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Putative function of ADAM9, ADAM10, and ADAM17 as APP α-secretase

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Abstract

The putative α-secretase cleaves the amyloid precursor protein (APP) of Alzheimer's disease in the middle of the amyloid β peptide (Aβ) domain. It is generally thought that the α-secretase pathway mitigates Aβ formation in the normal brain. Several studies have suggested that ADAM9, ADAM10, and ADAM17 are candidate α-secretases belonging to the ADAM (a disintegrin and metalloprotease) family, which are membrane-anchored cell surface proteins. In this comparative study of ADAM9, ADAM10, and ADAM17, we examined the physiological role of ADAMs by expressing these ADAMs in COS-7 cells, and both "constitutive" and "regulated" α-secretase activities of these ADAMs were determined. We tried to suppress the expression of these ADAMs in human glioblastoma A172 cells, which contain large amounts of endogenous α-secretase, by lipofection of the double-stranded RNA (dsRNA) encoding each of these ADAMs. The results indicate that ADAM9, ADAM10, and ADAM17 catalyze α-secretory cleavage and therefore act as α-secretases in A172 cells. This is the first report that to suggest the endogenous α-secretase is composed of several ADAM enzymes.

Keywords: α-Secretase; ADAM9; ADAM10; ADAM17; Alzheimer's disease; APP; A172 cells; RNAi

Alzheimer's disease (AD), the most prevalent neurodegenerative disease, is characterized by progressive dementia and cognitive disorders. Amyloid senile plaques are observed in the AD cerebral vasculature at the histopathological level [1,2]. Amyloid β peptide (Aβ), a major component of senile plaques [3], is generated from its precursor protein, termed amyloid precursor protein (APP), by enzymatic digestion involving β- and γ-secretase activities [4]. In the normal brain, the non-amyloidogenic secretory pathway involving α-secretase activity is predominant over the amyloidogenic pathway [2]. The β-secretase was identified as a membrane-bound aspartic protease named BACE1 (beta-site APP cleaving enzyme 1) by enzymology and genetics experiments [5–8]. The γ-secretase was considered to be a complex, containing presenilin as a major component [9–11]. Several lines of evidence suggest that the α-secretase activity is modulated by metal ions and metalloprotease inhibitors, and metalloprotease/disintegrin, ADAM9, ADAM10, or ADAM17, have been proposed as the α-secretase [12–14].

ADAM is a multi-functional gene family, some of which have been shown to play a role in diverse biological processes such as fertilization, myogenesis, neurogenesis, and the activation of growth factors/immune regulators [15,16]. The ADAM family has common characteristic domains, and some ADAMs have a consensus zinc-binding motif, HEXXH, in the catalytic domain. Therefore, it is thought that ADAMs are potentially active metalloproteases and their protease activity has actually been demonstrated by several groups [12–14,17–28]. For

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example, ADAM17, which is also known as TACE (TNF-α converting enzyme), has protease activity against multiple substrates including TNF-α, TGF-α, TNF receptor, interleukin-6 (IL-6), L-selectin, and APP [14,18–23]. *Drosophila* ADAM10, Kuzbanian, is also a sheddase, and has been found to release a soluble form of Delta, a Notch ligand [24]. Moreover, ADAM10 has been shown to process precursor TNF-α, type IV collagen, ephrin-A2, and APP [13,25–27]. ADAM9 (also MDC9 or meltrin γ) has been reported to shed the heparin-binding EGF-like growth factor (HB-EGF) [28], and we independently demonstrated that mouse ADAM9 (mADAM9) has an α-secretase activity in COS-7 cells [12]. To clarify further the role of ADAM proteins as α-secretases, we in the present studies examined whether the endogenous ADAM9, ADAM10, and ADAM17 can indeed specifically process APP.

Materials and methods

Antibodies. Anti-APP antibody (6E10), which recognizes residues 1–17 of the Aβ domain of full-length APP, was used to detect sAPPα (secreted form of APP by α-secretase). Anti-*myc* antibody was used to detect the expression of ADAMs in COS-7 cells. 6E10 antibody and anti-*myc* antibody were purchased from Senetek and Invitrogen, respectively.

Constructs. All expression constructs were engineered by PCR amplification of human ADAM9, ADAM10, and ADAM17, and the sequence of each plasmid was confirmed by DNA sequencing. Full-length human ADAMs were cloned into pSecDK vector, which was modified to delete the Igκ-chain leader sequence from pSecTag A vector (Invitrogen).

Comparison of α-secretase-like activities of ADAM9, ADAM10, and ADAM17 in COS-7 cells. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). COS-7 cells were transfected by a lipofection method using FuGENE 6 transfection reagent (Roche). One microgram of ADAM expressor plasmid was used per transfection. After 40–48 h transfection, the conditioned media were replaced by new serum-free media. Five hours after replacement of the media, ADAM-overexpressed cells were treated with/without 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (final concentration 30 nM). One hour after addition of TPA, the secreted proteins were harvested and concentrated with trichloroacetic acid (TCA). The samples were mixed in sample buffer containing β-mercaptoethanol and analyzed by SDS–PAGE and Western blotting. Cells were collected and sonicated in phosphate-buffered saline without magnesium and calcium [PBS(-)]. After centrifugation at 10,000*g* for 30 min, the protein concentration of the supernatant was determined with a DC protein assay kit (Bio-Rad). The supernatant was diluted in sample buffer containing β-mercaptoethanol and analyzed by SDS–PAGE and Western blotting. The amounts of sample applied to the gel were normalized to the protein concentration.

dsRNA preparation. 21-nucleotide RNAs with the same sequences as ADAM9 (CUCCUUGGAGAUUAACUAGUU and UUGAGGA ACCUCUAAUUGAUC), ADAM10 (AGACAUUAUGAAGGAU UAUUU and UUUCUGUAAUACUUCCUAAUA), and ADAM17 (GAGAAGCUUGAUUCUUUGCUU and UUCUCUUCGAACU AAGAAACG) were chemically synthesized (JBioS). For annealing short interfering RNAs (siRNAs), 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM Hepes–KOH at pH 7.4, and 2 mM magnesium acetate) for 1 min at 90 °C, followed by 1 h at 37 °C.

Effect of ADAM RNAi on α-secretase activity in A172 cells. A172 cells were cultured in DMEM supplemented with 10% FBS. A172 cells were transfected by a lipofection method using LipofectAmine Plus reagent (Invitrogen). Three microliter of ADAM dsRNAs (final concentration 24 nM) were used per transfection. After 3–30 h transfection, the conditioned media were replaced with new serum-free media. Eight hours after replacement of the media, the secreted proteins were harvested and concentrated with trichloroacetic acid (TCA). The samples were mixed with sample buffer containing β-mercaptoethanol and analyzed by SDS–PAGE and Western blotting. The amounts of sample applied to the gel were normalized to the number of cells.

Total RNA preparation. Human brain total RNA was purchased from Clontech. Total RNA from A172 cells was extracted by the standard acid guanidium–phenol–chloroform (AGPC) method. The integrity of the RNA was confirmed by agarose gel electrophoresis and stained with ethidium bromide.

Quantitative analysis of ADAM9, ADAM10, and ADAM17 mRNAs in brain. RT-PCR analysis was performed using total RNA from human brain or A172 cells. A reverse-transcription of the total RNA was conducted with each ADAM oligonucleotide and GAPDH using ThermoScript RT-PCR System (Invitrogen) according to the manufacturer's protocol. PCR amplification was carried out by 30 cycles; each cycle consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C. The sense and antisense oligonucleotides used in the PCR were as follows: GAPDH (452 bp) (sense) 5′-ACCACAGTCCATGCCATCAC-3′ and (antisense) 5′-TCC ACCACCCTGTTGCTGTA-3′; ADAM9 (463 bp) (sense) 5′-TTGCC ACAGACCCGGTATGT-3′ and (antisense) 5′-CTCTCCCATCATC GTGATTC-3′; ADAM10 (440 bp) (sense) 5′-ACACCAGCGTGCCA AAAGAG-3′ and (antisense) 5′-CCTCTACACCAGTCATCTGG-3′; and ADAM17 (451 bp) (sense) 5′-TTTCAAGGTCGTGGTGGT GG-3′ and (antisense) 5′-TTCCCCTCTGCCCATGTATC-3′. PCR products were assessed by 4.8% acrylamide gel electrophoresis and stained with ethidium bromide. The difference of efficiency of PCR with ADAM9, ADAM10, or ADAM17 oligonucleotides was normalized by PCR products, with each ADAM oligonucleotide using the above expressor plasmids encoding each ADAM sequence as a template.

Image analysis. The detected bands were quantified with software Scion Image (Scion).

Results and discussion

First, to investigate α-secretase activity, human ADAM9, ADAM10, and ADAM17 were cloned and expressed in COS-7 cells. As shown in Fig. 1, ADAM10 and ADAM17 have an α-secretase-like activity toward the endogenous APP in COS-7 cells, as previously reported [13,14]. Human ADAM9 also has an α-secretase-like activity when expressed in COS-7 cells, as previously reported in mouse ADAM9 (mADAM9) [12]. The protein sequence of human ADAM9 is 85% identical to the mouse ortholog and identity of the catalytic domain with mouse counterparts is 96%. Activation of these ADAMs by protein kinase C (PKC) via 12-*O*-tetradecanoylphorbol-13-acetate (TPA) results in increases in the amount of the secreted form of APP by α-secretase (sAPPα) in the medium. The results indicate that all these ADAMs have constitutive and regulatory APP α-secretase activities in vivo.

Next, to identify the endogenous α-secretase in A172 cells, RNA interference (RNAi) was induced by double-

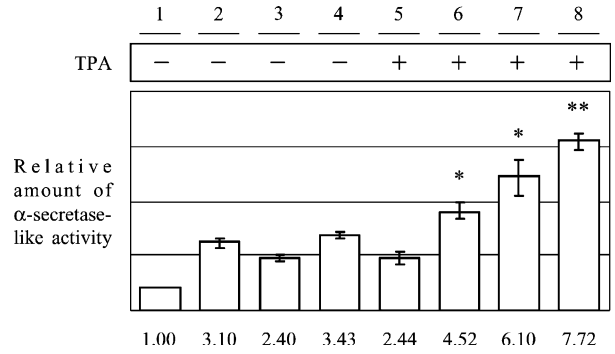

Fig. 1. Comparison of α-secretase-like activity of ADAM9, ADAM10, and ADAM17 in COS-7 cells. COS-7 cells were transiently transfected with ADAM9 (lanes 2 and 6), ADAM10 (lanes 3 and 7), ADAM17 (lanes 4 and 8) or pSecDK expression vector as a negative control (lane 1 and 5). Lanes 1–4 without TPA treatment; lanes 5–8 with 30 nM TPA. Densitometric quantification of the relative amount of sAPPα using Scion Image. The negative control (lane 1) was normalized to 1.0. The amounts of sample applied to the gel were normalized to the protein concentration in supernatant of the sonicated cells. The results are averages ± SD of three experiments. $*P < 0.05$ and $**P < 0.005$ relative to the activity given by each TPA untreated ADAMs, by Student's t test.

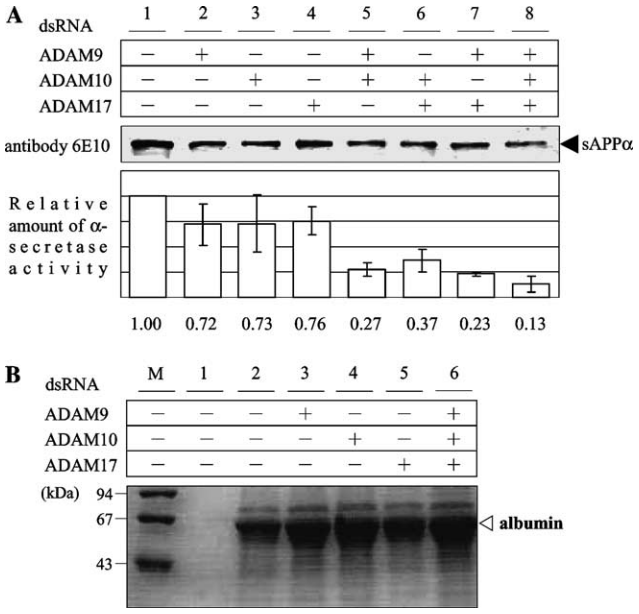

Fig. 2. Effect of ADAM RNAi on α-secretase activity in A172 cells. (A) ADAM dsRNA/dsRNAs was/were transfected into A172 cells. Non transfected cells served as a negative control (lane 1). Single dsRNAs (ADAM9: lane 2, ADAM10: lane 3, and ADAM17: lane 4), double dsRNAs (ADAM9 and ADAM10: lane 5, ADAM10 and ADAM17: lane 6, ADAM9 and ADAM17: lane 7), and triadic dsRNA were transfected (lane 8). (upper panel) Transfection/nontransfection of ADAM dsRNA(s) into A172 cells. (middle panel) Western blotting of total sAPPα in the medium with anti-APP antibody, 6E10. (lower panel) Densitometric quantification of the relative amount of sAPPα using Scion Image. Control (lane 1) was normalized to 1.0. The amounts of sample applied to the gel were normalized to the number of cells. The results are the averages ± SD of three experiments. (B) Media from transfection/nontransfection of ADAM dsRNA(s) cells were run on acrylamide gel and stained with CBB. Lane 1: DMEM without FBS, lane 2: untransfected, lanes 3–5: ADAM9, ADAM10, or ADAM17 dsRNA was transfected, respectively, and lane 6: triadic dsRNA was transfected. M: molecular weight marker. Densitometric quantification of the relative amount of albumin protein was done using Scion Image.

stranded RNA (dsRNA) encoding ADAM sequences. Recently it was been reported that specific gene-silencing with 21-nucleotide dsRNAs in mammalian cells provides a useful and reasonable tool [29]. A172 cells are exceptional human glioblastoma cells with an endogenous potent α-secretase activity. As shown in Fig. 2A, the gene-silencing of ADAM results in the suppression of α-secretase activity in A172 cells. A single application of RNAi decreased the amount of sAPPα in the medium by 25% compared with the control, and double RNAi and triple RNAi caused a 63–77% and 87% suppression of α-secretase activity, respectively. ADAM dsRNAs did not affect the secretion of albumin in A172 cells (Fig. 2B).

Lastly, to compare the expression of these ADAM mRNAs in brain, semi-quantitative RT-PCR was performed using human brain total RNA. The detected bands were quantified by image analyzer and the amounts were revised by the efficiency of PCR using the ADAM oligonucleotides (Fig. 3B). As shown in Fig. 3A, expression of ADAM10 mRNA was the lowest among three ADAMs and the relative amounts of ADAM9 and ADAM17 mRNA in brain were 1.71- and 1.25-fold, respectively.

In this study, we demonstrated that ADAM9, ADAM10, and ADAM17 have an α-secretase-like activity. The present results suggest that ADAM10 and ADAM17 carry out "constitutive α-cleavage," which is maintained constantly, or "regulated α-cleavage," which is modulated by activating the PKC cascade, respectively. Recently, it was reported the basal formation and secretion of sAPPα are not affected in cells derived from ADAM17 knock-out mice [14]. This report supports that our results. The α-secretase-like activity of ADAM9 was also activated by TPA in transiently expressed COS-7 cells; therefore ADAM9 also works as "regulated α-secretase." Together with previously reported data that both "constitutive α-cleavage" and "regulated α-cleavage" are by ADAM10 in HEK293 cells and that ADAM10 is not essential for α-secretase activity in fibroblasts [13,30], one possible explanation for this is that both "constitutive" and "regulated" α-cleavage of APP might depend on ADAM9, ADAM10, and/or ADAM17.

We showed that ADAM9, ADAM10, and ADAM17 act as α-secretases in A172 cells. A172 cells have a much more endogenous α-secretase activity than HEK293 and COS-7 cells. ADAM9, ADAM10, and ADAM17 mRNAs are highly expressed in A172 cells than in brain (data not shown). Our results provide the first proof that ADAM family members act as endogenous α-secretase(s)

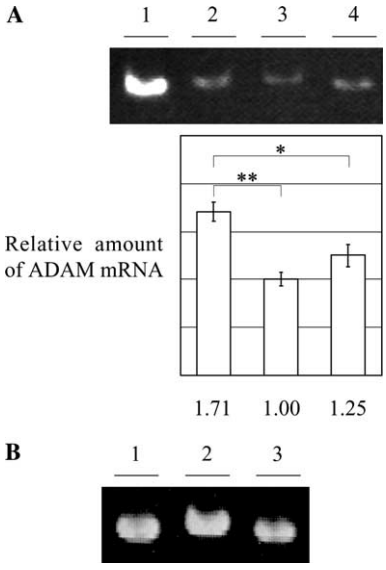

Fig. 3. Quantitative analysis of ADAM9, ADAM10, and ADAM17 mRNAs in brain. (A) (upper panel) Semi-quantitative RT-PCR was performed using human brain total RNA. Lane 1: GAPDH, lane 2: ADAM9, lane 3: ADAM10, and lane 4: ADAM17. The PCR products were run on acrylamide gel and stained with ethidium bromide. (lower panel) Densitometric quantification of relative amount of PCR products relative to GAPDH. ADAM10 was normalized to 1.0. The results are averages ± SD of seven experiments. $*P < 0.05$ and $**P < 0.005$ relative to the value given by ADAM9, by Student's t test. (B) PCR was performed using the equal ADAM expressor plasmid as a template to calculate the PCR efficiency of ADAM oligonucleotides. Lane 1: ADAM9, lane 2: ADAM10, and lane 3: ADAM17. The PCR products were run on acrylamide gel and stained with ethidium bromide.

and that the α-secretase may comprise more than one gene product.

Several investigators have studied the tissue distribution of ADAM9, ADAM10, and ADAM17. The mRNA of ADAM9 is ubiquitously expressed in human tissues [31]. ADAM10 mRNA is expressed in kidney, spleen, lymph node, thymus, liver, bone marrow, and brain [25,26]. Strong expression of ADAM17 mRNA is found in macrophages [18]. The tissue distributions of the mRNAs vary among these ADAMs. Our results that ADAM9 mRNA expression in human brain is higher than ADAM10 and ADAM17 mRNAs suggested that ADAM9 mainly plays a role as "constitutive" and/or "regulated" α-secretase. Moreover, ADAM9 has a splicing variant, which lacks the carboxyl-terminus, and the secreted short form of ADAM9 also has an α-secretase-like activity toward APP [32].

The stimulation of α-cleavage by APP secretase activity leads to a significant decrease in Aβ formation [33]. Therefore, the stimulation of α-secretase activity has been theorized to reduce Aβ production for the development of drugs to treat Alzheimer's disease.

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