Exchanging the Extracellular Domain of Amyloid Precursor Protein for Horseradish Peroxidase Does Not Interfere with \( \alpha \)-Secretase Cleavage of the \( \beta \)-Amyloid Region, but Randomizes Secretion in Madin-Darby Canine Kidney Cells*

(Received for publication, July 6, 1995, and in revised form, September 8, 1995)

Bart De Strooper, Katleen Craessaerts, Fred Van Leuven,§ and Herman Van Den Berghe

From the Experimental Genetics Group, Center for Human Genetics, Campus Gasthuisberg O & N, KULeuven, B-3000 Leuven, Belgium

Secretory processing and polarized sorting of horseradish peroxidase fused to the amyloid precursor protein transmembrane domain were compared with those of wild-type amyloid precursor protein in COS and polarized Madin-Darby canine kidney (MDCK) cells. The cellular and secreted forms of the chimeric protein were enzymatically active in colorimetric and cytochemical assays after reconstitution with heme and Ca\(^{2+}\). The peroxidase enzyme was secreted by a proteolytic process, similar to the parent amyloid precursor protein. In polarized MDCK cells, amyloid precursor protein was secreted exclusively in the basolateral compartment, while the peroxidase chimeric protein was secreted in both compartments. The basolateral sorting determinant for secretion must therefore be located in the extracellular domain of amyloid precursor protein. On the other hand, cell surface-associated peroxidase chimeric protein was similar to cell surface-associated wild-type amyloid precursor protein, mainly expressed at the basolateral side. The basolateral cell-surface expression, in contrast to the basolateral secretion, is therefore controlled by determinants in the cytoplasmic domain. Methylamine inhibited and bafilomycin slightly increased the basolateral secretion of both proteins, but both drugs strongly increased apical secretion. The default secretory pathway of COS cells and the basolateral (but not the apical) secretory pathway of MDCK cells are therefore comparable sensitive to methylamine and not to bafilomycin.

Amyloid precursor protein (APP)\(^1\) is the precursor of the \( \beta \)-amyloid (A\(_4\)) peptide, a 4-kDa protein that precipitates in the amyloid plaques in the brains of Alzheimer’s patients. The metabolism of the protein is complex and occurs along several possible pathways, all involving proteolytic cleavages of the precursor protein (reviewed in Ref. 1). The normal secretory processing of APP takes place in a late compartment of the default secretory pathway (2–4) by an \( \alpha \)-secretase cleavage in the \( \beta \)-amyloid domain of APP (5–8). This cleavage step yields the soluble extracellular domain of APP (APP\(_s\)) and precludes further amyloid formation. APP that escapes from this activity is transported to the cell surface and endocytosed (9). Alternative proteolytic cleavages, possibly in the endosomes, release the \( \beta \)-amyloid peptide and/or the p3-peptide (1). These fragments are secreted with APP, by all cell types studied until now. Interestingly, amyloid lesions in the brains of Alzheimer’s patients are always associated with polarized cell types, e.g. neurons and endothelial cells. This raises the question whether APP and its proteolytic products are transported and secreted in a polarized way, i.e. in neurons to the axons or dendrites and in epithelial cells to the apical or basolateral side. It was recently demonstrated that in primary cultures of hippocampal neurons, APP is transported first to the axons and later to the dendrites (10, 11). In polarized epithelial Madin-Darby canine kidney (MDCK) cells, APP and its various proteolytic products (APP\(_s\), \( \beta \)-A\(_4\), and p3) are strictly sorted to the basolateral side (12–15). It is therefore clear that APP is transported in a polarized way in polarized cells. While the study of APP sorting in hippocampal neurons is obviously more relevant to Alzheimer’s disease, the two functionally distinct domains of axons and dendrites cannot be independently studied or manipulated in vitro, which hampers the biochemical analysis of this model. In contrast, MDCK cells provide a powerful in vitro system to study polarized membrane trafficking of APP. They form a tight monolayer on tissue culture filters in vitro, which results in a complete physical separation of an upper (apical) and lower (basolateral) compartment. This allows us to label and analyze APP separately on the apical versus basolateral surface of the cells and in addition to follow the progressive accumulation of secreted APP, and \( \beta \)-A\(_4\)- and p3-peptides in the two corresponding compartments. Moreover, since APP is endogenously expressed by these cells, as kidney cells do in vivo, the study of this system is physiologically relevant.

Mutational analysis of the tyrosines in the cytoplasmic domain of APP has demonstrated one tyrosine residue (Tyr-653) to be critical for the basolateral surface sorting of the protein (13). Remarkably, the ectodomain of APP continued to be secreted into the basolateral compartment independently of the sorting of the integral membrane form (13, 15). This suggested two possibilities: either \( \alpha \)-secretase is only present in the basolateral pathway, or an independent basolateral sorting signal in the ectodomain of APP is responsible for its basolateral delivery. Both alternatives are not necessarily exclusive: the possible relationship between \( \alpha \)-secretase processing of APP and sorting remains to be further explored. Given that the

---

\* This work was supported by Grants 3.0069.89 and 3.0073.93 from the “Fonds voor Geneeskundig Wetenschappelijk Onderzoek,” by European Community Contracts BIOT-CT94-2065 and COF 94/12, and by a grant from the Inter-university Network for Fundamental Research (1991–1996). Part of this work was supported by Contract ETC-008 from the Action Program for Biotechnology of the Flemish Government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Postdoctoral Fellow of the “Naatonaal Fonds voor Wetenschappelijk Onderzoek.”

‡ To whom correspondence should be addressed. Tel.: 32-16-345862; Fax: 32-16-345871; E-mail: fredve@cc3.kuleuven.ac.be.

1 The abbreviations used are: APP, amyloid precursor protein; APP\(_s\), soluble APP; MDCK, Madin-Darby canine kidney; HRP, horseradish peroxidase.
aerial acid sequence specificity of α-secretase at the cleavage site in APP is low (3, 8, 16, 17), the question is raised how other integral membrane proteins in the default secretory pathway escape this enzymatic activity. Specific targeting of APP by undefined sorting signals to a "secretase compartment" (3) or specific binding of the secretase to a region of APP outside the βA4-amyloid peptide sequence, lending specificity to the cleavage process, are two possibilities that remain to be further explored.

The potential role of the cytoplasmic sorting determinants for polarized secretion and α-secretase processing of APP was further studied here with a reporter molecule containing the cytoplasmic domain of APP and the minimal structural information needed for α-secretase processing. Horseradish peroxidase (HRP) was chosen as a reporter protein because its enzymatic activity permits considerable signal amplification, and the end product can be visualized using either light or electron microscopy (18). We demonstrate that a membrane-bound chimeric protein of horseradish peroxidase fused to the transmembrane and cytoplasmic sequences of wild-type APP (HRP/βA4/TM/CD) is enzymatically active both in the culture medium and in fixed cells in situ. We show that the chimeric protein is processed by α-secretase, similar to wild-type APP. In polarized MDCK cells, however, the secretion of HRP/βA4/TM/CD, in contrast to the secretion of APP, is randomized, providing direct evidence that the basolateral sorting determinant for polarized secretion of APP resides in its extracellular domain. HRP/βA4/TM/CD that escaped α-secretase activity, on the other hand, is targeted to the basolateral surface, indicating that the cytoplasmic basolateral sorting determinants act independently of the extracellular domain of APP. Finally, we investigated the effect of alkalizing agents on α-secretase processing and sorting of HRP/βA4/TM/CD, demonstrating that the default secretory pathway in unpolarized COS cells and the basolateral pathway in MDCK cells (but not the apical pathway) are similarly sensitive to methylamine and not to bafilomycin as far as processing of APP and HRP/βA4/TM/CD is concerned.

MATERIALS AND METHODS

The extracellular domain of APP was replaced by horseradish peroxidase in a recombinant DNA construct (see Fig. 1). A cassette containing HindIII and PstI sites was cloned into the unique AspI site of the cDNA coding for the βA4-amyloid region and the transmembrane and cytoplasmic domains of murine APP695 (APP695(M)) (3) in the eukaryotic expression vector pSG5 (Stratagene). The HindIII/PstI fragment of BBG10 horseradish peroxidase (British Biotechnology Products Ltd., Abingdon, United Kingdom) engineered to mammalian codon usage (20) was cloned into the cassette. The resulting cDNA codes for a HRP/βA4/TM/CD fusion protein (see Fig. 1). The two small linker sequences needed to fuse the APP and HRP proteins are also displayed (see Fig. 1). In HRP/βA4/TM/CD, part of the cytoplasmic domain including the GY.NPTY sequence was deleted by introducing a stop codon at Ala-666 in APP695(M) (3).

The mouse APP695(M)/Y563* mutant, in which the complete cytoplasmic domain is deleted (see Fig. 1), was generated by site-directed mutagenesis in the pSG5 plasmid (21), mutating the codon for Tyr-653 toward a TAG stop codon, APP695(M)A626*, which terminates at the transmembrane domain, was made by introducing a cassette in the BbsI restriction site of APP695(M), changing codon 626 to a TAG stop codon. All constructs were analyzed by restriction analysis and by sequencing of the mutated sites (15). The stop codons were functionally active since carboxy-terminal antibodies did not react with the mutated proteins when expressed in COS-1 cells.

Transfection of pSG5 plasmids in COS-1 cells with DEAE-dextran has been described (3). For cytochemistry, COS-1 cells (150,000/slide) were seeded into chamber glass slides (Lab-Tek, Nunc) and transfected in situ the next day. After 2 days in culture, slides were washed with phosphate-buffered saline, fixed and permeabilized for 10 min in acetone, incubated for 60 min with Tris-buffered saline containing 1 mM Ca2+ and 5 μM hemin (Sigma), and stained for 15 min with 0.5 mg/ml diaminobenzidine in 50 mM Tris-HCl (pH 7.4) and 0.01% (w/v) H2O2.

Secreted apoperoxidase in cell culture medium was reconstituted with hemin (5 μM) in Tris-buffered saline and 1 mM Ca2+ (30 min, room temperature), and peroxidase activity was assessed spectrophotometrically at 450 nm by measuring the rate of conversion of 1,2-phenylenediamine. A standard curve was obtained by making dilutions from a 250 μM stock solution of apoperoxidase C (Sigma) in Tris-buffered saline containing 1 mM Ca2+ and 5 μM hemin. Results were expressed relative to arbitrary units.

RESULTS

The cDNA sequence coding for the extracellular domain of APP was replaced by cDNA coding for horseradish peroxidase (mammalian codon usage). In the resulting protein, the HRP enzyme is fused to the βA4-amyloid region and the transmembrane and cytoplasmic domains of APP (Fig. 1). The fusion protein (HRP/βA4/TM/CD) was expressed in COS-1 cells. In fixed and permeabilized cells, the enzymatic activity of the chimeric protein was detected by diaminobenzidine staining. Transfected COS cells or cells transfected with wild-type APP did not stain, demonstrating the specificity of the staining reaction. In different experiments, 15–30% of the cells stained positively, which approximates the expected transfection efficiency of this procedure (Fig. 2). The results demonstrate that HRP/βA4/TM/CD is functionally active and can be detected in situ. Staining was mainly concentrated in the Golgi region, in accordance with previously published studies using immunocytochemical detection of wild-type APP (24).

Secreted HRP activity in the culture medium increased exponentially with time. These kinetics were identical to those previously observed for the secretion of wild-type APP (3). Deletion of the cytoplasmic domain by introducing a stop codon at Ala-666 (numbering as in APP695) doubled the rate of secretion very similar to truncated wild-type APP (3). The doubling is not caused by differences in transfection or translation efficiency as assessed in parallel pulse-chase experiments, which demonstrated that both constructs yield equal amounts of APP precursor under the conditions used.

Methylamine strongly inhibited the secretion of HRP in a concentration-dependent way (Fig. 3 and Table I), while phorbol esters stimulated secretion of both "wild-type" and "truncated" HRP/βA4/TM/CD proteins (3, 22–29). Other known inhibitors of the secretory pathway such as monensin and
brefeldin A also strongly inhibited HRP/βA4/TM/CD secretion (Table I). Secreted HRP/βA4/TM/CD reacted with antibody R47 (6), which is directed against amino acids 1-16 of the βA4-amyloid peptide (data not shown). No reaction with antibodies against the cytoplasmic domain of APP (15) or with mononcloal antibody 4G8 against amino acids 17–24 of the βA4-amyloid peptide (30) could be demonstrated, providing further support to the conclusion that HRP release reflects authentic α-secretase processing.

The importance of the integral membrane domain for α-secretase processing of APP was further confirmed by investigating the proteolytic processing of deletion mutants of APP (Fig. 1). Deletion of the entire cytoplasmic domain of APP (APP695(M)Y653*) does not interfere with its normal secretion (Fig. 4A). Deletion of the cytoplasmic and transmembrane domains of APP (APP695(M)A626*) yields a soluble protein (Fig. 4B). However, Western blotting of the secreted products of the different constructs with monoclonal antibody 4G8 (30) revealed a strong signal only for APP695(M)A626* (Fig. 4B), demonstrating that this soluble APP mutant is not proteolytically processed.

HRP/βA4/TM/CD was stably expressed in MDCK cells using the Rous sarcoma virus promoter (15), and its polarized distribution was investigated using selective cell-surface biotinylation (Fig. 5A). More than 90% of the surface-expressed HRP/βA4/TM/CD was located at the basolateral side of the MDCK cells, which is similar to wild-type APP (13). In contrast, 41% of the total pool of secreted HRP/βA4/TM/CD was present in the apical compartment and 59% in the basolateral compartment, indicating that HRP/βA4/TM/CD is secreted essentially randomized (Fig. 5B). Wild-type (endogenous) APP was detected only in the basolateral compartment (12, 15).
We compared the effects of bafilomycin, a specific vacuolar proton pump inhibitor, and methylamine, a drug that accumulates in and alkalizes acid organelles, on the secretory processing and sorting of HRP/β4A/TM/CD in COS and MDCK cells. In COS cells, HRP/β4A/TM/CD secretion was somewhat increased by 100 nM bafilomycin, while secretion was strongly inhibited by 30 mM methylamine (Table I). In MDCK cells, the same effect was observed, but only for the basolateral pathway: HRP/β4A/TM/CD secretion was somewhat increased by bafilomycin, but was strongly inhibited by methylamine (Fig. 5C). Remarkably, a 4–5-fold increase in apical secretion was observed with both drugs (Fig. 5C).

**DISCUSSION**

This work, combined with previously observations (12–15), demonstrates unequivocally that two separate sorting mechanisms are responsible for the basolateral targeting of APP in polarized MDCK cells. The properties of these two mechanisms are summarized in Table I. One mechanism is responsible for the basolateral secretion of soluble APPβ and interacts with unknown determinant(s) that must be located in the extracellular domain and are thus deleted in the HRP/β4A/TM/CD chimeric protein, as demonstrated by the randomized secretion in the apical and basolateral compartments. More important, since the chimeric protein used in this study contained the entire β4A-amyloid peptide sequence, it follows that the β4A-amyloid region is not involved in the basolateral targeting of APPβ. The second mechanism is responsible for the polarized basolateral cell-surface expression of APP. This mechanism must act on the cytoplasmic domain of APP and directs HRP/β4A/TM/CD that escaped α-secretase cleavage to the basolateral surface (Table I). The results complement perfectly previous observations that demonstrated that deletions or mutations of the cytoplasmic domain of APP randomized its cell-surface distribution, but did not affect its polarized secretion (13, 15).

Although the sorting information in both domains works synergistically when intact APP is concerned, the mechanisms do act independently from each other (Refs. 13 and 15 and this work). While no precedent exists for a protein with a similar sorting behavior, the conservation of the sequences in the cytoplasmic domain of APP (13, 15) strongly suggests that the mechanism responsible for its basolateral cell-surface expression is identical to that operative on the low density lipoprotein receptor (31). Since membrane-bound and soluble HRP/β4A/TM/CD proteins are sorted differently, the present data further demonstrate that α-secretase is active in MDCK cells before the last sorting station, the basolateral endosome, is reached (3, 13, 15).

We have previously shown that pH and primary amines have profound differential effects on the α-secretase processing and targeting of APP, implying that metabolic disturbances can change the fate of APP (3, 15). Primary amines accumulate in acid compartments of the cell, resulting in their alkalization, and interfere with normal functions such as degradation in the lysosomes or receptor-ligand dissociation in the endosomes. In addition, methylamine inhibits the processing of proteins in the late compartments of the default secretory pathway in certain cell types (32, 33). The randomized secretion of APP from MDCK cells after treatment with alkalizing drugs (15) is most likely caused by the disruption of its interaction with the proposed basolateral sorting mechanism for soluble APPβ. A similar pH sensitivity of the polarized secretion of laminin and basal membrane proteoglycan has been demonstrated (34), as summarized in Table II. Methylamine, but not other alkalizing drugs, inhibited the secretion of APP in unpolarized COS cells (3, 35). In polarized MDCK cells, methylamine also inhibited APP secretion, but only in the basolateral compartment, and not in the apical compartment (15). Since bafilomycin and concanamycin, both specific inhibitors of the vacuolar proton ATPase (36), did not inhibit, but augmented secretion of the peroxidase chimeric protein from COS cells, the inhibition by methylamine of both HRP/β4A/TM/CD and APP secretion in COS cells cannot be caused by alkalization of the acidic compartment that is sensitive to bafilomycin. Alternatively, methylamine could block or divert the transport of HRP/β4A/TM/CD and APP and thereby prevent the normal processing in the α-secretase compartment (3). HRP/β4A/TM/CD secretion from COS cells is inhibited by methylamine to the same extent as by brefeldin A and monensin, drugs that are known to inhibit transport along the default secretory pathway. Surprisingly, in MDCK cells, methylamine blocks only the basolateral secretion of APP and HRP/β4A/TM/CD. Clearly, the methylamine-sensitive step is operative in the default secretory pathway of COS...
cells and in the basolateral (but not in the apical) pathway of MDCK cells. This adds more evidence to the emerging concept that different mechanisms are responsible for the apical and basolateral transport of proteins in MDCK cells (37, 38). Finally, the increase in apical secretion induced by methylamine (295), however, remains to be investigated.

Only recently was soluble HRP expressed in an active form in eukaryotic cells (19). We demonstrate here that HRP is enzymatically active when expressed as a chimeric integral membrane protein, which opens the possibility of using HRP as a marker in a variety of protein transport assays. Compared with alkaline phosphatase (39, 40), horseradish peroxidase offers several advantages as a reporter molecule (19). We anticipate using the HRP/j4A/4M/CD fusion protein to analyze the intracellular pathways as well as to screen for candidate a-secretases in a cellular environment (41). This is possible because we demonstrate here that the extracellular domain of APP, although implicated in the normal transport and targeting of APP, is not needed for normal a-secretase processing. This, together with the results obtained with the APP deletion mutants, demonstrated that the essential information for a-secretase processing is completely contained in the j4A-amyloid and transmembrane sequences, which constitute ~8% of APP (and HRP/j4A/4M/CD). The molecular explanation of the paradox that the nonspecific a-secretase is located in the default secretory pathway, but does not cleave all integral membrane proteins it encounters, must therefore be found in its interaction with and, hence, the structure of the integral membrane domain of APP (8). Another integral membrane protein, transforming growth factor a, is processed by a similar secretase mechanism, characterized by low sequence specificity and activated by protein kinase C (42). The cytoplasmic domain of transforming growth factor a, however, is absolutely needed for proteolytic cleavage, and replacement of the terminal valine in this domain prevents processing (43). In the case of APP and HRP/j4A/4M/CD, the cytoplasmic domain is not needed at all, indicating that at least two different “adapter” mechanisms are operating to permit transforming growth factor a and APP to interact with the putative nonspecific a-secretase proteolytic complex. This also implies that many other proteins could be processed by the same mechanism provided a suitable adapter protein is present.

Acknowledgments—We thank H. Peter (Ciba-Geigy, Basel, Switzerland) for generously providing bafilomycin and concanamycin and R. Robakis for antibodies.

REFERENCES
1. Haass, C., and Selkoe, D. (1993) Cell 75, 1039–1042
2. Sambamurti, K., Shioli, J., Anderson, J. P., Pappolla, M. A., and Robakis, N. (1992) J. Neurosci. Res. 33, 319–329
3. De Strooper, B., Umanz, L., Van Leuven, F., and Van Den Berghe, H. (1993) J. Cell Biol. 121, 295–304
4. Kuentzel, S., Ali, S., Altmann, R., Greenberg, B., and Raub, T. (1993) Biochem. J. 295, 367–378
5. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oldendorf, T., McClure, D., and Ward, P. J. (1990) Science 248, 1122–1124
6. Anderson, J. P., Esch, F. S., Keim, P. S., Sambamurti, K., Lieberburg, I., and Robakis, N. K. (1991) Neurosci. Lett. 128, 126–128
7. Wang, R., Meschia, J. F., Cotter, R. J., and Sisodia, S. S. (1991) J. Biol. Chem. 266, 16960–16964
8. Sisodia, S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6075–6079
9. Nordstedt, C., Caparosa, G. L., Thyberg, J., Gandy, S. E., and Greengard, P. (1993) J. Biol. Chem. 268, 609–614
10. Sacco, M., Ikonen, E., Tietz, N., and Simons, K. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5989–5993
11. Sisodia, S. S. (1993) Trends Neurosci. 16, 185–188
Exchanging the Extracellular Domain of Amyloid Precursor Protein for Horseradish Peroxidase Does Not Interfere with $\alpha$-Secretase Cleavage of the $\beta$-Amyloid Region, but Randomizes Secretion in Madin-Darby Canine Kidney Cells

Bart De Strooper, Katrien Craessaerts, Fred Van Leuven and Herman Van Den Berghe

*J. Biol. Chem.* 1995, 270:30310-30314.
doi: 10.1074/jbc.270.51.30310

Access the most updated version of this article at [http://www.jbc.org/content/270/51/30310](http://www.jbc.org/content/270/51/30310)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/270/51/30310.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 43 references, 19 of which can be accessed free at [http://www.jbc.org/content/270/51/30310.full.html#ref-list-1](http://www.jbc.org/content/270/51/30310.full.html#ref-list-1)