Title
Polarized sorting of beta-amyloid precursor protein and its proteolytic products in MDCK cells is regulated by two independent signals.

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Authors
Haass, C
Koo, EH
Capell, A
et al.

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Abstract. Progressive cerebral deposition of the amyloid (Aβ) β-protein is an early and invariant feature of Alzheimer's disease. Aβ is derived by proteolysis from the membrane-spanning β-amyloid precursor protein (βAPP). βAPP is processed into various secreted products, including soluble βAPP (APPs), the 4-kD Aβ peptide, and a related 3-kD peptide (p3). We analyzed the mechanisms regulating the polarized basolateral sorting of βAPP and its proteolytic derivatives in MDCK cells. Deletion of the last 32 amino acids (residues 664-695) of the βAPP cytoplasmic tail had no influence on either the constitutive ~90% level of basolateral sorting of surface βAPP, or the strong basolateral secretion of APPs, Aβ, and p3. However, deleting the last 42 amino acids (residues 654-695) or changing tyrosine 653 to alanine altered the distribution of cell surface βAPP so that 40-50% of the molecules were inserted apically. In parallel, Aβ was now secreted from both surfaces. Surprisingly, this change in surface βAPP had no influence on the basolateral secretion of APP, and p3. This result suggests that most βAPP molecules which give rise to APP, in MDCK cells are cleaved intracellularly before reaching the surface. Consistent with this conclusion, we readily detected intracellular APP, in carbonate extracts of isolated membrane vesicles. Moreover, ammonium chloride treatment resulted in the equal secretion of APP, into both compartments, as occurs with other non-membranous, basolaterally secreted proteins, but it did not influence the polarity of cell surface βAPP. These results demonstrate that in epithelial cells two independent mechanisms mediate the polarized trafficking of βAPP holoprotein and its major secreted derivative (APPs) and that Aβ peptides are derived in part from βAPP holoprotein targeted to the cell surface by a signal that includes tyrosine 653.

The β-amyloid precursor protein (βAPP) is a type I membrane-spanning glycoprotein which is ubiquitously expressed in mammalian cells. The cellular processing of βAPP has attracted great interest because progressive cerebral deposition of its ~40 residue amyloid β-protein (Aβ) fragment is an early and constant feature of Alzheimer's disease (AD). A central role for Aβ in the pathogenesis of AD is supported by the discovery of missense mutations within and immediately flanking the Aβ region of the βAPP gene in several families with autosomal dominant AD (AD; for review see Mullan and Crawford, 1993). Moreover, aggregated forms of Aβ 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). βAPP is encoded by a single gene on human chromosome 21 (Kang et al., 1987). Increased expression of βAPP in patients with trisomy 21 (Down's syndrome) may explain the invariant development of severe Aβ deposition and subsequent histopathology of AD in this disorder.

βAPP 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 βAPP includes at least two principal processing pathways: (a) cleavage within the Aβ region by an unidentified enzyme(s) called α-secretase (see Fig. 1 A) to release the large soluble ectodomain (APPs) (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 βAPP can serve as a substrate for α-secretase (Haass et al., 1992a; Roberts et al., 1994; Siso-
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 Aβ region, precluding release and deposition of intact Aβ. 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 βAPP 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 Aβ fragment is produced and released. The localization of the COOH terminus of Aβ in the hydrophobic transmembrane domain of βAPP predicted that a pathological alteration of membrane structure would be required to release this fragment. However, it has been shown that intact Aβ is constitutively secreted into the media of cultured cells under physiologic conditions, presumably after cleavage of βAPP by enzyme activities designated β- and γ-secretase (Fig. 1A; Bucsigliolo et al., 1993; Haass et al., 1992b; Shoji et al., 1992; for review see Haass and Selkoe, 1993). That such Aβ secretion also occurs normally in vivo has been proven by the identification of Aβ 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 (βAPP717) mutations increase production of long amyloid β protein. Neurobiol. Aging. 15:SS4).

In addition to Aβ, a related COOH-terminal fragment of molecular mass ~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 ~10-kD COOH-terminal fragment of βAPP that remains membrane bound after α-secretase cleavage (Haass et al., 1993, 1992b). Despite their secretion at nanomolar levels, Aβ 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 Aβ generation and the intracellular organelle(s) within which βAPP proteolysis occurs. Agents which inhibit the trafficking of βAPP through the Golgi (e.g., Brefeldin A) or alter intravesicular pH (e.g., ammonium chloride, chloroquine) block Aβ formation (Buxbaum et al., 1994; Haass et al., 1993; Koo and Squazzo, 1994; Shoji et al., 1992). Aβ cannot be detected within isolated lysosomes (Haass et al., 1993). Moreover, Aβ 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 Aβ is formed after βAPP matures through the Golgi and is cleaved at the NH2 terminus of the Aβ region in a non-lysosomal acidic compartment.

To understand the mechanism of βAPP trafficking and its relationship to Aβ production in greater detail, we examined βAPP 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 βAPP 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 βAPP undergoes highly polarized sorting, principally to the basolateral surface of MDCK cells, and that APPs, Aβ, 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), βAPP 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 Aβ in epithelial cells could prove directly relevant to the preferential localization of Aβ 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 βAPP cytoplasmic tail, using βAPP cDNA constructs containing a variety of deletions and substitutions. This analysis demonstrated two independent pathways mediating the polarized sorting of βAPP and its derivatives. One mechanism was dependent in part on cytoplasmic signals close to the membrane, including a critical tyrosine at residue 653 of βAPP653. This trafficking route regulates the localization of both surface-bound βAPP and, at least in part, secreted Aβ. The second mechanism appears to involve specialized signals within the ectodomain of βAPP. This mechanism directs βAPP 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 βAPP, help elucidate the mechanisms regulating βAPP trafficking and Aβ 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 filter chambers with pore sizes of 0.4 μm and 3 μm 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 βAPP653Y653A 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 μCi [35S]methionine per Transwell chamber for 15 min. Cells were washed 3× in DMEM, 10% FBS at 4°C, and chased for 0.5, 1, 4, 8, and 16h at 37°C. Media were collected and APP was immunoprecipitated.

Antibodies, Immunoprecipitation, and Gel Electrophoresis

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

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APPs, we used antibody 1736 (Haass et al., 1992b) raised to residues 595-611 (βAPP<sub>695</sub> numbering). The antibody was diluted 1:300. αβ and p3 were immunoprecipitated with antibody 1280 to βAPP 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 βAPP; Podlisny et al., 1991), antibody 1736, and antibody B5 (raised to amino acids 444-592 of βAPP 695; Ottersdorfer et al., 1990). The epitopes of all antibodies are shown in Fig. 1 A.

**Construction of Mutant βAPP cDNAs and Transfection**

All βAPP 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:

- CCTCAGATTACGTGCTGCTGC (Δ12 oligonucleotide)
- CCTCAGATTACGTGCTGCTGCTGCCTG (Δ22 oligonucleotide)
- CCTCAGATTACGTGCTGCTGCCTGCTGCC (Δ32 oligonucleotide)
- CCTCAGATTACGTGCTGCTGCCTGCTGCCCT (Δ42 oligonucleotide)

(underlined sequences = XbaI restriction site; bold letters = stop codon)

The following oligonucleotide was used as 5' primer:

GATGCGAATTCGCCAGAT (underlined sequence = EcoRI restriction site).

The PCR products were digested with EcoRI and XbaI and subcloned into EcoRI-SpeI-linearized CMV/βAPP<sub>695</sub> plasmid, generating the plasmids CMV/βAPP<sub>695</sub>A12, CMV/βAPP<sub>695</sub>A22, CMV/βAPP<sub>695</sub>A32, and CMV/βAPP<sub>695</sub>A42 (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):

ATGAA'IV_JGATGTGGCCTGTTTCTTCTT (Y653A oligonucleotide).

The Δ0.9-kb EcoRI-HindIII fragment of CMV/βAPP<sub>695</sub> was subcloned into M13mp18 and used as a single stranded template for mutagenesis. The Y653A oligonucleotide was used as a 5' primer, the 3' primer (see above), and a wild-type oligonucleotide (underlined sequences = XbaI restriction site; bold letters = stop codon).

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 APPs, 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 αβ generation upon butyrate treatment.

**Determination of the Cell Surface Distribution of βAPP in MDCK Cells**

The cell surface distribution of βAPP 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 βAPP 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 30,000 rpm (×233,000 g max) at 4°C in a 55 Ti rotor (Beckman Instruments, 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 in a Centricon 10 column, washed in STEN buffer (Weidemann et al., 1989) and again concentrated. The protein concentration of each fraction was determined 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 βAPP<sub>695</sub> 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 APPs 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 αβ Peptides**

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

**Results**

**The Surface Distribution of βAPP 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 side. To define sorting signals mediating the highly polarized surface expression of βAPP, we produced a set of βAPP mutants containing progressively larger deletions of the cytoplasmic tail (Fig. 1 B). MDCK cells were stably transfected with cDNAs encoding wild-type or truncated βAPP molecules, grown on polycarbonate filters in Transwell chambers, and then the cell surface distribution of βAPP was determined by an antibody-binding assay using a monoclonal antibody to the βAPP ectodomain (Haass et al., 1994a). MDCK cells expressing wild-type βAPP<sub>695</sub> (βAPP<sub>695</sub>) or βAPP<sub>695</sub> molecules missing the last 12 (βAPP<sub>695Δ12</sub>), 22 (βAPP<sub>695Δ22</sub>), or 32 (βAPP<sub>695Δ32</sub>) residues did not exhibit significant differences in the surface distribution of βAPP; 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 βAPP. However, deleting almost the entire cytoplasmic tail up to tyr653 (βAPP<sub>695Δ42</sub>; Fig. 1 B) resulted in a ~60:40 basolateral:apical distribution of βAPP (Fig. 2). This result suggested that a cytoplasmic signal sequence close to the transmembrane domain influences the cell surface distribution of βAPP. Since the βAPP<sub>695Δ42</sub> 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 distribution.
expression of βAPP (Fig. 2). To exclude the possibility that two partially redundant signals (Matter et al., 1992) within the cytoplasmic tail of βAPP 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 (~APP695Y653A A12). Determination of the cell surface expression of βAPP 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 βAPP in MDCK cells.

**Basolateral Secretion of APPs Occurs Independently of the Cytoplasmic Tail**

The above experiments examined only surface-bound holo-βAPP. To analyze the polarized release of the major secreted product, APPs, we metabolically labeled MDCK cells expressing various cDNA constructs, collected the media from both chambers and detected APP, by immunoprecipitation with antibody 1736 (Fig. 1 A), specific for α-secretase cleaved APP, (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 basolateral secretion of APPs. Even deleting almost the entire cytoplasmic tail (Δ42) or expressing the βAPP695Y653A or βAPP695Y653A Δ12 cDNAs still resulted in efficient basolateral secretion of APPs. These data indicate that the polarized secretion of APPs is independent of virtually the entire cytoplasmic sequence of βAPP 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 βAPP but not APPs polarity observed in the mutant-expressing cells is due to a change in the kinetics of APPs secretion into the apical and basolateral compartments, we performed pulse chase experiments using the MDCK cell line stably transfected with the βAPP695Y653A cDNA. During a cold chase, APP, molecules derived from the mutated cDNA construct and from the wild-type endogenous βAPP gene were secreted with very similar kinetics (Fig. 4). APPs, 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 APPs in the apical compartment occurs (Fig. 4). This result makes it unlikely that this mutation interferes with the kinetics of APPs secretion.

To confirm the hypothesis that polarized secretion of APPs involves sorting signals in the ectodomain rather than in the transmembrane or cytoplasmic domains, we examined secretion from a truncated βAPP cDNA construct (βAPP695/ S) that bears a stop codon at the α-secretase cleavage site (Hungh 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, was recovered from both chambers (Fig. 5, A and B). However, ~70% of total secreted APP, was still released into the basolateral chamber, clearly demonstrating that APP, 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 methionine99 of...
endog. 695 A12 A22 A32 A42 Y653A Y653A

Figure 3. Polarized secretion of APPs. (A) MDCK cells stably transfected with βAPP695, βAPP695Δ12, βAPP695Δ22, βAPP695Δ32, βAPP695Δ42, βAPP695ΔY653A, or βAPP695ΔY653A Δ12 were metabolically labeled with [35S]methionine. APPs was immunoprecipitated from the apical (A) and basolateral (B) chambers with antibody 1736. The upper band (751 APPs) represents endogenous APPs known to be secreted into the basolateral compartment (Haass et al., 1994a). The lower band corresponds to APPs derived from the transfected βAPP695 constructs. (B) Quantitation of the immunoprecipitations shown in A. Data are expressed as % of total secreted APPs released into the apical (A) or basolateral (B) compartment ± standard errors of the means (SEM); n = 6–12.

βAPP695 resulting in the preferential basolateral secretion of a truncated form of APPs (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 APPs Is Sensitive to Ammonium Chloride

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

Figure 4. Pulse chase experiment with a MDCK cell line stably expressing the βAPP695Y653A 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 APPs occurs within the apical compartment.

Figure 5. MDCK cells expressing the cDNA construct βAPP695-695/S secrete ~70% of their APPs into the basolateral compartment. (A) Immunoprecipitation of conditioned media with antibody 1736. Note that the endogenous APPs, 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 βAPP reaches the surface. However, it is important to note that full-length βAPP inserted at the basolateral surface also contributes to the pool of APP, because surface biotinylated βAPP is subsequently recovered as biotinylated APP, 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 βAPP₆⁹⁵ with a low dose of NH₄Cl did, in fact, equalize secretion of APP, (Fig. 6, A and B). This treatment did not alter the tight junctions, as shown by the exclusion of the radioactive tracer [³²P]inulin, nor did it change detectably the polarized phenotype of these cells, as shown by the preferential basolateral uptake of [⁴⁰S]methionine (Fig. 6 B). The distribution of surface βAPP 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 βAPP and of APP. Moreover, the selective NH₄Cl sensitivity of the polarized secretion of APP, strongly suggests a sorting mechanism capable of acting on soluble, non-membrane–bound forms of βAPP.

Detection of Intravesicular APP in MDCK Cells

Because the above data suggest the existence of soluble APP, molecules within intracellular vesicles, we prepared a PNS of MDCK cells stably transfected with βAPP₆⁹⁵. The PNS was centrifuged at 233,000 g (max) to pellet microsomal cell 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 bands corresponding to N- and N-plus O-glycosylated full-body B5 but not antibody C'/ (Fig. 7 A). However, the carbonate-supernatant also contained substantial amounts of a slightly smaller βAPP species reacting selectively with antibody B5 but not antibody C7 (Fig. 7 A, arrowhead) and migrating at the position expected for APP. To further confirm that the βAPP molecules detected by antibody B5 represent authentic APP, cleaved at the α-secretase site and do not correspond to random lysosomal degradation products of full-length βAPP, the same protein fractions were immunoblotted with antibody 1736 (Fig. 7 B). The latter finding again demonstrates that in MDCK cells two independent mechanisms determine the sorting of surface βAPP and of APP. Moreover, the selective NH₄Cl sensitivity of the polarized secretion of APP, strongly suggests a sorting mechanism capable of acting on soluble, non-membrane–bound forms of βAPP.

Figure 6. Polarized secretion of APP, is NH₄Cl sensitive. (A) Addition of 10 mM NH₄Cl during a 4-h metabolic-labeling period results in equal secretion of APP into both compartments. APP, was immunoprecipitated with antibody 1736. A, apical; B, basolateral. (B) Quantitation of the immunoprecipitations shown in A (APP, Secretion; third box). NH₄Cl has no effect on the polarized distribution of cell surface βAPP (Cell Surface APP) and no influence on the formation of tight junctions ([³²P]Met Uptake). 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.

Because the APP, protein in the carbonate supernatants is soluble, does not contain the C7 epitope, migrates with the appropriate M, in SDS-PAGE, and specifically reacts with antibody 1736, it very likely represents intracellular APP, created by α-secretase, as described previously by others (De Strooper et al., 1993; Kuentzel et al., 1993; Sambamurthi et al., 1992). It is unlikely that these APP, molecules were generated during the isolation procedure, since APP, 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
APP, by cultured cells occurs under these culture conditions (Haass et al., 1992a). The present results, therefore, strongly support the hypothesis that APP, can be generated within intracellular vesicles. Taken together, our observations suggest that intracellularly cleaved APP, and full-length βAPP are sorted for secretion and cell surface insertion, respectively, through two independent pathways.

Effects of Cytoplasmic Deletions on Polarized Secretion of Aβ

Having obtained evidence of distinct mechanisms for the polarized sorting of surface βAPP and APP, we next analyzed the polarity of Aβ and p3 peptides in MDCK cell lines expressing the mutant βAPP molecules described above. Deleting the last 12 amino acids of the cytoplasmic tail of βAPP (Fig. 1 B) had no effect on the release of Aβ 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 (βAPPΔ642; Fig. 1 B) resulted in an increased secretion of Aβ 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 Aβ and p3 secreted from cells expressing the βAPPΔ653A cDNA (Fig. 8). We conclude from these data that a tyrosine-containing sequence motif close to the transmembrane domain of βAPP mediates in part the polarity of Aβ secretion. These results correlate with the expression of the mutant βAPP molecules.

Figure 7. (A) Detection of APP, within cell lysates. Equal amounts (50 µg) 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 βAPP. Proteins were separated on 7% SDS-polyacrylamide gels. Arrowheads indicate the intravesicular APP. Arrows indicate membrane-bound immature (N'-glycosylated) and mature (N'/O' glycosylated) βAPP. The upper band identified by antibody 1736 represents endogenous 751 APP.
Figure 8. Polarized secretion of Aβ and p3. Aβ and p3 derived from cells expressing βAPPsos, βAPPsosΔ12, βAPPsosΔ22, βAPPsosΔ32, βAPPsosΔ42, and βAPPsosY653A during a 12-h metabolic-labeling period are predominantly detected in the basolateral compartment. Although cells expressing βAPPsosΔ42 and βAPPsosY653A still target Aβ into the basolateral compartment, Aβ can also be readily detected within the apical compartment. Note the relative increase of Aβ 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 βAPP 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 Aβ into the apical compartment is not as marked as the observed redistribution of surface βAPP.

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 Aβ species. We showed previously that this characteristic 4-kD Aβ doublet produced by MDCK cells is composed of peptides beginning predominantly at Aβ 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 βAPP, we radiosequenced the peptides in the doublet species derived from the βAPP695-Δ42 transfected cell line. We found an elimination of the normal radioactive phenylalanine peaks at sequencing cycles 4, 19, and 20, indicating an absence of Aβ species beginning at Asp 1. In this regard, it is important to note that Aβ purified from amyloid deposits in AD brain begins predominantly at Asp 1 (Roher et al., 1993). Moreover, it should be noted that alternative Aβ peptides could be generated within different subcellular compartments than typical Aβ starting at aparatate 1. Therefore, the results in Fig. 8 may not reflect the sorting of typical Aβ but rather that of alternative Aβ-like peptides.

Discussion

βAPP is an attractive model protein for identifying sorting signals because, unlike transmembrane proteins studied to date (Rodriguez-Boulan 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 Aβ 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 Aβ. Analyzing the sorting of wild-type and mutant βAPP 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 βAPP in MDCK cells involving two populations of βAPP molecules and two independent mechanisms (Fig. 9). Surface-bound βAPP is sorted via a pathway that is dependent, at least in part, on a tyrosine-containing signal at 653 within the cytoplasmic domain of βAPP that is distinct from the putative internalization signal at residues 684-687. However, the majority of secreted APP, molecules is sorted largely independently of signals in the βAPP 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 βAPP trafficking, because MDCK cells transfected with the non-membrane-inserted 695/S truncation construct lose some of the preferential basolateral sorting of APP.
A Dual Trafficking Mechanism Regulates Sorting of Cell Surface βAPP/αβ and βAPP/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 (LGP 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 LGP 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 Giersch, 1991; Eberle et al., 1991).

We describe here the unexpected finding that surface-bound βAPP molecules can be targeted in part to the apical cell surface by deleting the cytoplasmic tail of βAPP 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 βAPP and these other cell surface proteins may lie in the fact that the βAPP 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 βAPP. 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 NH2-Cl-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 βAPP substrate to work efficiently, because MDCK cells expressing the soluble βAPP695/S molecule missort ~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 NH2-Cl sensitive compartment (Fig. 9). Our MDCK studies also demonstrate for the first time that βAPP 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 βAPP on the apical cell surface would be expected, something which is clearly not the case.

Implications for the Cellular Mechanism of Aβ 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 Aβ peptides (in particular, the Aβ 1 species), presumably by altering the internalization signal. This finding is consistent with a proposed mechanism of Aβ generation that involves the endocytosis of cell surface βAPP (Koo and Squazzo, 1994; Haass, C., D. B. Teplow, and D. J. Selkoe, manuscript in preparation). However, because production of Aβ and localization of cell surface βAPP do not coincide precisely, it is possible that additional cleavages resulting in Aβ 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 βAPP 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 Aβ are generated within different cellular compartments and that precise analysis of Aβ polarity may therefore be difficult, as cytoplasmic deletions seem to lead to a selective inhibition of secretion of the Aβ 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. Craessenaerts, I. Dewachter, D. Mocchhs, B. Greenberg, F. Van Leuven, and H. Van Den Bergh. 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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