Membrane-anchored metalloprotease MDC9 has an α-secretase activity responsible for processing the amyloid precursor protein

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MDC9, also known as meltrin γ, is a membrane-anchored metalloprotease. MDC9 contains several distinct protein domains: a signal sequence followed by a prodomain and a domain showing sequence similarity to snake venom metalloproteases, a disintegrin-like domain, a cysteine-rich region, an epidermal-growth-factor-like repeat, a transmembrane domain and a cytoplasmic domain. Here we demonstrate that MDC9 expressed in COS cells is cleaved between the prodomain and the metalloprotease domain. Further, when MDC9 was co-expressed in COS cells with amyloid precursor protein (APP695) and treated with phorbol ester, APP695 was digested exclusively at the α-secretory site in MDC9-expressing cells. When an artificial α-secretory site mutant was also co-expressed with MDC9 and treated with phorbol ester, APP secreted by α-secretase was not increased in conditional medium. Inhibition of MDC9 by a hydroxamate-based metalloprotease inhibitor, SI-27, enhanced β-secretase cleavage. These results suggest that MDC9 has an α-secretase-like activity and is activated by phorbol ester.

Key words: Alzheimer's disease, hydroxamate.

INTRODUCTION

Metalloprotease/disintegrin/cysteine-rich proteins (MDCs, also called 'a disintegrin and metalloprotease' or ADAMs) are membrane-anchored proteins with several domains including a metalloprotease domain, a disintegrin-like domain, a cysteine-rich sequence, an epidermal growth factor-like sequence, a transmembrane domain and a cytoplasmic domain [1]. At least 21 members of the ADAMs family have now been identified and sequenced [2–11]; together with snake venom metalloproteases they make up the reprolysin family of zinc metalloproteases. Members of the metzincin superfamily of proteolytic enzymes, including the reprolysin and matrix metalloprotease families, contain the distinctive zinc-binding consensus sequence HEXXH [12,13]. However, only a subset of ADAMs, including ADAM 12 ('meltrin α'), contain this motif and are therefore considered to be potentially active metalloproteases. Protease activity has actually been demonstrated so far for only a few members of the ADAMs family, namely ADAM 17 ('TACE'), ADAM 12 and the *Drosophila* ADAM 10 homologue KUZ [7,8,14–20].

Several lines of evidence suggest that metalloproteases are involved in the processing of the amyloid precursor protein (APP). APP is usually degraded within amyloid β (Aβ) by a putative α-secretase in normal brain; a 90–100 kDa, soluble, non-amyloidogenic N-terminal fragment of APP (sAPP) is secreted from the cells [20–25]. The α-secretase is suspected to be a metalloprotease and the α-secretase cleavage is activated by phorbol esters such as PMA and phorbol dibutyrate [26,27].

We report here that the metalloprotease domain of MDC9 (meltrin γ) is proteolytically active and that full-length MDC9 has an α-secretase activity for APP.

MATERIALS AND METHODS

Expression and purification of mMDC9s in COS cells

Full-length mouse MDC9 (mMDC9-FL) and metalloprotease-domain deletion mutant (mMDC9-ΔMP) cDNA fragments were released from their stop codons by PCR by using site-directed mutagenesis primers. These DNA fragments were cloned into pSecTag (Invitrogen, San Diego, CA, U.S.A.). COS-7 cells were grown in Dulbecco's modified Eagle's medium with 4.5 mg/ml glucose and 10 % (v/v) fetal bovine serum at 37 °C in air/CO_2 (19:1). pSecTag/mMDC9-FL and pSecTag/mMDC9-ΔMP were transfected into COS-7 cells by the electroporation method [28]. After 72 h of incubation, the transfected cells were collected and sonicated in buffer A [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/1 % (v/v) Triton X-100/0.1 % (v/v) Nonidet P40]. After centrifugation at 8000 ***g*** for 5 min, the protein concentration of the supernatant was determined with a DC protein assay kit (Bio-Rad, Richmond, CA, U.S.A.). The supernatant was diluted in 2 × SDS buffer [125 mM Tris/HCl (pH 6.8)/30 % (v/v) glycerol/2 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/0.2 % Bromophenol Blue]. The samples were subjected to SDS/PAGE [10 % (w/v) gel] and then transferred to a PVDF membrane (Finetrap NT-32; Nihon Eido, Tokyo,

Abbreviations used: Aβ, amyloid β; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; MMP, matrix metalloproteinase; sAPP, secreted form of APP.

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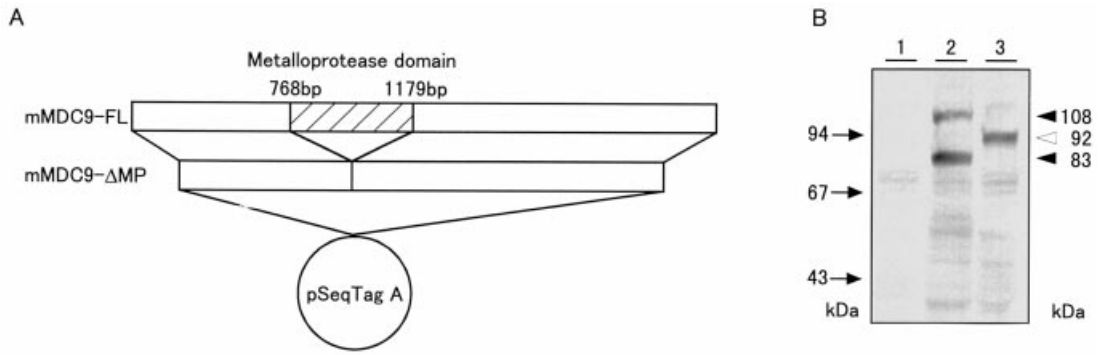

Figure 1 Expression of full-length and deletion mutant mMDC9 proteins

Cell lysates from COS cells transfected with various constructs were subjected to SDS/PAGE and immunoblotted with anti-*myc* antibody. (**A**) mMDC9 constructs were wild-type (mMDC9-FL, upper panel) and a metalloprotease-domain deletion mutant (mMDC9-ΔMP, lower panel), lacking codons 256–393 (from 768 to 1179 bp). (**B**) Analysis of the mMDC9 protein expressed by COS cells. Lane 1 shows cells transfected with a negative-control plasmid (pSecTag A with no insert). Lanes 2 and 3 are from cells transfected with a full-length mMDC9-FL and a metalloprotease domain deletion mutant, mMDC9-ΔMP, respectively. Samples were analysed by SDS/PAGE (10% gel) (20 μg of protein per lane).

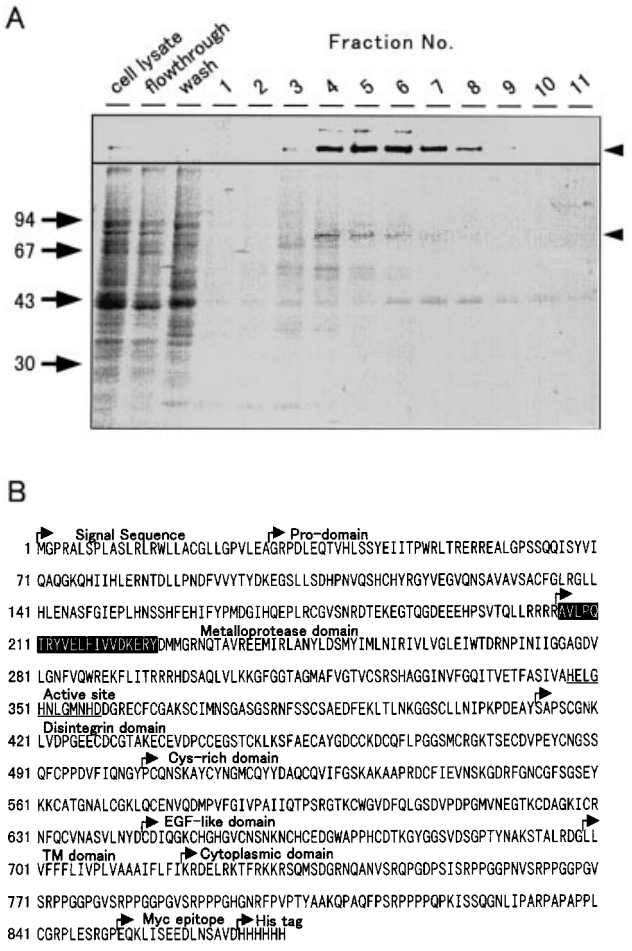

Figure 2 Purification of mMDC9-ΔPro and amino acid sequence of mMDC9-FL

(**A**) Expressed mMDC9-FL was purified by Ni/nitrilotriacetate column chromatography, subjected to electrophoresis (15 μl of each fraction) and stained with Coomassie Brilliant Blue R-250 (lower panel). mMDC9-FL was immunostained with anti-*myc* antibodies (upper panel) after transfer to PVDF membrane. The arrowheads indicate mMDC9-ΔPro. The positions of molecular mass markers are indicated at the left. (**B**) Amino acid sequence of mMDC9-FL and each domain was shown in this figure. The sequence in white on a black background correspond to the N-terminal amino acid sequence of purified mMDC9-ΔPro.

Japan). The membrane was incubated overnight with anti-*myc* antibody (Invitrogen) at 4 °C and bands were revealed with a VECTASTAIN *Elite* ABC kit (Vector Laboratory, Burlingame, CA, U.S.A.) and a POD immunostain set (Wako, Osaka, Japan).

Purification and N-terminal amino acid sequence determination of mMDC9-FL

The mMDC9-FL was transfected into COS-7 cells, which were collected after 80 h and sonicated in buffer A. The homogenate was subjected to centrifugation, first at 8000 ***g*** for 10 min at 4 °C, then at 100000 ***g*** for 30 min at 4 °C. The supernatant was applied to an Ni/nitrilotriacetate column (Qiagen, Hilden, Germany) equilibrated with buffer N [20 mM Tris/HCl (pH 7.5)/0.5 M NaCl/10 mM imidazole] and washed with 20 ml of 20 mM imidazole in buffer N. Proteins were eluted with 120 mM imidazole in buffer N (flow rate 1 ml/min); 2 ml fractions were collected. The fractions were dialysed overnight against buffer A and the protein concentrations of all fractions were determined with the DC protein assay kit. The fractions were diluted in 2 × SDS buffer and samples were subjected to SDS/PAGE (10% gel) and then transferred to a PVDF membrane. The membrane was incubated with anti-*myc* overnight at 4 °C and proteins were then revealed. The mMDC9-FL fraction was subjected to SDS/PAGE (10% gel) and transferred to PVDF membrane (Trans-Blot®; Bio-Rad). The membrane was stained with 0.1% Amido Black in 50% (v/v) methanol/10% (v/v) acetic acid. The protein band to be examined was cut out and washed with Milli Q. Amino acid sequences were determined with an Applied Biosystems model 492-140C amino acid sequencer (Foster City, CA, U.S.A.).

APP695 cleavage assay *in vitro*

Human APP695-WT (wild-type APP) with the p91023 vector was used to transfect COS-7 cells by the electroporation method [28]. After 72 h of incubation, the transfected cells were collected and sonicated in buffer A containing 10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64'), 10 μM leupeptin and 100 μM PMSF. After centrifugation at 8000 ***g*** for 5 min, the protein concentration of the supernatant (APP-lysate) was determined with a DC protein assay kit. Purified mMDC9-FL was incubated with APP-lysate (15 μg of protein) containing 5 mM

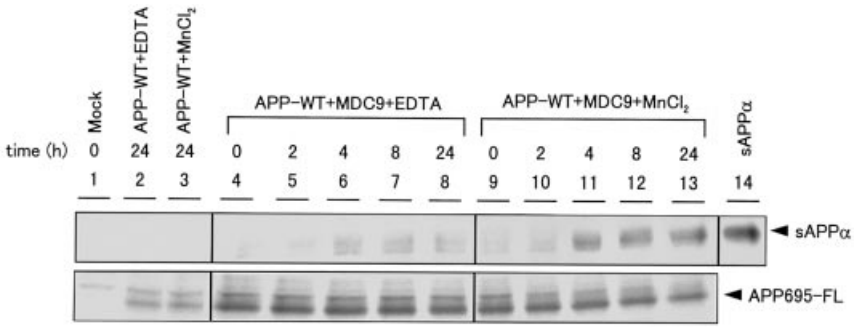

Figure 3 APP was cleaved by MDC9

Lane 1 is COS cell lysate transfected with vector only; lanes 2 and 3 are APP-lysates incubated with EDTA or $MnCl_2$ at 37 °C for 24 h. Purified mMDC9 was incubated with APP-lysate in the presence of EDTA (lanes 4–8) or $MnCl_2$ (lanes 9–13) at 37 °C for the indicated durations. Lane 14 was sAPPα in cultured medium. Each mixture was subjected to SDS/PAGE and immunoblotted with anti-Aβ1–16 (upper panel) or anti-22C11 antibody (lower panel).

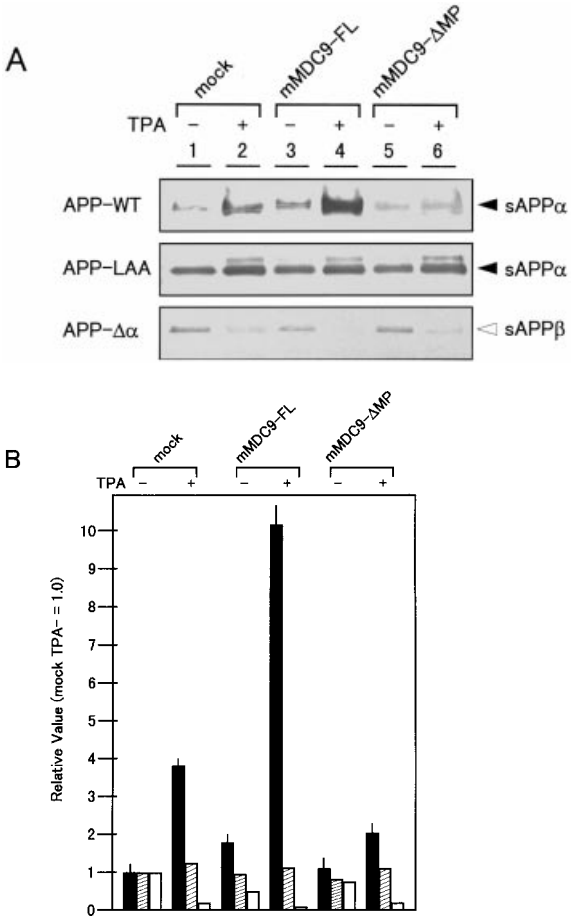

Figure 4 sAPPs secreted into the condition medium

(**A**) Medium from COS cells transfected with various plasmids was concentrated, subjected to SDS/PAGE and immunoblotted with anti-Aβ1–16 or anti-sAPPβ antibody. APP-WT was co-transfected with mMDC9 plasmids (top panel, immunoblotted with anti-Aβ1–16). The APP-LAA mutant (middle panel, immunoblotted with anti-Aβ1–16) and APP-$\Delta\alpha$ mutant (bottom panel, immunoblotted with anti-sAPPβ) were treated similarly. Lanes 1 and 2, cells co-transfected with an APP plasmid and pSecTag A with no insert; lanes 3 and 4, cells co-transfected with an APP plasmid and mMDC9-FL; lanes 5 and 6, cells co-transfected with an APP plasmid and mMDC9-ΔMP. Lanes 1, 3 and 5, without treatment with PMA (TPA); lanes 2, 4 and 6, with PMA (30 nM). Samples were analysed by SDS/PAGE (10% gel) (20 μg of cell lysate protein per lane). (**B**) Densitometric quantification showed an increased secretion of anti-Aβ1–16- and anti-sAPPβ-immunoreactive products. Key: black bars, sAPPα-WT; hatched bars, sAPPα-LAA; white bars, sappβ-$\Delta\alpha$. For APP-WT only, means $\pm$ S.E.M. are shown for three independent experiments ($P < 0.05$).

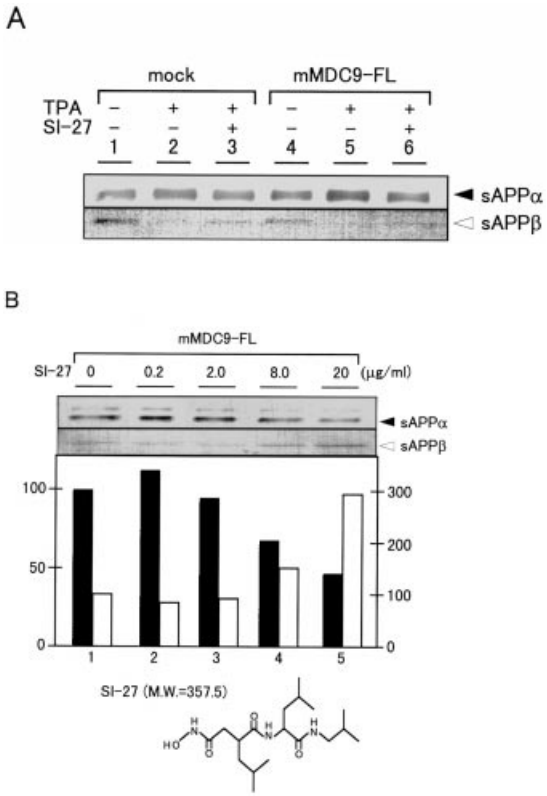

Figure 5 Inhibition of sAPPα secretion by SI-27

(**A**) Medium from COS cells transfected with APP-WT and vector (lanes 1–3) or mMDC9-FL (lanes 4–6) was concentrated, subjected to SDS/PAGE and immunoblotted with anti-Aβ1–16 (upper panel) or anti-sAPPβ (lower panel) antibody. Lanes 1 and 4, without treatment with PMA (TPA) and SI-27; lanes 2 and 5, with PMA (30 nM); lanes 3 and 6, with PMA (30 nM) and SI-27 (20 μg/ml). (**B**) Dose-dependent effects of SI-27 on secretion of APP. Lane 1, with PMA (30 nM) only; lanes 2–5, with PMA (30 nM) and SI-27 (0.2, 2.0, 8.0 and 20 μg/ml respectively). The lower graph of densitometric quantification showed decreased secretions of sAPPα (filled bars, scale on the left) with increased secretions of sAPPβ (open bars, scale on the right) immunoreactive products. Both scales are based on a value of 100% in the absence of SI-27. The chemical structure of SI-27 (molecular mass 357.5 Da) is shown at the bottom.

$MnCl_2$ or EDTA at 37 °C for 0, 2, 4, 8 and 24 h. Only APP-lysate for the negative control was incubated containing 5 mM $MnCl_2$ or EDTA at 37 °C for 24 h. The reaction was stopped with 2 × SDS buffer and the mixture was then boiled at 100 °C for 5 min, subjected to SDS/PAGE (10% gel) and then transferred

to a PVDF membrane (Finetrap NT-32). The transferred membrane was incubated overnight with anti-22C11 (Boehringer Ingelheim) or anti-Aβ1–16 antibodies [26] at 4 °C and proteins were then revealed.

Co-expression of mMDC9 and APP695s in COS cells

Various human APP695 cDNA fragments (APP-WT, APP-LAA and APP-$\Delta\alpha$, artificial APP mutants) were constructed with the p91023 vector. We replaced the sequence HHQK of Aβ 13–16 with LHHAA to confer added resistance to α-secretory cleavage. We also constructed the deletion mutant APP-$\Delta\alpha$ lacking 19 residues from Arg^{601} to Asp^{619} (Aβ 5–23). The APP plasmid and mMDC9-FL or mMDC9-ΔMP plasmid were used to transfect COS-7 cells with or without a hydroxamate-based metalloprotease inhibitor SI-27 (final concentration up to 20 μg/ml) by the electroporation method [28]. After 12 h of incubation, transfected cells were transferred to serum-free medium with or without PMA (final concentration 30 nM) and/or SI-27 (final concentration 20 μg/ml or as indicated). After an additional 24 h of incubation, the transfected cells were harvested, sonicated in buffer A and centrifuged at 8000 ***g*** for 5 min. The protein concentration of the supernatant was determined. The conditioned medium (8 ml) was concentrated by the addition of trichloroacetic acid and centrifuged at 10000 ***g*** for 15 min. The precipitate was washed with diethyl ether/ethanol (1:1, v/v) and then dissolved in 300 μl of 2 × SDS buffer. The samples were subjected to SDS/PAGE (10 % gel) and then transferred to a PVDF membrane. The transferred membrane was incubated with anti-APP antibodies overnight at 4 °C and proteins were revealed. The anti-APP antibodies used were anti-22C11 (Boehringer Mannheim), anti-Aβ1–16 [26] and anti-sAPPβ (T. C. Saido, unpublished work). The bands detected were quantified with an Imagemaster ver. 2.0 (Pharmacia).

RESULTS AND DISCUSSION

Expression of mMDC9-FL and mMDC9-ΔMP in COS cells

Figure 1(A) shows the mMDC9-FL and mMDC9-ΔMP cDNA constructs. The immunoblot (Figure 1B) indicates the molecular masses of mMDC9-FL (871 amino acid residues) and mMDC9-ΔMP (734 residues; Δ 256–359) to be 108 and 92 kDa respectively. These sizes are slightly larger than those calculated from the primary sequences (95 and 80.3 kDa) and most probably reflect glycosylation, as in ADAM 12 polypeptides [29,30]. mMDC9-ΔMP was detected as a single band but mMDC9-FL revealed two bands (108 and 83 kDa). These results indicate that mMDC9-FL was cleaved between the prodomain and the metalloprotease domain but mMDC9-ΔMP was not. We therefore conclude that this cleavage was due to MDC9 autolytic activity. We regard the lower 83 kDa band as being a cleavage product of the 108 kDa full-length band corresponding to the deletion of the prodomain (23 kDa; 205 residue residues).

N-terminal amino acid sequence of mMDC9-ΔPro and APP cleavage assay *in vitro*

We attempted to determine the N-terminal sequence of the mMDC9 cleavage product (mMDC9-ΔPro). Figure 2(A) shows the SDS/PAGE patterns of proteins eluted from the Ni/nitrilotriacetate column. The 83 kDa mMDC9-ΔPro was eluted from fractions 4–7 with approx. 60 mM imidazole. mMDC9-ΔPro was identified by immunoblot analysis with anti-*myc* antibody. The N-terminal amino acid sequence of the 83 kDa protein was determined as AVLPQTRYVELFIVVDKERY. This sequence agrees completely with mMDC9-FL residues 206–225 (Figure 2B). This cleavage occurred after the furin-like cleavage site ($RRRR^{205}$-AVLP). Previous reports demonstrated that ADAM 12 is inactive until the prodomain is cleaved [18] and MDC9 is cleaved by recombinant furin between the prodomain and the metalloprotease domain [31]. We therefore conclude that full-length MDC9 can be cleaved and activated by autolysis or by furin-like protease in COS cells.

Next we tried to determine whether purified mMDC9-FL has an α-secretase activity. Figure 3 clearly demonstrates that mMDC9 had an α-secretase activity *in vitro*. The purified mMDC9 (Figure 2A, fraction 5) was incubated with APP-lysate with or without $MnCl_2$ (Figure 3, upper panel, lanes 4–8 and 9–13) at 37 °C. sAPPα was detected in lane 11 after 4 h with $MnCl_2$ and the amount was gradually increased (Figure 3, upper panel, lanes 11–13). In contrast, we could not detect sAPPα in the control lysates, even after 24 h (Figure 3, upper panel, lanes 2 and 3). In the absence of $MnCl_2$, however, sAPPα-like band(s) were detected (Figure 3, upper panel, lanes 6–8). We regard these bands as a product formed by slightly active MDC9. In addition, we found that the amount of APP695-WT was decreased in a time-dependent manner (Figure 3, lower panel). The upper band of full-length APP695-WT was full-length endogenous APP770 in COS cell lysate, and this APP770 also decreased after 4, 8 and 24 h with $MnCl_2$ (Figure 3, lower panel, lanes 11–13). These results strongly suggest that MDC9 had a metal-dependent α-secretase activity *in vitro*.

α-Secretase activity of mMDC9

To investigate mMDC9 as a potential α-secretase, MDC9s (mMDC9-FL or mMDC9-ΔMP) and APP analogues (APP-WT, APP-LAA or APP-$\Delta\alpha$) were co-expressed in COS cells. APP expression in cell lysates and conditioned medium was evaluated by immunoblot analysis with anti-22C11 antibody. Secreted APPα and APPβ were specifically detected by using anti-Aβ1–16 or anti-sAPPβ antibodies respectively (Figure 4A). Densitometric quantification showed an increased secretion of the anti-Aβ1–16 immunoreactive product (sAPPα) from APP-WT by stimulation with phorbol ester (Figure 4A, top panel, and Figure 4B). This result agrees with previous reports that MDC9 is activated by PMA [31,32]. The expression of MDC9 in COS cells also enhanced sAPPα secretion by phorbol ester treatment. Of three secretases, only α-secretase could be activated by phorbol esters. These results satisfy the necessary conditions for an α-secretase.

Next we transfected APP695-LAA cDNA in COS cells. We replaced the HHQK of Aβ 13–16 with LHHAA to confer greater resistance to α-secretory cleavage. Figure 4(A) (middle panel) indicates the failure of phorbol activation of α-secretory cleavage. An activation of endogenous APP770 secretion was observed. The deletion mutant APP-$\Delta\alpha$, lacking the α-secretory cleavage site, did not resist cleavage (Figure 4A, bottom panel). With APP-$\Delta\alpha$ (Δ Arg^5–Asp^{23} of Aβ), cleavage occurred between Glu^{593} and Val^{594} of APP695. Because anti-sAPPβ detected only the VKM sequence of the C-terminus of sAPPβ, the sAPP detected in lane 1 should be endogenous sAPPβ. PMA decreased β-cleavage as shown previously (Figure 4A, bottom panel, lane 2); transfection of MDC9 (putative α-secretase) clearly suppressed β-cleavage (lane 3), but not in the protease-deficient mutant (lane 5).

Inhibition of sAPPα secretion by a metalloprotease inhibitor

Figure 5(A) (upper panel) shows that a metalloprotease inhibitor, SI-27, inhibited the PMA-induced increase in sAPPα secretion.

Several reports have indicated that the secretion of sAPPα was triggered with PMA (Figure 5A, upper panel, lanes 2 and 5) [26,27] but the addition of SI-27 (20 μg/ml) suppressed the secretion of sAPPα (Figure 5A, upper panel, lanes 3 and 6). In contrast, the secretion of sAPPβ was inhibited by the treatment with PMA (Figure 5A, lower panel, lanes 2 and 5). In the presence of SI-27, however, sAPPβ secretion was increased (Figure 5A, lower panel, lanes 3 and 6). As shown in Figure 5(B), increasing concentrations of SI-27 (0, 0.2, 2.0, 8.0 and 20 μg/ml), had reciprocal effects on sAPPα and sAPPβ secretion (Figure 5B). The design of several metalloprotease inhibitors has been based on the structural requirements of the active site of metalloproteases for its substrates. Hydroxamate-based inhibitors have been used to inhibit metalloproteases. They are usually broad-spectrum matrix metalloproteinase (MMP) inhibitors and can also inhibit the shedding of membrane proteins [27]. SI-27 inhibited MMPs (IC_{50} values for MMP-3, MMP-2 and MMP-9 are 0.17, 0.036 and 0.090 μM respectively) as well as other metalloproteases (IC_{50} values for thermolysin and leucine aminopeptidase are 6.27 and 15 μM respectively) (A. Okuyama, unpublished work). Amour et al. [19] reported that hydroxamate derivatives strongly inhibited one ADAM, recombinant TACE. Although there is no evidence that SI-27 directly inhibits the ADAM family of metalloproteases, it is possible that SI-27 could inhibit ADAM.

In the present study we examined the role of MDC9 in the regulated α-secretion of APP. The MDC9 expressed in COS cells increased the secretion of basal sAPPα (Figure 4A, lanes 1 and 3) and enhanced sAPPα secretion in the PMA-treated condition (Figure 4A, lanes 2 and 4). The addition of SI-27 inhibited the MDC9-induced secretion of sAPPα. A previous report demonstrated that the stimulation of sAPPα release by PMA was not observed in ADAM 17 knock-out cells [20]. However, basal sAPPα secretion was still observed, indicating that ADAM 17 is not α-secretase but has a similar specificity to that of α-secretase. The mRNA content of ADAM 17 is low in human adult brain [7], but the mRNA for MDC9 is ubiquitously expressed in human tissues. In conclusion, we believe that MDC9 is an α-secretase responsible for processing APP.

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