A novel mechanism for the regulation of amyloid precursor protein metabolism

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Introduction

Alzheimer’s disease (AD)* is the most prevalent cause of dementia in the elderly. AD is in part characterized pathologically by the presence of extracellular plaques consisting of deposits of the amyloid β-peptides (Aβs) derived from β-amyloid precursor protein (APP). APP is cleaved by three proteolytic activities, α-, β-, and γ-secretases (Mills and Reiner, 1999; De Strooper and Annaert, 2000). α-Secretase cleaves APP within the Aβ peptide sequence, whereas β-secretase cleaves APP at the NH2-terminus of the Aβ peptide sequence. APPα and APPβ are the NH2-terminal fragments generated by α- and β-secretases, respectively, whereas the remaining fragments are cleaved at the COOH terminus of the Aβ peptide sequence by γ-secretase. The presence of mutant genes that encode presenilins (PSs) PS1 and PS2 have been linked to early onset familial Alzheimer’s disease (FAD) (Rogaev et al., 1995; Sherrington et al., 1995), and an increased deposition of Aβ in plaques has been associated with these mutations (Sherrington et al., 1995; Scheuner et al., 1996). There are several proteins that interact with the PSs (Van Gassen et al., 2000). Among these is the modifier of cell adhesion protein (MOCA) (Kashiwa et al., 2000; Chen et al., 2001). Although MOCA has 40% sequence homology with DOCK180 and contains SH3 and Crk binding domains, its function was unknown. Because MOCA binds PS1 and because PS1 has been associated with γ-secretase activities in APP processing (Wolfe and Haass, 2001), it was asked if MOCA is involved in the regulation of APP metabolism.

Results

MOCA reduces APP and Aβ secretion

To investigate the effect of MOCA on APP protein secretion, a rat nerve cell line called B103 (Schubert et al., 1974) was stably transfected with APP695 and again with a plasmid harboring the full-length MOCA cDNA. B103 cells normally express little or no APP (Schubert et al., 1989) and no MOCA. The expression of MOCA was detected in transfected clones of B103 (APP695/MOCA) at levels similar to or less than MOCA expressed in the mouse hippocampus (Fig. 1, A and D). Secreted proteins from the stably transfected cells containing either an empty vector or the MOCA gene were first stud-
ied by Western blotting using antibody 6E10, which recognizes APP and amino acid residues 1–17 of human Aβ (Kim et al., 1990). The release of secreted APP (APPs) was decreased dramatically in all independently isolated clones expressing MOCA relative to clones containing empty vector (Fig. 1A). To determine the specificity of the MOCA effect on APP secretion and eliminate the possibility of clonal variation, several control experiments were performed. First, a transcriptional change was ruled out because the abundance of APP mRNA was not altered by Northern blot and reverse transcription (RT)-PCR analysis (Fig. 1B). Second, transient transfection of MOCA into B103 (APP695) cells led to the reduction of APP secretion in an uncloned population (Fig. 1D). Third, the secretion of APP was not affected by overexpression of DOCK180, a protein which has a 40% homology with MOCA (Fig. 2D). Fourth, the secretion of endogenous APP751 was reduced in HEK293T cells expressing MOCA (Fig. 1D). Lastly, since APP is a type I integral membrane protein we asked if MOCA alters the expression or secretion (shedding) of six other proteins. The effect of MOCA expression on the secretion of another large extracellular protein, β-laminin, was examined; no difference was observed between cells transfected with APP and APP plus MOCA (Fig. 1A). Neural cell adhesion molecule (N-CAM) is involved in both homotypic and heterotypic cell–cell adhesions and expressed as several membrane-bound and secreted isoforms through an alternative splicing mechanism (Gower et al., 1988). No effect on the levels of intracellular N-CAM expression and/or N-CAM secretion was observed in B103 cells expressing MOCA (Fig. 1A). A single band with molecular weight ~130 kd was detected intracellularly, whereas the secreted N-CAM was aggregated into higher molecular weight complexes (>300 kd) as described by others (Tavella et al., 1994). Notch-1 is involved in crucial cell fate decisions during development and the processing of Notch is similar to γ-secretase–mediated cleavage of APP (Greenwald, 1998; De...
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Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). Fig. 1 C shows that the expression of (intracellular) full-length (mol wt/H11601 300 kd), and truncated transmembrane forms (mol wt/H11601 120 kd) of Notch are only slightly altered in B103 cells expressing MOCA. Finally, previous studies have shown that the processing or metabolism of amyloid precursor-like protein (APLP) and cadherins are regulated by PSs (Naruse et al., 1998; Marambaud et al., 2002). No difference in E-cadherin and APLP2 expression was observed, nor was there a decrease in APLP2 secretion. In contrast, the expression level of N-cadherin was significantly increased in MOCA-expressing B103 and 293T cells (Fig. 1 E). The above data show that MOCA expression leads to the reduced secretion of APP but not other type-1 membrane proteins.

Because PS1 is associated with γ-secretase cleavage of APP (Wolfe and Haass, 2001) and in situ hybridization shows that MOCA and PS1 are expressed in overlapping areas of the brain and are colocalized on cells expressing both proteins (Kashiwa et al., 2000), it was asked if the effect of MOCA on APP secretion is altered by PS1 expression or activity. The endogenous expression of PS1 was detected in B103 cells using an antibody specific to rat PS1 (Fig. 2 A) shows the abundant processed NH2-terminal fragments of PS1 (mol wt/H11601 19–21 kd). A very weak band with a mol wt/H11601 90 kd was also observed, which may be a complexed form of PS1 (unpublished data). To block PS1-related γ-secretase activities, several inhibitors, which are specific for γ-secretase, were used. All of these agents have been shown to decrease Aβ production with concomitant increases in the levels of the corresponding COOH-terminal fragments at the concentrations we used. γ-Secretase inhibitor II is a transition-state analogue, which selectively inhibits the γ-secretase cleavage of APP and Notch-1 (Wolfe et al., 1998, 1999; De Strooper et al., 1999; Berezovska et al., 2000). Several dipeptidyl aldehydes (γ-secretase inhibitor III [Z-LL-CHO], γ-secretase inhibitor IV [2-naphthoyl-VF-CHO], γ-secretase inhibitor V [Z-LF-CHO], and calpain inhibitor III [Z-VL-CHO, Calp III]) were also tested (Higaki et al., 1995; Figueiredo-Pereira et al., 1999; Sinha and Lieberburg, 1999). No effect of γ-secretase inhibitors II and V on APP secretion.
was found in B103 cells expressing MOCA. APP secretion was partially restored by γ-secretase inhibitors III and IV and by Calp III in B103 cells expressing MOCA and wild-type cells. As controls, MG132 and lactacystin dramatically affected APP secretion and intracellular expression (see the following). All of the secretase inhibitors were functional because they all increased the accumulation of COOH-terminal stubs (Fig. 2 B). There was much less accumulation of these molecules in MOCA-expressing cells, probably because of the limited substrate (APP) availability. Similar results were also observed in HEK293 cells stably transfected with MOCA (unpublished data). In addition, the effect of the overexpression of human PS1 on APP secretion was studied (Fig. 2 C). The full-length wild-type or a mutant PS1 gene was stably transfected into the B103 (APPsw/MOCA) cells, and APP, assayed. Fig. 2 C shows that APP, release was further reduced relative to cells expressing MOCA alone in four independently isolated clones, which coexpress MOCA and wild-type (WT) PS1 or the mutant (FAD) PS1 (L392V). The effect of MOCA on APP secretion is summarized in Fig. 2 D, which also shows human PS1 overexpression (Fig. 2 D, bottom).

To determine the effect of MOCA expression on Aβ peptide production, we transfected the MOCA gene into a B103 cell line, which contains the FAD Swedish form of APP (APPsw) and secretes Aβ peptides into the medium (Xu et al., 1999). The levels of APPsw secretion were significantly decreased in cells expressing MOCA (Fig. 3 A). The intracellular levels of APPsw were also decreased by MOCA (Fig. 3 B). Therefore, the effects of MOCA on APPsw secretion were similar to those on wild-type APP (Fig. 1). Aβ secretion into the culture medium was then measured by immunoprecipitation and radioautography (Fig. 3 C) and independently by the sandwich ELISA (Fig. 3 D). The level of Aβ in the medium derived from B103 (APPsw/MOCA) cells was considerably decreased compared with B103 (APPsw) cells (Fig. 3, C and D). We could not detect the intracellular expression of Aβ in any of the B103 (APPsw) or B103 (APPsw) cell lines using the techniques employed to assay extracellular Aβ. The above data show that MOCA decreases the secretion of both APP and Aβ.

**Reduced APP secretion is due to APP degradation**

The decreased secretion of APP caused by MOCA may be due to a blockage of the secretion pathway, which will cause an intracellular accumulation of APP, a decrease in its rate of synthesis, or an increase in the rate of APP degradation. To distinguish between these alternatives, we first examined the intracellular levels of APP. Full-length APP was reduced in B103 cells containing APPsw/MOCA relative to APPsw cells as defined by two antibodies, 6E10 and 22C11 (Fig. 4, A and B). In addition, the level of expression of the COOH-terminal fragment of APP was diminished in cells stably transfected with MOCA as determined by immunoprecipitation with the CT-15 antibody, which recognizes the last 15 COOH-terminal amino acid residues of APPsw (Fig. 4 C). These data rule out the possibility that MOCA expression is enhancing the intracellular accumulation of APP and suggest that the decreased APP secretion caused by MOCA expression is not due to a direct blockage of the secretion pathways. Because there is no increased intracellular accumulation of APP in cells expressing MOCA, we investigated the effect of MOCA on the turnover of APP by pulse–chase experiments. Antibodies 6E10 and CT-15 specifically immunoprecipitate the intracellular APP holoprotein, whereas antibodies GID and 22C11 performed less well (Fig. 5 A). 6E10 was used in...
the following experiments. B103 cells expressing either APP695/vector or APP695/MOCA were labeled for 10 min in [35S]methionine-containing media and chased for 15, 30, 45, and 60 min in “cold,” serum-free conditioned media. In control B103 (APP695/vector) cells, the APP protein level decreased gradually (Fig. 5 B). In contrast, the intracellular APP protein level in B103 (APP695/MOCA) cells rapidly decreased. The initial levels after the 10-min labeling period were also lower than controls in cells expressing MOCA, a result which would be predicted for nascent proteins can be very rapidly degraded after synthesis (Schubert et al., 2000). There was a temporal increase in the amount of APP protein in the medium of both cell types (Fig. 5 B), but the overall level of APP accumulation in the medium is decreased by the expression of MOCA (Figs. 1–3). Therefore, it is likely that MOCA facilitates the degradation of APP. Finally, the data in the following paragraphs rule out the remaining alternative that MOCA expression down-regulates APP synthesis, since in the presence of proteasome inhibitors the levels of extracellular APP are similar in cells expressing MOCA and controls.

Proteasomes mediate APP degradation

To independently verify the data on APP degradation, we studied the effect of protease inhibitors on the secretion and the intracellular accumulation of APP. Leupeptin inhibits lysosomal proteases, whereas MG132 inhibits membrane protein degradation through the ubiquitin-proteasome pathway (Soriano et al., 1999). Both APP secretion and the intracellular accumulation of APP were significantly increased in the APP695/MOCA cells by 10 μM MG132; no effect was observed in cells treated with 100 μM leupeptin (unpublished data). More strikingly, the secretion of APP in cells expressing MOCA was enhanced in concert with the increasing du-

Figure 4. **APP is not accumulated intracellularly in cells expressing MOCA.** The intracellular levels of APP were detected in whole cell lysates by antibodies 6E10 (A) and 22C11 (B). (C) The cell lysates from the corresponding cells were immunoprecipitated with the antibody CT-15 and visualized by Western blot analysis. (D) Actin controls for protein loading.

Figure 5. **MOCA alters the rate of intracellular APP protein degradation.** (A) Antibody specificity was tested by immunoprecipitation of [35S]methionine-labeled protein using various antibodies including 6E10 (lane 1), 22C11 (lane 2), GID (lane 3), and CT-15 (lane 4). Both the APP695 holoprotein in transfected B103 cells bearing the APP695 gene and the endogenous APP751 protein in HEK293 cells were immunoprecipitated by 6E10 and CT-15. (B) The turnover rate for intracellular APP holoprotein in APP695/vector and APP695/MOCA (clone 8) cells was determined. The cells were labeled for 10 min with [35S]methionine and chased for the indicated time periods with cold medium. The cell lysates were collected and immunoprecipitated with antibody 6E10 (arrow is APP). The level of APP secretion was also determined at the same time. The optical density of APP expression was quantitated by NIH image, and the data are presented as the percentage change after normalization to the initial levels of APP (intracellular) or to the highest levels of APPs (n = 3). (C) The effects of the protease inhibitor MG132 on the secretion of APP. Cells were incubated in the presence and absence of MG132 (10 μM) for the indicated time periods. The identical amount of protein collected from the serum-free condition medium was subjected to SDS-PAGE analysis, and the APP levels were determined by Western blot analysis. The secretion of APP was increased in APP695/MOCA (clone 8) cells treated by MG132 as a function of time. The optical density of APP secretion was quantitated from lower exposure images by NIH image, and the data are presented as the percentage increase in APP, plus or minus the standard error of the mean (n = 3).
rion of MG132 treatment and was nearly restored to control levels after 16 h (Fig. 5 C). These data again show that the APP degradation is affected by MOCA. They also confirm the Northern and RT-PCR data, showing no effect of MOCA on APP transcription, and rule out the possibility that the expression of MOCA reduces APP synthesis.

To confirm the above data on protein degradation and identify the mechanism which might be involved in the MOCA regulation of APP degradation, the effects of additional protease inhibitors were tested. Cells were treated with chloroquine (50 μM), NH₄Cl (5 mM), phosphoramidon (2 μM), N-acetyl-leucyl-norleucinal (ALLN) (27 μM), N-Ac-Leu-Leu-methioninal (ALLM) (27 μM), lactacystin (20 μM), clasto-lactacystin β-lactone (20 μM), or epoxomicin (10 μM) for 4 and 16 h, and the secretion and the intracellular levels of APP were measured by Western blotting. Lactacystin, β-lactone, and epoxomicin, which specifically target the proteasome and do not inhibit lysosomal protein degradation (Craiu et al., 1997; Fenteany and Schreiber, 1998; Meng et al., 1999), effectively restored the level of APP degradation in MOCA-containing cells (Fig. 6 A). Similar effects were also observed in cells treated with the peptide aldehydes, ALLN and ALLM, which inhibit proteasomes but also inhibit lysosomal cysteine proteases and calpains (Sherwood et al., 1993; Zhang et al., 1999). In contrast, two lysosomal protease inhibitors, chloroquine and ammonium chloride (Caporaso et al., 1992), did not reverse the MOCA effects on APP secretion. As another negative control, phosphoramidon, a metalloprotease inhibitor, did not affect APP secretion, consistent with the previous reports (Parvathy et al., 1998). The effects of these inhibitors on the intracellular levels of APP were also tested and were comparable to the corresponding effects on APP secretion (Fig. 6 B). These data were confirmed by a kinetic pulse–chase analysis. Cells with and without MOCA were labeled for 10 min with [35S]methionine and chased in complete medium in the presence of 10 μM MG132. Fig. 7 A shows that nascent APP molecules are stabilized by proteasome inhibition and that the rate of loss of intracellular APP in cells expressing MOCA becomes similar to that of MOCA-deficient cells. These data strongly support the involvement of proteasomes in the regulation of APP degradation by MOCA.

To determine if MOCA itself is affected by proteasome inhibition, MOCA expression patterns were studied as a function of time by Western blotting and confocal microscopy in the presence of MG132. The accumulation of intracellular MOCA increased after MG132 treatment (Fig. 7 B). Confocal images showed that under normal conditions MOCA is widely dispersed in the cytoplasm of B103 nerve cells (Fig. 7, C-2). MG132 treatment caused an increase in MOCA accumulation in the cytoplasm, and after 16 h treatment with MG132, there was MOCA accumulation in the perinuclear areas (Fig. 7, C-5). These data suggest that like many other proteins MOCA metabolism is regulated by the proteasome pathway.

**MOCA reduces cell-substratum adhesion**

The profound decrease in APP secretion caused by MOCA should have biological consequences. APP has multiple functions, but its ability to mediate cell-substratum adhesion has been well-documented (Schubert et al., 1989; Chen and Yankner, 1991; Schubert and Behl, 1993; Jin et al., 1994; Beher et al., 1996; Coulson et al., 1997). Because the inhibition of APP synthesis by antisense nucleotides blocks cell-substratum adhesion (Coulson et al., 1997), it would be predicted that the expression of MOCA has a similar effect and that this phenotype would be reversed by proteasome inhibitors, which restore APP accumulation and secretion. This is indeed the case when the ability of B103 nerve cells, expressing APP, MOCA, or both, to adhere to the extracellular matrix protein laminin...
Figure 7. **Proteasome inhibitors block the degradation of nascent APP.** (A) Pulse–chase analysis of intracellular APP in cells expressing MOCA or no MOCA in the presence of MG132 (10 μM). Cells were labeled for 10 min with [35S]methionine and then chased with normal medium. The rate of intracellular decline was determined relative to the initial level of APP in each cell type. The data are the mean of triplicate determinations plus or minus the standard error of the mean. (B) Western blotting of MOCA in the presence of MG132 (10 μM) at indicated times. (C) Confocal images of cells show an increase in MOCA accumulation in B103 (APP695/MOCA) cells. The expression of MOCA was analyzed with a MOCA antibody and visualized by staining with Texas red–conjugated secondary antibodies. Control, secondary Texas Red–conjugated antibody stain only (C-1); no MG132 (C-2); MG132-treated cells (4 h) (C-3); MG132-treated cells (8 h) (C-4); MG132-treated cells (16 h) (C-5).

Figure 8. **The expression of MOCA decreases cell-substratum adhesion.** Exponentially growing cells were labeled with 3H-leucine overnight, and their rate of adhesion to laminin was determined as a function of time as described in Materials and methods. (A) ○, B103; ×, B103 APP695/Δ, B103 MOCA C1 3; □, B103 APP695/MOCA C1 8. (B) ○, B103/APP695Cl 1 with empty vector; ×-B103/APP695 with empty vector plus LC; ▵-B103/APP695/MOCA C1 8 plus LC; Δ-B103/APP695/MOCA. In some cells, 25 μM lactacystin (LC) was added to the cultures 5 h before the adhesion assay. The data are presented as the mean plus or minus the standard error of the mean for triplicate determinations. The experiment was repeated three times with similar results.

Discussion

The above data show that MOCA decreases the secretion of APP and Aβ and also reduces the rate of cell adhesion. These results reflect the acceleration of intracellular APP

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degradation for the following reasons. (a) There is no accumulation of intracellular APP nor is there a decrease in APP mRNA in cells expressing MOCA. (b) In pulse–chase experiments, the intracellular APP protein level is rapidly reduced in cells expressing physiological levels of MOCA relative to cells not expressing MOCA. (c) The level of APP secretion and the remaining COOH-terminal fragments of APP are decreased by MOCA to a similar extent. (d) Several proteasome inhibitors increase the accumulation of intracellular APP and reverse the effect of MOCA on APP secretion and cell adhesion; lysosomal protease inhibitors are ineffective. (e) Pulse-labeled APP is also stabilized by proteasome inhibitors in MOCA-expressing cells, and unlike in the absence of proteasome inhibitors, its rate of intracellular decline is indistinguishable from that in cells not expressing MOCA.

APP is a type I membrane-spanning protein whose secretion is regulated by a variety of factors including growth factors, neurotransmitters, phorbol esters, extracellular matrix molecules, and stress (Schubert et al., 1989; Mills and Reiner, 1999; De Strooper and Annaert, 2000). The mechanisms involved in the regulation of APP secretion include alterations in APP phosphorylation (Caporaso et al., 1992), the modification of protein glycosylation (Galbete et al., 2000), alternative in gene splicing (Shepherd et al., 2000) and transcription (Giallèlla et al., 1994), and also changes in protein degradation (Checler et al., 2000). Although these mechanisms are diverse, it is likely that they are shared with many type I membrane proteins. APP is probably the most studied molecule of this class because of its medical importance.

The intracellular sites of APP metabolism still remain controversial with α-secretase cleavage described at the plasma membrane, in the Golgi, or in the post-Golgi secretory vesicles (Mills and Reiner, 1999). Similarly, β- and γ-secretase activities have been identified in the trans-Golgi network (Xu et al., 1997), the endoplasmic reticulum/intermediate compartment (Cook et al., 1997), and the endosome/lysosome system (Haass et al., 1992). Our data do not distinguish between these alternatives but show that secretion of APP and Aβ are both reduced in the presence of MOCA. Because we do not observe an intracellular accumulation of APP or intermediate breakdown products of APP, the effect of MOCA on APP processing most likely occurs before APP reaches the cell surface. The pleotropic effects of MOCA on APP degradation may occur in the ER, which is consistent with the subcellular localization of both PS1 and MOCA (Doan et al., 1996; Kashiwa et al., 2000; Xia et al., 2000).

Proteins destined for membranes or secretion are translocated into the ER, folded, assembled, and transported to a cellular destination or secreted (Hurtley and Helenius, 1989). Incorrectly folded proteins, unassembled subunits of multimeric complexes, and mutated proteins are rapidly eliminated from the cell; misfolded proteins are translocated to proteasomes and degraded (Suzuki et al., 1998). However, in the case of unfolded proteins the conventional route may not be taken because excessive accumulation of these macromolecules within the ER lumen might lead to their aggregation and precipitation, thereby blocking the secretory pathway (Klausner and Sitia, 1990). Because APP binds to the molecular chaperones Bip/G-RP78 and HSC73 and misfolded proteins bound to Bip/GRP78 are degraded, it has been suggested that APP can be retained in the ER as a nascent polypeptide and degraded (Yang et al., 1998; Kouchi et al., 1999). This idea is consistent with data suggesting that the degradation pathway for APP in the ER participates in APP secretion and is distinct from γ-secretase cleavage (Bunnell et al., 1998) and that the proteasome is involved in APP processing (Hare, 2001). Because APP secretion is restored by proteasome inhibitors and intracellular accumulation of APP is not observed in the absence of proteasome inhibitors, the secretion pathway is not blocked by MOCA. Consistent with previous data (Bunnell et al., 1998), we were unable to isolate a ubiquitin-conjugated APP complex even after the treatment with proteasome inhibitors, and we did not observe any APP intermediates caused by MOCA expression.

Proteasome inhibitors increase the stability of both total and pulse-labeled APP in cells (Figs. 6 and 7) and restore the secretion rate to near control levels in MOCA-expressing cells (Fig. 5 C and Fig. 6). They also increase the intracellular accumulation of MOCA over long periods of time (Fig. 7). Although it cannot be formally ruled out in any studies that proteasome inhibitors block an unknown function to generate the resultant phenotype, we feel that this is unlikely in the experiments described here for two reasons. First, in an experiment where cells are pulse labeled for 10 min and chased in the presence of a proteasome inhibitor there was a rapid inhibition of APP breakdown, bringing it up to the rate of loss of intracellular APP in non-MOCA cells due to secretion (Fig. 7). The effect occurred well before any significant accumulation of other proteins like MOCA. The accumulation in proteins in the ER in the presence of proteasome inhibitors can sometimes indirectly inhibit the secretory pathway; in our case, APP secretion is enhanced. Second, a variety of structurally diverse proteasome inhibitors reversed the effect of MOCA on APP secretion, whereas γ-secretase inhibitors, lysosomal protease inhibitors, and a metalloprotease inhibitor have no effect. These results strongly suggest that proteasomes are involved in MOCA-induced APP degradation. These data also suggest that nascent APP may pass through an ER environment in which complexes for both protein degradation and protein assembly coexist. In the absence of MOCA, the precursor protein follows the secretory pathway, whereas the expression of MOCA directs a significant fraction of APP to proteasomes where it is degraded. This novel MOCA-mediated pathway presents yet another way in which the expression of specific proteins may be controlled.

Aβ amyloid peptides are also generated from various cellular compartments, including the ER, the Golgi apparatus, the trans-Golgi networks, lysosomes, and endosomes, through either a constitutive secretion pathway or through an endocytotic pathway in which cell surface APP moves to the lysosomes or endosomes where Aβ is produced (Mills and Reiner, 1999; De Strooper and Annaert, 2000). Aβ secretion is decreased by MOCA in our studies, probably because of the rapid degradation of APP, therefore reducing the APP source for the generation of Aβ. Because the overall production of Aβ is reduced by MOCA, MOCA expression may help to reduce Aβ production in the central nervous system. It follows that the loss of MOCA function could lead to AD.

PS1 controls several aspects of APP metabolism (Sisodia,
2000) and protein breakdown (Niwa et al., 1999; Katayama et al., 2000). PS1 is also required for \(\gamma\)-secretase cleavage of Notch-1. However, the proteolytic cleavage of APP and Notch are differentially facilitated (Capell et al., 2000). The PS1-dependent \(\gamma\)-secretase processing of APP appears to be nonselective and occurs at multiple sites within the APP transmembrane domain. This is in contrast to the highly selective PS1-dependent processing of Notch (Yu et al., 2001).

Our data show that, unlike PS1, MOCA has a minimal effect on Notch-1 degradation and that of five additional membrane proteins (Fig. 1). We also studied the effects of several selective \(\gamma\)-secretase inhibitors on APP secretion modulated by MOCA (Fig. 2 B). Consistent with previous observations, there was little effect of these \(\gamma\)-secretase inhibitors on APP secretion in the absence of MOCA. In contrast to the proteasome inhibitors, among the \(\gamma\)-secretase inhibitors tested, only \(\gamma\)-secretase inhibitors III and IV and Calp III partially revert the effect of MOCA on APP secretion. \(\gamma\)-Secretase inhibitors II, which has no effect, is thought to be an aspartyl protease inhibitor, which selectively inhibits the \(\beta\)-secretase cleavage of APP and Notch-1 proteolysis (Wolfe et al., 1998; Niwa et al., 1999; Katayama et al., 2000) and protein breakdown (Niwa et al., 1999; Katayama et al., 2000) and protein breakdown (Niwa et al., 1999; Katayama et al., 2000).

The regulation of neurite outgrowth (Jin et al., 1994), the expression of MOCA during development could regulate cell migration and axon pathfinding. Therefore, the aberrant expression of MOCA or its loss could have pathological consequences in addition to those caused by altered A\(\beta\) secretion.

### Materials and methods

#### Antibodies and chemicals

Monoclonal antibody 22C11, which recognizes amino acid residues 66–81 of APP, was purchased from Roche. Anti-GID, a rabbit polyclonal antibody against amino acid residues 175–186 of APP, was provided by Dr. Greg Cole (University of California, Los Angeles, CA) and has been well-characterized (Schubert et al., 1989). CT-15, a rabbit polyclonal antibody against the last 15 COOH-terminal amino acid residues of APP, was provided by Dr. Edward Koo (University of California, San Diego, CA). Monoclonal antibody 6E10, which recognizes amino acid residues 1–17 of human A\(\beta\), was obtained from Dr. Richard Kiscak (New York State Institute for Basic Research, Staten Island, NY) or purchased from Chemicon. Antibodies against amino acid residues 81 of APP were provided by Schlesinger and colleagues (Schlesinger, 1992). Anti–mouse IgG agarose (Roche) were then added to each sample and incubated at 4°C overnight. 25 µl of anti–mouse IgG agarose (Roche) were then added to each sample and incubated at 4°C for 2 h on a rocker platform. The immunoprecipitates were collected by centrifugation and washed four times with the washing.
buffer (0.1% Triton, 20 mM Hepes, 150 mM NaCl, 10% glycerol). The agarose beads were resuspended in 30 μl SDS-PAGE sample buffer and boiled for 3 min to release the proteins. After 2 min of centrifugation, the supernatants were separated on 10% Tris-glycine gels. For Aβ production, cells were trypsinized and transferred onto Zeta membranes (Bio-Rad Laboratories, Inc.). Northern hybridization was performed in a UltraHyb buffer (Ambion) with an APP cDNA fragment probe labeled with \(^{32}\)P-dCTP using a rediprime kit (Amersham Pharmacia Biotech). RT reactions were performed for each RNA sample using 1 μg of total RNA in RT buffer composed of 10 mM DTT, 20 μM each of dATP, dCTP, dGTP, and dTTP, and 1 μl of oligo(dT). The solution was heated to 65°C for 5 min and then cooled to 37°C for 10 min, and then incubated in the presence of 25 U of AMV RT at 42°C for 1 h. Master mixes for the PCR reactions were used for each sample. The PCR reaction mixture contained forward and reverse primers (10–20 pmol each), dNTPs (200 μM each as final concentration), 1× PCR buffer, Taq DNA polymerase (0.5 U) (Roche), and 1 μl of the RT mixture as the source of cDNA. The primers used for PCR reaction were as follows: 5’-ATGCGATTCCGACATCAGC-3’ (forward) (nt 1,931–1,956) and 5’-CTAGTTTCTGCATGTCTCAGAAGA-3’ (reverse) (nt 2,235–2,212) for the APP gene (sequence data available from GenBank/EMBL/DDBJ under accession no. Y00264). Amplification was performed at 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min, for 35 cycles. The PCR reactions with primers and RNAs but without the RT reaction were conducted as controls. After amplification, each sample was electrophoresed on a 1.5% agarose gel visualized by ethidium bromide staining.

**Adhesion assay**

The cell adhesion assays were performed as described previously (Schubert et al., 1989). Exponentially B103 (APP695/Vector) and B103 (APP 695/H9262) cells were labeled with [H]leucine for 15 h. The cells were pipetted from the culture dishes and washed three times by centrifugation with Hepes buffered medium containing 0.4% BSA (Calbiochem). No trypsin or chelating reagents were used. Aliquots of 0.2 ml containing 5 × 10^6 cells were pipetted into 35-mm plastic Petri dishes coated with 5 μg mouse laminin and 2 ml of the above medium. At the indicated times, the dishes were swirled 10 times, the cells did not attach to uncoated dishes in 2% BSA in PBS for 1 h, and incubated with primary antibodies followed by fluorescein-conjugated secondary antibodies (Molecular Probes). The cells were then mounted under glass coverslips with antifading media containing 4% N-propyl gallate (Sigma-Aldrich). The cells were examined with a Carl Zeiss Microimaging, Inc. LSM 5 PASCAL laser scanning microscope. 0.3-μm-thick serial optical sections of the cells were recorded using the Carl Zeiss Microimaging, Inc. LSM 5 Image Examiner software to obtain images with pixel intensity within a linear range.

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