A novel mechanism for the regulation of amyloid precursor protein metabolism

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Modifier of cell adhesion protein (MOCA; previously called presenilin [PS] binding protein) is a DOCK180-related molecule, which interacts with PS1 and PS2, is localized to brain areas involved in Alzheimer’s disease (AD) pathology, and is lost from the soluble fraction of sporadic Alzheimer’s disease (AD) brains. Because PS1 has been associated with γ-secretase activity, MOCA may be involved in the regulation of β-amyloid precursor protein (APP) processing. Here we show that the expression of MOCA decreases both APP and amyloid β-peptide secretion and lowers the rate of cell-sustratum adhesion. In contrast, MOCA does not lower the secretion of amyloid precursor-like protein (APLP) or several additional type 1 membrane proteins. The phenotypic changes caused by MOCA are due to an acceleration in the rate of intracellular APP degradation. The effect of MOCA expression on the secretion of APP and cellular adhesion is reversed by proteasome inhibitors, suggesting that MOCA directs nascent APP to proteasomes for destruction. It is concluded that MOCA plays a major role in APP metabolism and that the effect of MOCA on APP secretion and cell adhesion is a downstream consequence of MOCA-directed APP catabolism. This is a new mechanism by which the expression of APP is regulated.

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 APPγ 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 AB (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. 1 A). 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. 1 B). Second, transient transfection of MOCA into B103 (APP695) cells led to the reduction of APP secretion in an uncloned population (Fig. 1 D). Third, the secretion of APP was not affected by the overexpression of DOCK180, a protein which has a 40% homology with MOCA (Fig. 2 D). Fourth, the secretion of endogenous APP751 was reduced in HEK293T cells expressing MOCA (Fig. 1 D). 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. 1 A). 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. 1 A). A single band with molecular weight ~130 kd is 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...
Amyloid precursor metabolism | Chen et al. 81

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 300 kd), and truncated transmembrane forms (mol wt 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 expressing an antibody specific to rat PS1. Fig. 2 A shows the abundant processed NH2-terminal fragments of APP (mol wt 19–21 kd). A very weak band with a mol wt 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.
Effect of MOCA on Aβ-secretion. (A) The levels of secreted and intracellular APPsw were examined in several B103 clones stably transfected with both APPsw and MOCA by Western blot analysis. The optical density of APPsw secretion was quantitated by NIH image, normalized to the APPsw vector alone level, and the data are presented as the percentage change plus or minus the standard error or the mean (n = 3). (B) Intracellular APP was assayed by Western blotting with antibody 6E10 and quantitated as above. (C) The [35S]methionine-labeled (16 h) secreted Aβ peptides were immunoprecipitated with 6E10 antibody, separated in SDS–polyacrylamide gels, and visualized by exposure to X-films. The optical density of Aβ secretion was quantitated by NIH image, and the data are presented as the percentage of change plus or minus the standard error or the mean (n = 3). (D) The secreted Aβ42 peptides were measured by using a sandwich ELISA kit (Biosource International). Data from four separate experiments were combined and are presented as mean ± SEM pgm of Aβ per mg cellular protein (n = 16/condition).

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 a 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 (L927V). 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/MOCA) 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 blockade 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 protease 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-
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...
are compared. Fig. 8 A shows that the expression of APP<sub>695</sub> in B103 cells, which normally lack APP, increases the rate of cell-substratum adhesion to laminin. The expression of MOCA alone in B103 cells lacking APP<sub>695</sub> has no effect on adhesion. In contrast, cells expressing both APP and MOCA adhere to laminin at a rate which is indistinguishable from cells expressing no APP. However, when cells expressing APP and MOCA are exposed to the proteasome inhibitor lactacystin for 5 h before the adhesion assay there is an increase in the rate of adhesion to a level indistinguishable from cells expressing APP but no MOCA (Fig. 8 B). Lactacystin does not alter the rate of adhesion of cells which do not express MOCA or wild-type B103 cells. The adhesion data are in agreement with those which show that MOCA expression increases APP breakdown and decreases its secretion (Figs. 1, 2, and 5) and that APP accumulation and secretion can be restored by proteasome inhibitors (Figs. 5 and 6).

**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 breakdown and suggest that MOCA expression may contribute to the pathology of AD.

![Proteasome inhibitors block the degradation of nascent APP](image.png)

Fig. 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 (APP<sub>695</sub>/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).

![The expression of MOCA decreases cell-substratum adhesion](image.png)

Fig. 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) O, B103; x, B103 APP<sub>695</sub>; Δ, B103 MOCA Cl 3; □, B103 APP<sub>695</sub>/MOCA Cl 8. (B) O, B103/APP<sub>695</sub>/MOCA Cl 1 with empty vector; x-B103/APP<sub>695</sub>/MOCA Cl 1 with empty vector plus LC; Δ-B103/APP<sub>695</sub>/MOCA Cl 8 plus LC; Δ-B103/APP<sub>695</sub>/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.
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 alternations 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 (Haas 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 Söltia, 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 “γ-secretase” cleavage of Notch-1. However, the proteolytic cleavage of APP and Notch are differentially facilitated (Capell et al., 2000). The PS1-dependent γ-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 γ-secretase inhibitors on APP secretion modulated by MOCA (Fig. 2 B). Consistent with previous observations, there was little effect of these γ-secretase inhibitors on APP secretion in the absence of MOCA. In contrast to the proteasome inhibitors, among the γ-secretase inhibitors tested, only γ-secretase inhibitors III and IV and Calp III partially revert the effect of MOCA effect on APP secretion. γ-Secretase inhibitor II, which has no effect, is thought to be an aspartyl protease inhibitor, which selectively inhibits the γ-secretase cleavage of APP and Notch-1 proteolysis (Wolfe et al., 1998; 1999; De Strooper et al., 1999; Berezovska et al., 2000; Esler et al., 2000). The other γ-secretase inhibitors including III, IV, V, and the potent calpain inhibitor III are dipeptidyl aldehydes targeting cysteine proteases and may block proteasome enzymes, accounting for the inconsistency of their effects on APP secretion affected by MOCA. Finally, the additive effect of PS1 on MOCA-decreased APP secretion was demonstrated (Fig. 2 C). This observation is consistent with the fact that PS1 lowers the secretion of APP in yeast (Evin et al., 2000). MOCA also functions effectively in the presence of mutant PS1. Together, the above data show that the effect of MOCA is very different from that of the PS1-associated γ-secretase activity. MOCA contains an SH3 domain, interacts with the Crk adaptor protein, and shares a 40% homology with DOCK180 (Kashiwa et al., 2000). Because DOCK180 interacts with Rac and other small G-proteins, MOCA may also interact with small G-proteins involved in protein breakdown or mediate phosphorylation events between proteins involved in APP trafficking and metabolism.

APP is a potent cell adhesion molecule which binds to both heparin and other extracellular matrix molecules (Schubert et al., 1989; Beher et al., 1996; Wu et al., 1997). Cells which express APP adhere more rapidly to extracellular matrix proteins than cells which do not (Fig. 8) (Schubert and Behl, 1993). Conversely, the inhibition of APP synthesis by antisense techniques blocks cell-substratum adhesion (Coulson et al., 1997). The expression of MOCA in B103 nerve cells, which do not make APP, has no effect on cellular adhesion to laminin, but reduces the rate of adhesion of cells which express APP (Fig. 8). Therefore, the effect of MOCA on cell-substratum is tightly coupled to APP expression. When the rapid breakdown of APP caused by MOCA is blocked by proteasome inhibitors, there is a return to the normal rate of adhesion for B103 cells expressing APP (Fig. 8 B). The simplest explanation for these results is that in the presence of MOCA newly synthesized APP is degraded at such a rapid rate that very little reaches the cell surface so that it is unable to participate in adhesive interactions. Because one adhesion-dependent biological activity of APP is 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 Aβ 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 Aβ, was obtained from Dr. Richard Kiscsk (New York State Institute for Basic Research, Staten Island, NY) or purchased from neuron. An affinity purified polyclonal antibody, which recognizes amino acid residues 2,012–2,027 of MOCA, was obtained in rabbits (Kashiwa et al., 2000). A monoclonal antibody, recognizing the NH2-terminal amino acid residues 21–80 of human PS1, and a polyclonal antibody, recognizing the amino acid residues 303–331 of mouse PS1, were purchased from Chemicon and Oncogene, respectively. β-Laminin antibody (sc-6018), N-CAM antibody (sc-1307), N- and E-cadherin antibody from Transduction Laboratories, Notch antibody (sc-6014) were purchased from Santa Cruz Biotechnology, Inc., and APLP was from Dr. Gopal Thinakaran (University of Chicago, Chicago, IL). The following chemical reagents were bought from Sigma-Aldrich: chloroquine, NH4Cl, phosphoramidon, ALLN, ALLM, lacticystin, clasto-lactacytin β-lactone, leupeptin, and MG132. Epsomoxzion was bought from Bioremol. γ-Secretase inhibitors II, III, IV, V and calpain inhibitor III were purchased from Calbiochem.

Cells and transfection

The neuronal cell line B103 (Schubert et al., 1974) was grown in DME supplemented with 10% heat-inactivated FBS. B103 cells were stably transfected with APP695 using G418 selection (Schubert et al., 1989) and with various plasmids by Lipofectamine 2000 (Invitrogen) using purumycin selection for MOCA or hygromycin for PS1. The stably transfected cells were subsequently cloned and screened for protein expression by Western blot analysis.

Western blotting, metabolic labeling, immunoprecipitation, and HSA

For Western blotting, cells were washed twice with ice-cold PBS and lysed in lysis buffer (1% Triton, 50 mM Hapes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 10 mM Na3P04, 25 μM protease inhibitors [Complete Mini; Roche]). For secreted protein analysis, semiconfluent cultures (106 cells in 100-mm tissue culture dishes) were washed twice with serum-free medium and incubated for 20 h in 1 ml serum-free medium. The growth conditioned medium was then de-salted by passage through a Sephadex G25 column, and 10% of the material was used to determine protein content. For a given experiment, equal amounts of protein (usually 15 μg) were lyophilized and resuspended in 50 μl of sample buffer. Protein concentrations were determined by Coomassie Plus (Pierce Chemical Co). The same amount of protein from each sample was separated on Novex precast 10% polyacrylamide gels (Invitrogen) and transferred to Immobilon membranes (Millipore). The membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h at room temperature. After overnight incubation at 4°C with primary antibodies, the antigens were detected with HRP-conjugated secondary antibodies (Bio-Rad Laboratories) using an ECL kit (Amersham Pharmaica) and exposed to film. For pulse–chase experiments, cells were grown to 80% confluency and incubated in methionine-free DME for 90 min. Cells were labeled with [35S]methionine (400 μCi/ml in methionine-free DME plus 5% dialyzed FBS) for 10 min at 37°C, and the medium was replaced with serum-free DME plus 10% supplement (Sigma-Aldrich). The media were collected, and cells were lysed after different time periods. Protein concentrations were determined, and the same amounts of protein were immunoprecipitated with antibodies 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 analysis, 10–20% acrylamide Tricine gels and longer incubation times were used. The gels were dried and subjected to autoradiography and quantified by NIH Image. Aβ production was also measured by a sensitive fluo-

References


