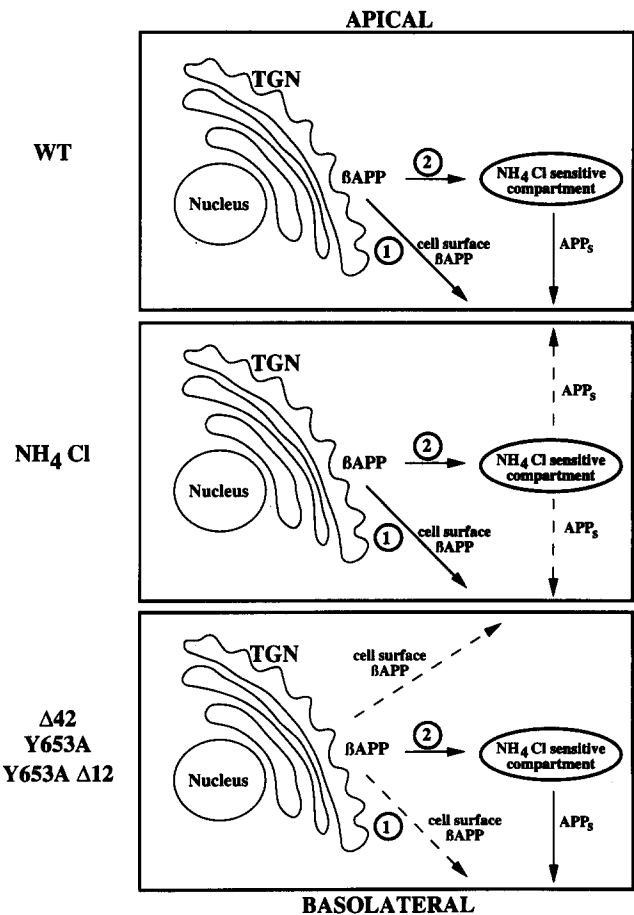


Figure 9. A hypothetical model illustrating the dual pathways regulating polarized sorting of cell surface β APP (pathway 1) and APP_s (pathway 2). During normal metabolism of wild-type β APP (*wt*), cell surface-bound β APP and APP_s are processed through NH₄Cl-insensitive (1) and NH₄Cl-sensitive (2) pathways, respectively. NH₄Cl essentially obliterates the polarized secretion of APP_s but has no effect on the polarity of cell surface β APP (*NH₄Cl*). Deletion of the last 42 amino acids or exchange of Tyr 653 to Ala result in a redistribution of cell surface β APP but have no effect on the polarized secretion of APP_s (Δ 42, Y653 A, Y653A Δ 12). Dashed lines indicate pathways affected by either NH₄Cl treatment (*middle box*) or the expression of the mutant cDNA constructs (*lower box*), respectively.

face-bound β APP is sorted via a pathway that is dependent, at least in part, on a tyrosine-containing signal at 653 within the cytoplasmic domain of β APP that is distinct from the putative internalization signal at residues 684-687. However, the majority of secreted APP_s molecules is sorted largely independently of signals in the β APP cytoplasmic domain, most likely via a mechanism using signals within its extracellular region in a manner consistent with a soluble, non-membrane-bound molecule. This sorting pathway is NH₄Cl sensitive (Fig. 9). The latter signals may act in concert with cytoplasmic sorting signals during normal β APP trafficking, because MDCK cells transfected with the non-membrane-inserted 695/S truncation construct lose some of the preferential basolateral sorting of APP_s.

A Dual Trafficking Mechanism Regulates Sorting of Cell Surface β APP/ β and β APP_{p3}

Basolateral targeting signals have been widely demonstrated within the cytoplasmic tails of a variety of transmembrane proteins expressed in polarized cells. Removal of the tails of these proteins, such as the polymeric immunoglobulin receptor, the Fc receptor, the low density lipoprotein receptor (LDL-R) and lysosomal glycoprotein 120 (LPG 120) (for review see Hopkins, 1991), results in a redistribution to the apical membrane. Hunziker et al. (1991) found a strong correlation between the ability of the Fc receptor, LDL-R, and LPG 120 to accumulate within clathrin-coated pits and their targeting to the basolateral plasma membrane. This finding suggested that basolateral targeting signals might overlap with coated pit-mediated reinternalization signals. Indeed, in follow up studies, a second tyrosine-containing targeting signal was identified that was colinear with the reinternalization signal within the cytoplasmic tail of LDL-R (Matter et al., 1992). This second signal efficiently mediated basolateral sorting but not reinternalization. It appears that tyrosine-containing signals are common motifs for intracellular sorting mechanisms (Matter et al., 1992; Rodriguez-Boulan and Powell, 1992). For example, tyrosine seems to play an important conformational role within the reinternalization signal, Asn-Pro-x-Tyr (x = any amino acid). This signal adopts a reverse-turn conformation in aqueous solution and is inactivated when the critical tyrosine is mutated to alanine (Bansal and Gierasch, 1991; Eberle et al., 1991).

We describe here the unexpected finding that surface-bound β APP molecules can be targeted in part to the apical cell surface by deleting the cytoplasmic tail of β APP or mutating a single cytoplasmic tyrosine (653), without affecting the strong basolateral secretion of APP, and the related p3 fragment (Fig. 9). The first part of these data agrees with previous results on a variety of cell surface proteins whose polarized sorting depends on cytoplasmic tyrosines (Hopkins, 1991; Rodriguez-Boulan and Powell, 1992). However, studies of these polarized proteins have demonstrated that deletion of the basolateral targeting signal results in a marked (up to 80%) redistribution to the apical cell surface (Hunziker et al., 1991; Hunziker and Mellman, 1989; Matter et al., 1992; Peters et al., 1990). The difference between the polarized sorting of β APP and these other cell surface proteins may lie in the fact that the β APP ectodomain contains additional signal(s) that are capable of mediating basolateral sorting. The latter signal(s) may act in concert with cytoplasmic tyrosine 653 to direct the almost complete basolateral sorting of membrane-bound β APP. In this regard, it is interesting to note that the human transferrin receptor, which is targeted to the basolateral compartment, also contains additional signals, so that deleting its cytoplasmic tail results in only a partial redistribution of the protein to the apical surface (Dargemont et al., 1993).

The polarized secretion of APP, and p3 is not affected by tyrosine 653 or any other cytoplasmic signal, consistent with the existence of a second, independent trafficking mechanism that is NH_4Cl -sensitive and thus resembles that for sorting other soluble proteins. Although this second pathway is clearly independent of the first, it initially requires membrane-bound β APP substrate to work efficiently, because MDCK cells expressing the soluble β APP695/S molecule

missort $\sim 30\%$ of the resultant APP, into the apical compartment (Fig. 5). The fact that only a minority of the APP, molecules are missorted suggests that both extracellular and cytoplasmic signals mediate complete basolateral targeting. Our results on APP, polarity lead to the conclusion that the majority of APP, in the MDCK cells is generated intracellularly, as suggested by studies of other cell types (De Strooper et al., 1993; Kuentzel et al., 1993; Sambamurti et al., 1992), and is secreted through an NH_4Cl sensitive compartment (Fig. 9). Our MDCK studies also demonstrate for the first time that β APP can be cleaved intracellularly in the secretory pathway as well as after its insertion into the plasma membrane in the same cell type (this report and Haass et al., 1994a).

An alternative interpretation of our results on APP, polarity would be that apical transport vesicles do not contain α -secretase. However, we find this possibility very unlikely, because under these conditions, an accumulation of membrane-bound full-length β APP on the apical cell surface would be expected, something which is clearly not the case.

Implications for the Cellular Mechanism of $A\beta$ Production

Mutations of the distal tyrosine residues, especially at 687, do not perturb polarized cell surface targeting, but rather result in the reduction of typical $A\beta$ peptides (in particular, the Asp 1 species), presumably by altering the internalization signal. This finding is consistent with a proposed mechanism of $A\beta$ generation that involves the endocytosis of cell surface β APP (Koo and Squazzo, 1994; Haass, C., D. B. Teplow, and D. J. Selkoe, manuscript in preparation). However, because production of $A\beta$ and localization of cell surface β APP do not coincide precisely, it is possible that additional cleavages resulting in $A\beta$ peptides may occur within the secretory pathway. This possibility is supported by recent evidence suggesting the occurrence of intracellular β -secretase cleavage in MDCK cells transfected with the mutant β APP isoform containing the codon 670/671 ("Swedish") mutations (Mullan et al., 1992; Lo et al., 1994). It is also interesting to speculate that different species of $A\beta$ are generated within different cellular compartments and that precise analysis of $A\beta$ polarity may therefore be difficult, as cytoplasmic deletions seem to lead to a selective inhibition of secretion of the $A\beta$ peptide starting at Asp 1.

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Note Added in Proof. After this paper was submitted, we learned that data from another laboratory (De Strooper, B., K. Craessaerts, I. Dewachter, D. Moechars, B. Greenberg, F. Van Leuven, and H. Van Den Berghe. 1995. Basolateral secretion of amyloid precursor protein in MDCK cells is disturbed by alterations of intracellular pH and by introducing a mutation associated with familial Alzheimer's Disease. *J. Biol. Chem.* In press.) are in agreement with the results presented here.

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