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The Metalloprotease Meprin β Generates Amino Terminal-truncated Amyloid β Peptide Species*

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Background: Meprin β cleaves the amyloid precursor protein.
Results: Meprin β-mediated cleavage of the amyloid precursor protein leads to an increase of amyloid β production and to the generation of an N-terminal truncated amyloid β variant.
Conclusion: Meprin β can generate N-terminal truncated amyloid β peptides.
Significance: Our data indicate that meprin β is a novel protease in amyloid β generation.

The amyloid β (Aβ) peptide, which is abundantly found in the brains of patients suffering from Alzheimer disease, is central in the pathogenesis of this disease. Therefore, to understand the processing of the amyloid precursor protein (APP) is of critical importance. Recently, we demonstrated that the metalloprotease meprin β cleaves APP and liberates soluble N-terminal APP (N-APP) fragments. In this work, we present evidence that meprin β can also process APP in a manner reminiscent of β-secretase. We identified cleavage sites of meprin β in the amyloid β sequence of the wild type and Swedish mutant of APP at positions p1 and p2, thereby generating Aβ variants starting at the first or second amino acid residue. We observed even higher kinetic values for meprin β than BACE1 for both the wild type and the Swedish mutant APP form. This enzymatic activity of meprin β on APP and Aβ generation was also observed in the absence of BACE1/2 activity using a β-secretase inhibitor and BACE knock-out cells, indicating that meprin β acts independently of β-secretase.

Late-onset Alzheimer disease (AD)[4] is the most common, progressive, and incurable form of dementia. In brains of patients, loss of neurons and synapses occurs as a result of the accumulation of amyloid β (Aβ) peptides and hyperphosphorylated forms of microtubule associated protein Tau (1). Although the deposition of Aβ peptides in extracellular, insoluble amyloid plaques in post-mortem brain samples correlates poorly with the cognitive ability of patients at the time of death (2, 3), the concentration of soluble Aβ peptides assayed by biochemical methods is strongly associated with cognitive ability (3–5). This and the discovery of mutations in the amyloid precursor protein (APP) and the presenilin (*PSEN*) genes, which cause autosomal dominant early-onset forms of AD (familial AD) by an increased generation of Aβ peptides, support the "amyloid hypothesis of AD," which states that abnormal accumulation of Aβ peptides in the brain is the primary event that causes AD. The Aβ peptide is generated from APP during the normal cellular metabolism (6, 7). The major β-site APP cleaving enzyme (β-secretase, BACE1) generates the N terminus of Aβ from the full-length APP (8). This cleavage produces a membrane-bound APP carboxyl-terminal fragment (CTF) serving as a substrate for the γ-secretase complex generating Aβ peptides (9). Aβ is a generic name for a variety of peptides, the majority of which are 1–40 amino acids in length. Two different N termini of Aβ, Asp in p1 ($Aβ_{1-x}$) and Glu in p11 ($Aβ_{11-x}$), are generated as a result of BACE1-dependent cleavage of APP (10). However; N-terminally truncated Aβ variants have been found in the cerebrospinal fluid of AD patients, starting with the alanine in p2 position (Aβ2–40) that cannot be attributed to BACE activity (11–14).

We have recently shown that APP is processed by the metalloprotease meprin β, generating novel, soluble N-terminal APP fragments (15). Meprin β is a type I transmembrane protein of the astacin protease family displaying a wide expression pattern in humans (16–18). To date, only a few *in vivo* substrates of meprin β have been identified, *e.g.* interleukin-1β and VEGF-A

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[4] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β; N-APP, N-terminal APP; APP, amyloid precursor protein; CTF, carboxyl-terminal fragment; EGFP, enhanced GFP; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DMSO, dimethyl sulfoxide; MEF, mouse embryonic fibroblast; sAPP, soluble APP; AICD, APP intracellular domain.

(vascular endothelial growth factor A) (17, 19–21). Using a proteomic approach, based on peptide libraries and native proteins, we discovered several new substrates, including APP, and identified a unique cleavage specificity for meprin β, with a preference for acidic amino acid residues (22). Here, we examined the role of meprin β in overall Aβ production. Our results demonstrate that meprin β processes APP by generating truncated Aβ peptides starting in p2 position independent of BACE1.

EXPERIMENTAL PROCEDURES

The HEK293T cell line has been purchased from Invitrogen. All common chemicals have been purchased from Carl Roth Chemicals and Sigma unless stated otherwise. Cell culture medium and accompanying reagents have been purchased from Invitrogen and Lonza. Cell culture plastics have been obtained from Techno Plastic Products.

Identification of Aβ Cleavage Sites—Substrate peptides (SEVKMDAEFR; SEVNLDAEFR) were purchased from Bachem Distribution Services GmbH (Weil am Rhein, Germany). Cleavage of peptides by recombinant meprin β (23) was performed in a molar ratio of 400:1 at 37 °C for 120 min and inactivated by following heating at 65 °C for 10 min. Samples were further analyzed by MALDI-TOF (Centre Commun de Microanalyse des Protéines of the Institut Fédératif de Recherche 128, Lyon, France).

Activity Assays Using Fluorogenic Peptides to Validate Catalytic Properties of Meprin β—To test the enzymatic efficiency of meprin β for different APP substrates, we used quenched fluorogenic peptides (see Fig. 1) that were obtained from Bachem Distribution Services GmbH (Weil am Rhein, Germany). The enzyme activity was measured with the fluorescent spectrometer Varioskan Flash (Thermo Scientific). Data were analyzed using Skan It Software for Varioskan Flash (version 2.4). Enzyme was buffered in 50 mM HEPES, pH 7.5, and used in a final concentration of 1×10^{-9} M. Final substrate concentration ranged from 5 μM to 100 μM. Fluorescence of the substrate was detected every 12 s for 120–240 min at 37 °C. The proteolytic activity was related to the emission at 405 nm with an excitation at 320 nm. The activity was determined by the slope of the initial linear range of the curve. Kinetics (K_m, k_{cat}, and k_{cat}/K_m) were calculated using the GraFit software (version 4.0, Erithacus Software, Ltd., Staines, UK).

Quantitative Real-time PCR—Total RNA extracted from 20 human brain samples (10 AD brains, 10 age-matched, non-demented normal brains) was transcribed into cDNA using random primers and SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The obtained cDNA was subjected to quantitative real-time PCR measurement using the StepOnePlus RT-PCR system (Applied Biosystems, Darmstadt, Germany). Amplification reaction consisted of a hold of 10 min at 95 °C and 40 cycles with subsequent recording of primer melting curves. The primer sequences for amplification of the target gene Mep1b were meprin_fwd: tgctgatcatcacccttgtc, and meprin_rev: cggagtcaaatttggtcgat. The transcript level was normalized to the transcript level of ARF1 (ADP-ribosylation factor 1). As reference samples, we used commercially available adult human renal RNA (Agilent Technologies, Waldbronn, Germany), adult human brain RNA (BD Biosciences), and Stratagene universal human reference RNA (Agilent Technologies, Cedar Creek, TX).

Statistical analysis was performed using the Mann-Whitney test (non-parametric, one-tailed *t* test). Tissue samples were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience (Amsterdam, The Netherlands). All material has been collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by The Netherlands Brain Bank.

Transient Transfections of HEK293T cells with APP751 and Meprin β—HEK293T cells were grown in DMEM (Invitrogen), containing 4.5 g/liter D-glucose, 2 mM L-glutamine, sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (PAA Laboratories). HEK293T cells were transiently transfected with the following cDNAs: 1 μg of pcDNA3 (Invitrogen) and 1 μg of APP751wt-pCI-neo; 1 μg of pcDNA3 and 1 μg of meprin β-pIRES2-EGFP; 1 μg of APP751wt-pCI-neo and 1 μg of meprin β-pIRES2-EGFP using FuGENE HD transfection reagent (Roche), according to the manufacturer's instructions. After 24 h, cells were incubated with serum-free medium overnight. To investigate the specificity of Aβ generation, a γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) (Merck), was used at a final concentration of 5 μM. To inhibit β-secretase cleavage in these cells, the BACE IV inhibitor (Merck) was used at a final concentration of 1 μM. Actinonin, an inhibitor of meprin β, was used at a final concentration of 100 μM to check whether the meprin β-mediated cleavage can be blocked. If no inhibitor was used, the cells were treated with 1% DMSO as a vehicle control.

Treatment of APP751 wt Overexpressing Cells with Actinonin—24 h after transfection of HEK293T cells with 2 μg of cDNA containing APP751wt-pCI-neo, 800 μl of serum-free medium with or without 10 μM actinonin was added to the cells and incubated for 24 h. Aβ was immunoprecipitated from the collected medium using the IC-16 antibody.

γ-Secretase Activity Assay—HEK293T cells stably overexpressing APP695 C-terminally fused to the yeast DNA binding domain Gal4 were treated for 2 h with 100 μM actinonin or 5 μM DAPT. The generation of the APP intracellular domain (AICD)-Gal4 fusion proteins under the presence of the different inhibitors was analyzed by Western blotting in cell lysates using an anti-C-terminal APP antibody, 369 (24).

Mass Spectrometric Analysis of Aβ Generated in HEK293T Cells Overexpressing APP751 and Meprin β—HEK293T cells were transfected in 6-cm cell culture dishes using 2 μg of pcDNA3 and 2 μg of APP751wt-pCI-neo; 2 μg of pcDNA3 and 2 μg of meprin β-pIRES2-EGFP; and 2 μg of APP751wt-pCI-neo and 2 μg of meprin β-pIRES2-EGFP. As a control, co-transfected cells were treated with 5 μM DAPT or DMSO overnight. Aβ was immunoprecipitated from conditioned medium with antibody 4G8 and protein G-Sepharose. In another approach, we used PS70 cells, stably expressing meprin β, glutaminyl cyclase, and APP751 wt. To increase APP expression, the cells

were additionally infected with an APP containing adenovirus. The cells were treated with 5 μM DAPT, 100 μM actinonin, 1 μM BACE inhibitor IV, or 1% DMSO overnight. Aβ was immunoprecipitated from conditioned medium with antibody W0–2 and protein G-Sepharose.

Sepharose was washed twice in phosphate-buffered saline (PBS) and twice in 100 mM ammonium acetate. Aβ was eluted twice with 300 μl of 50% acetic acid and vacuum-dried. The sample was resuspended in 10 μl of 33% acetonitrile containing 0.1% trifluoric acetic acid and ultrasonicated. MALDI-MS analysis was carried out on sinapinic acid matrix with an UltraflexII TOF/TOF (Bruker Daltonics).

Treatment of APP-overexpressing Cells with Exogenous Meprin β—Chinese hamster ovary (CHO) cells stably overexpressing APP751 wt, 7WD10, were incubated with serum-free medium and treated with active meprin β or its inactive form, an E90A mutant at final concentrations of 0.3 nM, 0.6 nM, 0.8 nM (prepared in 50 mM HEPES buffer, pH 7.5) for 5 and 16 h. As a control, HEPES buffer was added. Following the indicated time points, cell medium was collected and analyzed using SDS-PAGE and Western blotting.

Generation of BACE1/2-KO Mouse Fibroblasts (MEFs) Overexpressing Human APP695 Isoform and Human Meprin β—BACE1/2 double KO MEFs have been described previously (25). They were grown in DMEM, containing 4.5 g/liter D-glucose, 2 mM L-glutamine, sodium pyruvate, minimum essential medium non-essential amino acids (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. To generate an APP695-containing retrovirus, 7 μg of APP695-pLHCX and 7 μg of pVSV-G plasmid DNAs were co-transfected into retrovirus packaging cells GP2-293, by using TurboFect *in vitro* transfection reagent (Fermentas) according to the manufacturer's instructions. Cell medium containing the retrovirus was collected 48 h after transfection and used to infect BACE1/2 double KO MEFs. 24–48 h after the infection, cells were incubated with cell culture medium containing 25 μg/ml hygromycin B (Invitrogen) as a selection antibiotic. A clone with high expression of APP was used in further experiments.

To obtain meprin β expression in these clones, a meprin β-containing retrovirus was generated in analogy to APP695 using a pLBCXmeprin β HA tag construct. Blasticidin in a final concentration of 2 μg/ml was used as a selection antibiotic.

Analysis of Aβ Production in BACE1/2-KO Mouse Fibroblasts Overexpressing Human APP695 and Human Meprin β—BACE1/2 double KO MEFs stably expressing human APP695 isoform and human meprin β were incubated with fresh medium with or without 5 μM DAPT, 10 μM actinonin, or 0.1% DMSO overnight. The conditioned medium was collected, and Aβ peptides were immunoprecipitated and analyzed and compared with synthetic Aβ1–40 peptide from Genosphere Biotech (Paris, France).

SDS-PAGE, Western Blotting, and Immunoprecipitations—Cells were washed with PBS, and cell lysates were prepared with Nonidet P-40 lysis buffer (500 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.02% sodium azide) and a mixture of protease inhibitors (Complete, Roche Applied Science). Equal amounts of total protein, determined by BCA protein assay (Pierce Chemicals, Rockford, IL), were used for analysis. Cell lysates were used for detection of full-length APP (APP), membrane-bound meprin β, and tubulin using an anti-C-terminal APP antibody, 369 (24), anti-meprin β antibody, MEP1B (R&D Systems), or anti-tubulin antibody, respectively.

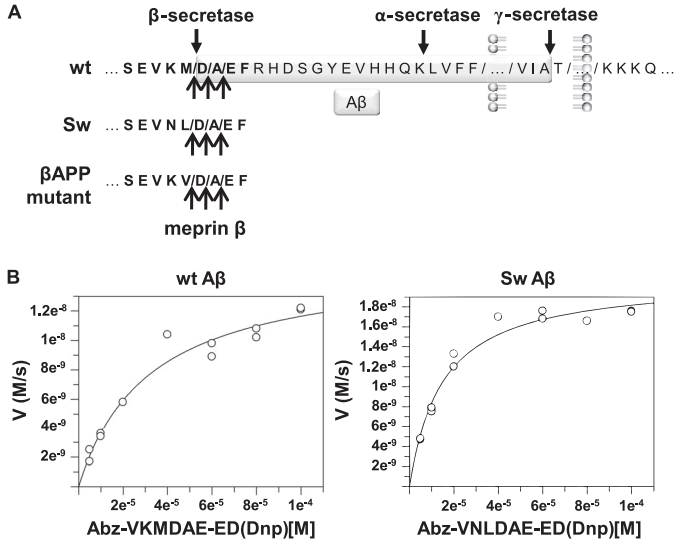
Collected cell medium was used for detection of soluble APP (sAPP), soluble meprin β, and total Aβ using an anti-N-terminal APP antibody, 22C11 (26), MEP1B or IC16 antibody (27), respectively. All samples were mixed with 2× Laemmli sample buffer (28), heated at 95 °C for 5 min, and resolved on 4–12% NuPage (Novex, Invitrogen) gradient gel electrophoresis (SDS-PAGE) by using MES running buffer (Invitrogen). Following Western blotting, the resulting nitrocellulose membrane (Millipore, Bedford, MA), containing transferred proteins, was blocked in PBS containing 5% nonfat milk and 0.2% Tween 20.

When indicated, total Aβ was immunoprecipitated from cell medium with the IC16 antibody (27) and protein A-agarose beads (Invitrogen) and resolved on a 14% sodium dodecyl sulfate polyacrylamide gel and subsequently probed on a PVDF (polyvinylidene fluoride) membrane. Final Aβ detection was carried out with the IC16 antibody.

Signal detection in all Western blotting experiments was carried out using HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) assay solutions (Millipore) and LAS-3,000mini (Fujifilm, Duesseldorf, Germany). Western blots were quantified by using NIH ImageJ (version 1.44). Stastical analysis was performed using GraphPad Prism software (version 5, GraphPad Software, San Diego, CA).

RESULTS

Kinetics of Aβ Generation by Meprin β—We have recently demonstrated that the N-terminal extracellular domain of APP is a substrate for the metalloproteinase meprin β. Moreover, we observed that the level of full length APP decreases after meprin β overexpression, which might indicate further meprin β cleavage sites within the APP sequence (15). To further determine specific meprin β cleavage sites within the APP amino acid sequence, three peptide substrates derived from APP representing β-secretase cleavage sites were analyzed by MALDI-TOF after meprin β incubation (Fig. 1*A*). Peptide sequences were used corresponding to the APP wild type (wt) and to the APP Swedish (swe) cleavage sites, respectively. A third substrate was investigated that, due to a missense mutation (M/V), is not cleaved by the β-secretase (10, 29). MALDI-TOF analysis revealed meprin β-mediated cleavage of the APP wt and APP swe peptide sequences indicative for Aβ generation (Fig. 1*B*). Although meprin β shows cleavage activity at the aspartate in p1 correlating with the BACE1 cleavage site, more importantly, and for the first time, we present a protease that cleaves the peptide at the alanine in p2 within all three peptides. Additionally, meprin β cleaves the peptide bond in p3, resulting in an N-terminal glutamate residue. To further analyze the proteolytic efficiency of meprin β for APP wt and APP swe amino acid sequences, the hydrolysis of two fluorogenic substrates and the kinetic parameters K_m, k_{cat}, and k_{cat}/K_m were determined (Fig. 1, *B* and *C*). Compared with BACE1 (30–32), meprin β exhibited a 10^4- and 10^3-fold higher catalytic efficiency toward the APP wt and the swe substrate under these experimental conditions. Due to a decreased K_m indicating increased affinity



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kinetic parameters	wt VKMDAE	Sw VNLDAE
K_m (M)	3.2 x 10^{-5}	1.5 x 10^{-5}
Std. error K_m	5.87 x 10^{-6}	1.93 x 10^{-6}
V_{max} (M)	1.53 x 10^{-8}	2.1 x 10^{-8}
Std. error V_{max}	1.1 x 10^{-9}	8.0 x 10^{-10}
K_{cat} (s^{-1})	15.3	20.9
K_{cat}/K_m ($M^{-1}s^{-1}$)	4.8 x 10^5	1.41 x 10^6

FIGURE 1. **Catalytic properties and cleavage sites of meprin β within different APP peptides.** *A*, the amino acid sequence of APP with the wild type Aβ region (highlighted in a *gray box*) is presented, indicating the α-, β-, and γ-secretase cleavage sites. The peptide sequences analyzed are displayed in *letters in boldface type*. *Black arrows* indicate the cleavage sites analyzed by MALDI-TOF. Sequences are shown in the one letter code. *Sw*, Swedish mutant. *B*, kinetic parameters of Aβ cleavage by meprin β. The calculated kinetic constants for the wt β-site fluorogenic substrate (Abz-VKMDAE-EDDnp) are displayed in the *left panel*, for the Swedish mutated β site (Abz-VNLDAE-EDDnp) on the right. *Abz*, aminobenzoic acid; *EDDnp*, ethylenediamine 2.4-dinitrophenyl; *M*, molar mass; *v*, velocity. *C*, fluorogenic APP substrates for kinetic calculations. The enzyme concentration used in all assays was 1×10^{-9} M, and substrate concentrations varied as indicated in *B*. *Std.*, standard.

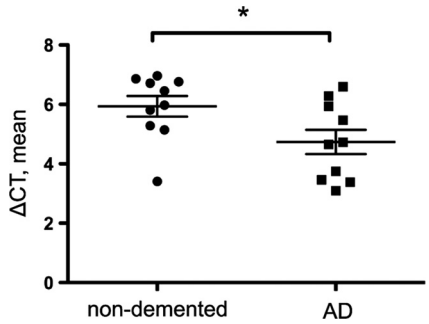

FIGURE 2. **Meprin β mRNA levels are increased in brains of AD patients compared with age-matched control individuals.** Quantitative real-time PCR of total RNA extracted from 10 age-matched healthy individuals and 10 AD patients. Statistical analysis revealed significantly higher levels of meprin β mRNA in the brain samples obtained from AD patients. To compare levels of meprin β RNA, ΔC_T, mean values were analyzed. Statistical analysis was performed using the Mann-Whitney test (non-parametric, one-tailed *t* test, statistical significance, *, $p < 0.05$).

toward a substrate, the k_{cat}/K_m for the APP swe substrate was even higher with a value of 1.4×10^6 M^{-1} s^{-1} compared with a k_{cat}/K_m of 4.8×10^5 M^{-1} s^{-1} for the APP wt peptide substrate (Fig. 1*C*).

Meprin β Expression in Non-demented and Alzheimer Disease Brains—To investigate whether the expression levels of meprin β might be altered in AD, we examined meprin β RNA levels in human brain of 10 age-matched healthy individuals and 10 AD patients. To quantify the expression levels of meprin β in human brain, we performed quantitative real-time PCR (Fig. 2) and to compare levels of meprin β RNA, ΔCT, mean values were analyzed. Statistical analysis revealed significantly higher levels of meprin β RNA in the brain samples obtained from AD patients (Fig. 2).

Meprin β Overexpression Causes Increased Generation of Aβ Peptides in HEK293T Cells—To examine the effects of meprin β on Aβ production in mammalian cells, wt human APP751 and human meprin β were transiently overexpressed in HEK293T cells. Immunoprecipitation and subsequent SDS-PAGE followed by Western blotting revealed that co-expression of human APP and human meprin β in HEK293T cells led to a significant increase in total Aβ secretion (Fig. 3*A*, *lane 3*). Treatment with DAPT, a γ-secretase inhibitor, demonstrated that the peptides generated by meprin β activity are produced in a γ-secretase-dependent manner (Fig. 3*A*, *lane 4*). Moreover, by using the hydroxamate actinonin, a potent active site zinc binding inhibitor of human meprin β (33) but not of BACE1, we could show that the increased Aβ production in meprin β-overexpressing cells was dramatically reduced after meprin β inhibition (Fig. 3*A*, *lane 5*). We wanted to exclude an inhibitory effect of actinonin on γ-secretase by performing an *in vitro* γ-secretase activity assay conducted in cells stably overexpressing APP695 C-terminally fused to the DNA binding domain Gal4. Our data clearly demonstrate that actinonin had no side effect on γ-secretase activity as AICD production was not affected by actinonin (Fig. 4). In contrast to the effect of actinonin, inhibition of the β-secretase with the BACE inhibitor IV did not cause any significant reduction in Aβ production, indicating that the increased Aβ release is most likely due to the activity of a metalloprotease (Fig. 3*A*, *lane 6*). To exclude that the observed effects are due to the solvent used for the inhibitors, the other cells were also treated with DMSO, which did not influence the Aβ production (Fig. 3*A*, *lanes 1–3*).

Analysis of the signals revealed an ~8-fold increase in Aβ secretion after co-expression of meprin β compared with APP overexpression alone, which was significantly reduced after treatment with the γ-secretase inhibitor DAPT to levels comparable in meprin β non-transfected cells. In co-expressing cells, treated with the meprin β inhibitor actinonin, a dramatically decrease in Aβ production could also be observed (Fig. 3*B*).

The role of meprin β in Aβ generation was further characterized by using the meprin β inhibitor actinonin to suppress endogenous meprin β activity (Fig. 5). APP overexpressing cells were treated with 10 μM actinonin during a 24-h period. Our results demonstrate a significant reduction of secreted Aβ levels following the inhibition of endogenous meprin β by actinonin. In accordance, APP levels increased due to meprin β inhibition. Because 10 μM actinonin do not inhibit γ-secretase activity, these results indicate that even endogenous levels of

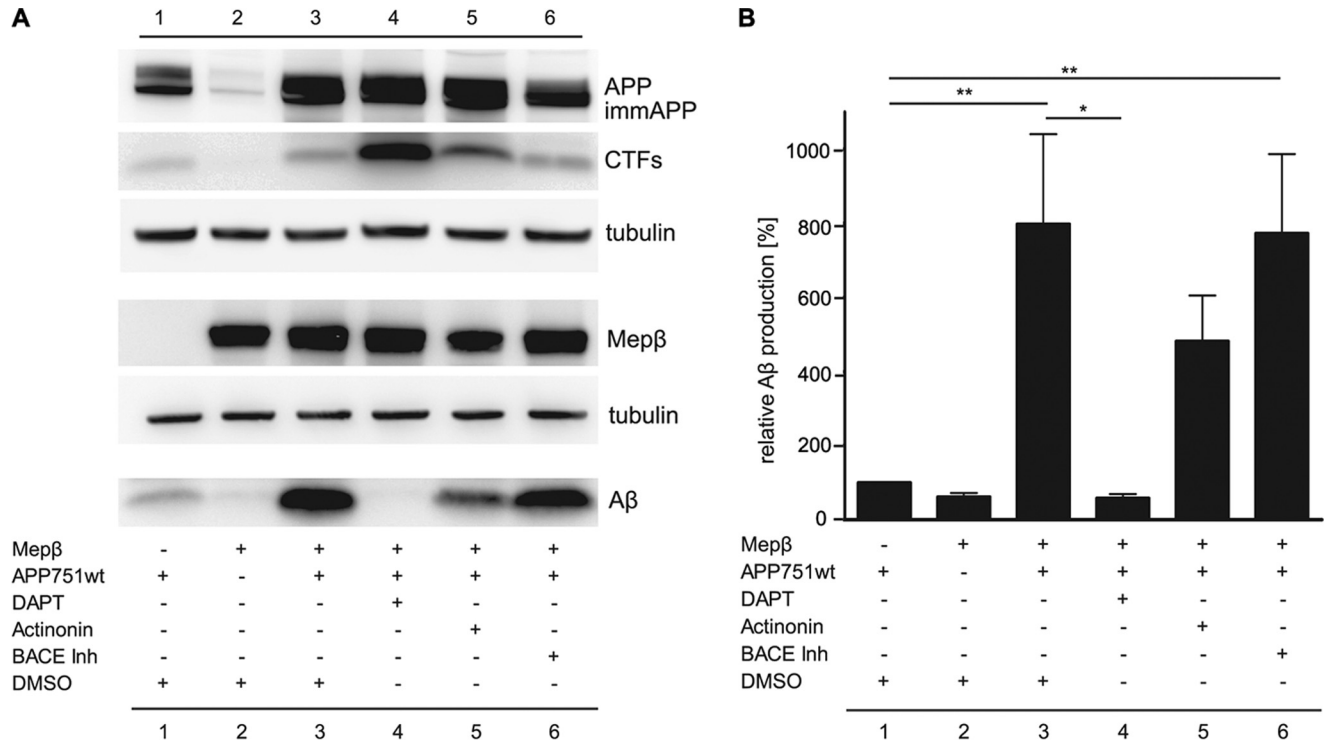

FIGURE 3. **Overexpression of meprin β and APP751 wt in HEK293T cells leads to an increase in Aβ production.** HEK293T cells transiently overexpressing human APP751 wt isoform and/or human meprin β were treated with 100 μM actinonin, a meprin β (*Mepβ*) inhibitor; 5 μM DAPT, a γ-secretase inhibitor; 1 μM BACE inhibitor IV (*BACE Inh*), a β-secretase inhibitor, or DMSO as a vehicle control, overnight. The amount of secreted Aβ was detected in cell culture medium by immunoprecipitation/Western blotting with the IC-16 antibody. *A*, protein levels of APP, meprin β, and tubulin were detected in the corresponding cell lysates. *B*, diagram showing Aβ production, depicted as percentage of APP751 wt single transfected cells. Comparison between APP751 wt overexpressing and APP751 wt and meprin β co-expressing cells revealed a significant increase in Aβ secretion due to meprin β activity. The Aβ production was decreased after treatment with the γ-secretase inhibitor DAPT, suggesting that meprin β produced Aβ is dependent on γ-secretase activity. Treatment with the meprin β inhibitor actinonin but not with a β-secretase inhibitor decreased Aβ levels, demonstrating that the increased Aβ generation in APP and meprin β co-expressing cells was dependent on meprin β but not BACE1 activity (graph shows mean ± S.E. ($n = 5$); statistical significance: *, $p < 0.05$; **, $p < 0.01$; one-way analysis of variance).

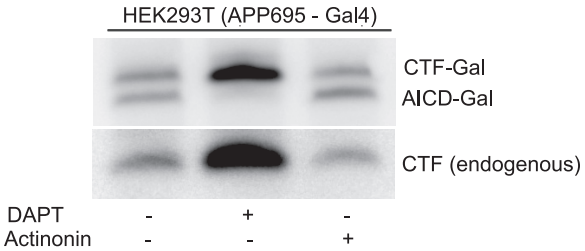

FIGURE 4. **Actinonin has no effect on γ-secretase activity.** HEK293T cells stably expressing APP695, which is C-terminally fused to the DNA binding domain Gal4, were treated with 100 μM actinonin or 5 μM DAPT for 2 h. AICD generation was detected in cell lysate using an anti-APP antibody, 396, and compared with untreated cells. Due to Gal4, the AICDs are stabilized, and signal detection is improved. After DAPT treatment, AICD generation was abolished, and the amount of CTFs fused to Gal4 but also endogenous CTF levels were increased. After treatment with actinonin, no difference in AICD generation could be observed, and CTF levels were comparable with untreated cells. Therefore, actinonin seems to have no effect on γ-secretase activity.

metalloproteases like meprin β, might contribute to a minor fraction of Aβ production in cell cultures.

Mass Spectrometry Analysis of Meprin β Generated Aβ Species—To gain further information about the cleavage sites of meprin β within APP and to verify the production of Aβ peptides through meprin β, we used MALDI-MS analysis.

For this approach, Aβ was immunoprecipitated from tissue culture supernatants of HEK293T cells, transiently overexpressing APP751 wt or meprin β or both, using the monoclonal antibody 4G8, which recognizes amino acid residues 17–24 of Aβ. In cells overexpressing APP751 wt alone, Aβ1–40 could be detected (Fig. 6*A*). Whereas cells overexpressing meprin β alone did only show diminished Aβ secretion derived of endogenous APP (Fig. 6*B*).

In cells co-expressing APP and meprin β, Aβ1–40, and additionally, an amino-terminal truncated Aβ2–40 variant could be detected (Fig. 6*C*). After DAPT treatment, all Aβ production could be abolished, suggesting that γ-secretase is also required for meprin β-mediated Aβ generation (Fig. 6*D*). Finally, addition of the vehicle DMSO to cells overexpressing APP and meprin β did not affect the observed Aβ species (Fig. 6*E*). To verify that the additional peak of an amino-terminal truncated form of Aβ is due to meprin β activity and not an effect of transient transfection, we used PS70 cells that stably overexpress meprin β. The cells were infected using an APP-adenovirus and treated with DAPT, actinonin, BACE inhibitor IV, or DMSO, as a vehicle control, overnight. Aβ was immunoprecipitated from supernatants, using the monoclonal antibody W0-2, which recognizes amino acid residues 5–8 of Aβ. In cells infected with the APP-adenovirus, a normal Aβ1–40 pattern could be detected (Fig. 6*F*). In cells which additionally express meprin β, Aβ1–40 and the additional peak, representing the amino-terminal truncated Aβ2–40 variant, could also be detected, the latter due to meprin β cleavage (Fig. 6*G*). After DAPT treatment, all Aβ production could again be abolished

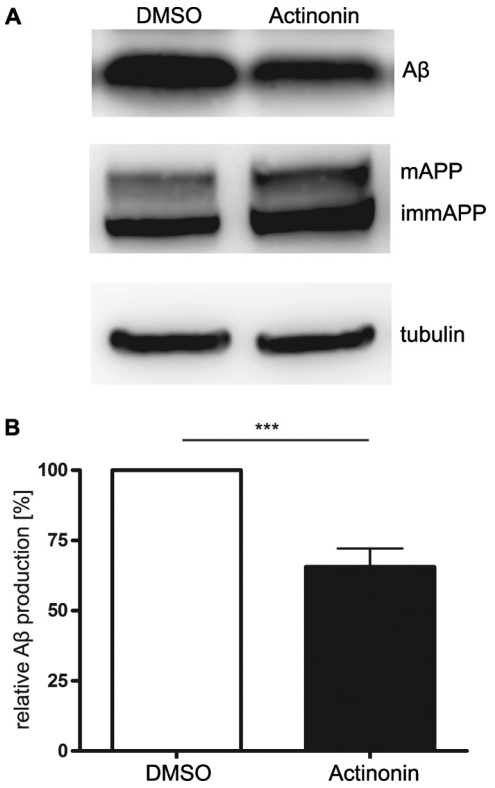

FIGURE 5. **Meprin β inhibition leads to a decrease in Aβ secretion.** HEK293T cells transiently overexpressing APP751 wt were treated with 10 μM actinonin, a meprin β inhibitor, for 24 h in serum-free medium. *A*, total secreted Aβ was detected in the samples of cell culture medium by immunoprecipitation using the IC-16 antibody. Protein levels of carboxyl-terminal APP fragments, CTFs, mature (*mAPP*), immature APP (*immAPP*), and tubulin were detected in the samples of cell lysates using an anti-APP antibody, 369, and an anti-tubulin antibody, respectively. *B*, the Aβ signals, obtained with the IC-16 antibody, were quantified and depicted as percentage of vehicle control. Actinonin treatment caused a statistically significant reduction in Aβ secretion (graph shows mean ± S.E. ($n = 5$); ***, $p = 0.0008$, *t* test).

(Fig. 6*H*). After actinonin treatment, the signal for Aβ2–40 disappeared, suggesting, that this Aβ variant is produced by meprin β (Fig. 6*I*). To investigate whether meprin β can produce Aβ without β-secretase activity within the cell, we treated the cells with BACE inhibitor IV. Even after β-secretase inhibition, we were able to detect a strong signal for Aβ1–40. Additionally, we detected a prominent peak of the amino-terminal truncated Aβ2–40 variant and further truncated Aβ variants (Fig. 6*J*). This provides evidence, that when β-secretases are inhibited, meprin β can still produce Aβ1–40, Aβ1–39, and Aβ2–40. Taken together, these data indicate, that meprin β is capable of generating Aβ1–40, mimicking a β-secretase activity. Additionally, we were also able to show that meprin β generates an N-terminal truncated form of Aβ2–40 in living cells.

Meprin β Overexpression in BACE 1/2 Knock-out Cells Induces Aβ Generation—To further prove whether meprin β cleaves APP directly or whether meprin β activity is indirectly mediated through BACE1, we used BACE1/2 knock-out MEF cells stably overexpressing the human APP695 isoform. Indeed, we were able to detect Aβ in the absence of BACE1 and BACE2 in meprin β-overexpressing cells after immunoprecipitation of conditioned medium. As expected, Aβ secretion was not detectable in BACE1/2-deficient cells overexpressing only APP695 (Fig. 7*A*). To analyze the involvement of γ-secretase in the meprin β-induced peptide release, cells were incubated with DAPT overnight, which resulted in a complete inhibition of meprin β-mediated Aβ generation with a concomitant increase in APP-CTFs (Fig. 7*C*). To inhibit meprin β activity, cells were incubated with actinonin, which again resulted in decreased Aβ secretion compared with control cells. Treatment with DMSO as a vehicle control neither influenced the expression of APP nor the Aβ production. Quantification of four independent experiments demonstrated a dramatic increase in Aβ secretion in the presence of meprin β, which could be reduced by either actinonin or DAPT treatment (Fig. 7*B*). Meprin β expression was not influenced by the different treatments, indicating that the observed effects were not due to diminished meprin β expression. These data suggest that a small portion of APP can be cleaved by meprin β at a cleavage site identical or close to the known β-secretase cleavage site, as meprin β is able to produce a peptide with approximately the same size as Aβ even in the absence of β-secretase. In addition, as described for the HEK293T cells, γ-secretase cleavage is required to generate meprin-cleaved Aβ.

Soluble Meprin β Does Not Influence Aβ Secretion but Influences APP N-terminal Cleavage—To analyze whether membrane bound or secreted meprin β might be responsible for the induction in Aβ secretion, we incubated exogenous soluble meprin β with APP751 stably overexpressing 7WD10 cells (Fig. 8). After direct loading of tissue culture supernatant, we were able to detect the previously described N-APP20 fragment when cells were incubated with the active, soluble meprin β enzyme (Fig. 8, *lower panel*) (15). As a control, we applied exogenously a soluble inactive E90A mutant of meprin β revealing no detectable increase in N-APP20. Although we were able to confirm that meprin β activity generates the N-APP20 fragment, we were unable to detect any increase in Aβ secretion in cells treated either with active or inactive meprin β (Fig. 8). These results provide evidence that Aβ secretion is mediated by membrane-bound meprin β rather than through shedded meprin β.

DISCUSSION

Recently, we were able to demonstrate that the metalloprotease meprin β can cleave the amyloid precursor protein in its N-terminal region (15). The work presented here extends this investigation by showing that meprin β, although in a smaller extend than BACE1, can also generate different Aβ species with several cleavage sites identical or proximate to the known β-secretase cleavage site.

To date, the most convincing hypothesis to explain the development of Alzheimer disease involves the amyloid cascade (34–36). Numerous publications have demonstrated that aggregated but soluble Aβ species have a detrimental effect on neural homeostasis and plasticity (36). For human meprin β, a striking preference for aspartate and glutamate residues around the cleavage site in native substrates has been revealed, demonstrating an exceptionally high specificity for a metalloprotease (22). Recently, APP was found to be a substrate of human meprin β, using a cell culture-based degradomic approach (15). Together, these observations identify meprin β as a protease candidate for the generation of Aβ peptides, exhibiting acid

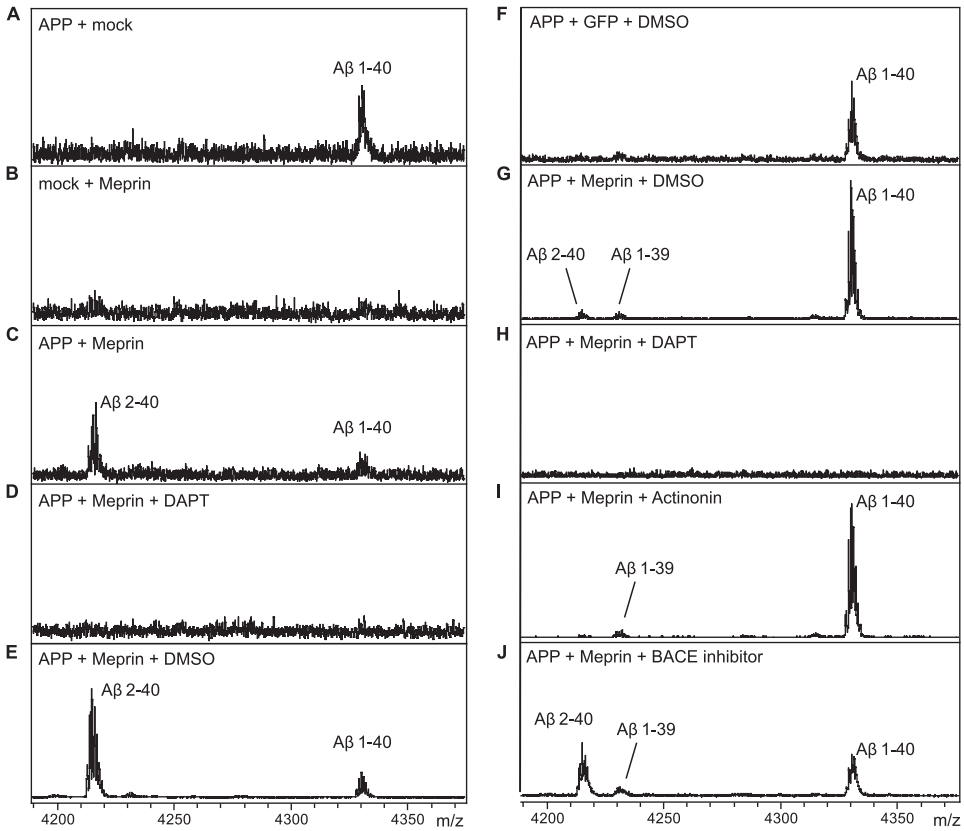

FIGURE 6. **MALDI-MS spectra of secreted Aβ from APP751 wt and meprin β single and co-expressing cells.** HEK293T cells were co-transfected with APP751 wt and meprin β and single-transfected as control. Additionally, co-expressing cells were treated with 5 μM DAPT, or DMSO overnight. With the monoclonal antibody 4G8, immunoprecipitated Aβ was analyzed using MALDI MS analysis. Shown are the mass spectra of one representative measurement of three independent experiments. *A*, mass spectrum showing Aβ1–40 peptides detected in the medium of APP751 wt single-transfected cells. *B*, in meprin β single transfected cells, no Aβ signal could be detected. *C*, after APP and meprin β co-transfection, in addition to the Aβ1–40 signal, an amino-terminal truncated Aβ2–40 peptide could be observed. *D*, both Aβ1–40 and Aβ2–40 production could be abolished using DAPT, a γ-secretase inhibitor. *E*, DMSO did not influence the production of both Aβ species. In another approach, PS70 cells stably expressing meprin β (or GFP as a control) were additionally infected with an APP cDNA containing adenovirus. Additionally, cells were treated with 5 μM DAPT (*H*), 1 μM BACE inhibitor IV (*I*), 100 μM actinonin (*J*), or DMSO (*F* and *G*) as a vehicle control overnight. With the monoclonal antibody W0–2, immunoprecipitated Aβ was analyzed using MALDI MS analysis. Shown are the mass spectra of Aβ peptides of the different treated cells. *F*, in cells expressing APP and GFP, the mass spectrum shows Aβ1–40, Aβ1–39, representing a normal Aβ pattern. *G*, in cells expressing APP and meprin β, the Aβ1–40 peak increased and an additional peak representing Aβ2–40 appears due to meprin β cleavage. *H*, after DAPT treatment of cells co-expressing APP and meprin β, no Aβ signal could be detected, suggesting that also meprin β cleaved Aβ is dependent on γ-secretase cleavage. *I*, treatment of these cells with actinonin, an inhibitor for meprin β, resulted in a loss of the Aβ2–40 species, proofing the specificity of meprin β toward the Aβ sequence. *J*, treatment with BACE inhibitor IV did not abolish the Aβ1–40 or the Aβ2–40 signal ($n = 1$). Theoretical and observed masses for Aβ1–40 and Aβ2–40 are 4328.2/4328.3 and 4213.1/4213.3 Da, respectively. Aβ2–40 was corroborated by LIFT sequencing.

amino acid residues in p1, p2, and p3 position. Aβ processing by BACE1 has only been observed at the aspartates in p1 and p11, implicating a different catalytic activity of an alternative enzyme for cleavage at the alanine residue in p2 (10, 29). Indeed, in comparison with BACE1, MALDI-TOF analysis of Aβ peptides incubated with meprin β revealed two cleavage sites: at the aspartate in p1, also known for the β-secretase BACE1 and at the following alanine in p2.

A role for meprin β in APP processing was further supported by kinetic studies. The data we present indicate that the cleavage of an artificial APP substrate by meprin β was ~10^4-fold more efficient for the APP wild type and 10^3-fold for the Swedish mutant compared with BACE1 (Fig. 1) (30–32). However, in BACE1 knock-out mice, Aβ production in the brain is at the detection limit (8, 25, 37). Hence, BACE1 is the major protease responsible for the liberation of Aβ peptides in AD patients. Nevertheless, we were able to detect increased mRNA expression of meprin β in AD patients compared with non-demented age matched control subjects, indicating a possible role of meprin β in the disease process (Fig. 2). The specificity of meprin β for Aβ peptide release was supported by treatment with the hydroxamate actinonin, known to be a potent inhibitor for human meprin β (38). This compound acts as a chelator, thereby binding to the zinc within the active site of metalloproteases. Hence, BACE1 and γ-secretase, both aspartyl proteases, should not be affected. As γ-secretase cleavage is of critical importance for meprin β-mediated Aβ generation, we additionally proved that actinonin had no inhibitory effect on the activity of γ-secretase (Fig. 4). Actinonin significantly decreased Aβ levels in APP overexpressing cells with either endogenous meprin β expression or after meprin β overexpression, indicating that a metalloprotease might be involved in APP processing in a smaller percentage than BACE1. The concentration of actinonin used in our study (10–100 μM) was demonstrated to be not sufficient for significant inhibition of matrix metalloproteinases able to process exogenously added Aβ (39), providing evidence for specific inhibition of meprin β in our assay. In addition, by using a catalytically inactive mutant

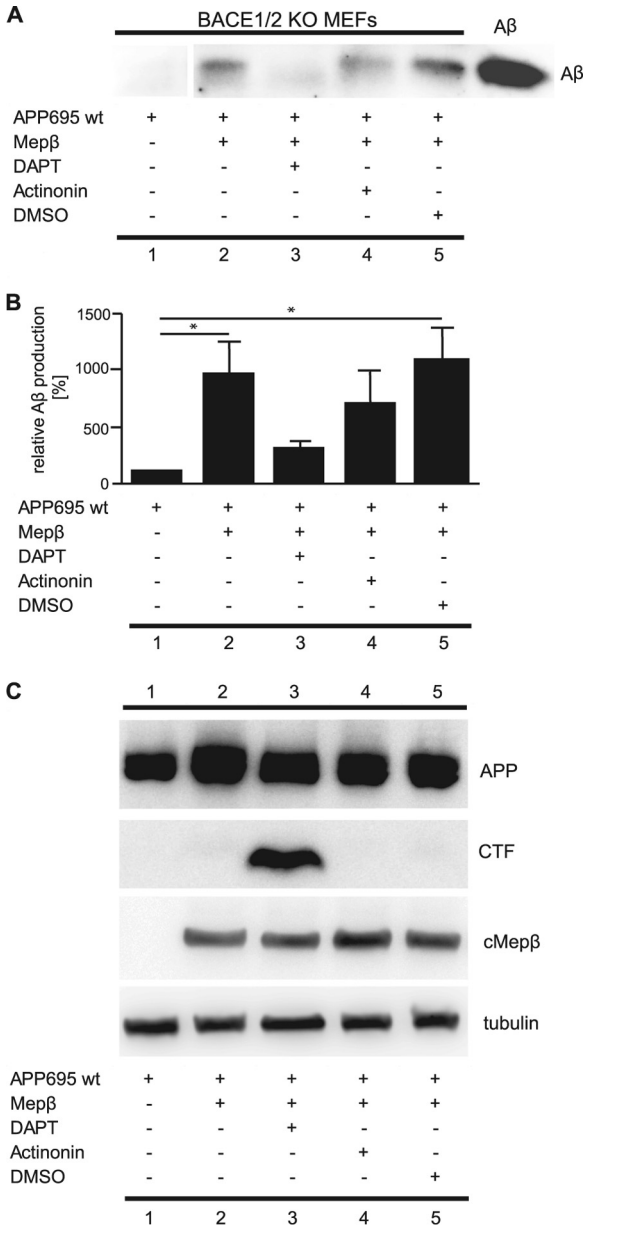

FIGURE 7. **Production of Aβ in BACE1/2 KO cells stably co-expressing the APP695 wt isoform and meprin β.** BACE1/2 knock-out MEFs stably co-expressing APP695 wt and meprin β were generated. Where indicated, the cells were treated with 10 μM actinonin, a meprin β inhibitor; 5 μM DAPT, a γ-secretase inhibitor or with DMSO as a vector control overnight. *A*, total secreted Aβ was detected in the samples of cell culture medium by immunoprecipitation with the IC-16 antibody. As an Aβ standard peptide, a synthetic Aβ1–40 peptide was used (Genosphere Biotechnologies). All samples were analyzed on the same Western blot but in a different order and rearranged for better understanding. *B*, the Aβ signals, obtained with the IC-16 antibody, were quantified and depicted as percentage of APP695 wt single-expressing cells (graph shows mean ± S.E. ($n = 4$); statistical significance: *, $p < 0.05$; *t* test). *C*, protein levels of carboxyl-terminal APP fragments, CTFs, APP, and tubulin were detected in the samples of cell lysates. Meprin β expression was analyzed using the MEP1B antibody. In comparison with cells stably overexpressing APP695 wt alone, cells stably co-expressing APP695 wt and meprin β showed a significant increase in Aβ secretion due to meprin β activity even in the absence of β-secretases due to a knock-out of BACE1 and BACE2. The Aβ production could be decreased using DAPT, a γ-secretase inhibitor, again suggesting that also meprin β cleaved Aβ is dependent on γ-secretase activity.

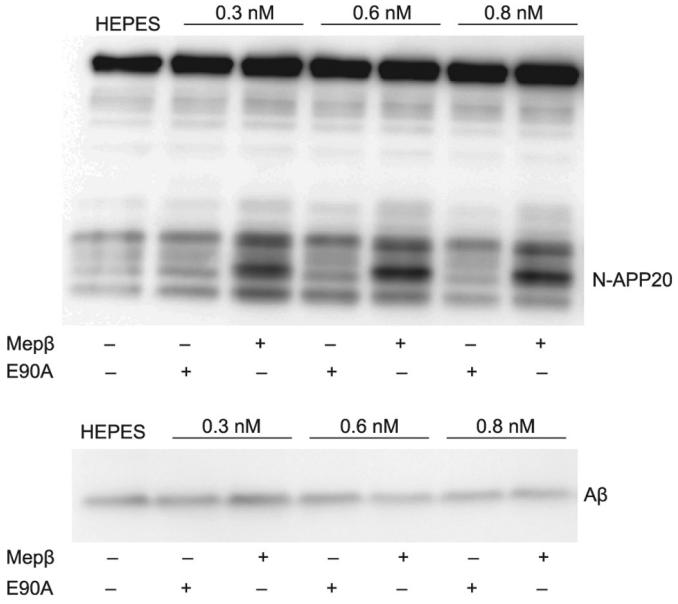

FIGURE 8. **Exogenous meprin β generates N-APP20 fragments, whereas it has no effect on Aβ.** Treatment of APP-overexpressing cells with different concentrations of exogenously added inactive (E90A) and active meprin β (Mepβ) was carried out for 5 h in serum-free medium. Cell culture medium was used to detect total, sAPP, and N-APP20 fragments with anti-N-APP antibody, 22C11 (*anti-N-APP Ab*). Total extracellular Aβ was detected in the samples of cell culture medium with IC-16 antibody, raised against a peptide containing the first 16 amino acids of the Aβ sequence. As a control, medium was treated with HEPES buffer only. Anti-N-APP antibody, 22C11, was used to detect total sAPP and N-APP20 fragments, resulting from sAPP cleavage carried out by the active meprin β. Only total sAPP was detected following the incubation of cell culture medium with the inactive meprin β.

of meprin β, no cleavage of the APP N-terminal domain could be observed (Fig. 8). To provide further evidence for Aβ generation by meprin β, we performed mass spectrometric analysis to identify the Aβ species released from cells due to meprin β expression. We were able to show that meprin β not only cleaves APP at the β-secretase cleavage site after co-incubation of both proteins *in vitro* but also in cell culture experiments with co-expression of APP and meprin β (Fig. 6). Additionally, we were able to identify meprin β as the enzyme responsible for the cleavage of Aβ at the alanine in p2 position. Although the experiments presented here clearly suggest that meprin β is involved in a small portion of Aβ generation, we wanted to investigate whether meprin β directly or indirectly influences APP cleavage. Because BACE1 acts as the main β-secretase *in vivo*, we analyzed whether meprin β shows β-secretase activity toward APP in the absence of BACE1 and BACE2. Therefore, we generated BACE1/2 knock-out cells overexpressing APP695 wt and meprin β and clearly demonstrated Aβ generation by meprin β in the absence of BACE1/2 activity (Fig. 7). This was further supported by mass spectrometry analysis showing that Aβ could be produced even after inhibition of β-secretase (Fig. 5).

Taken together, our data indicate that meprin β is an enzyme capable of cleaving APP in a β-secretase manner, also at position 672 to generate the previously documented 2–40 Aβ peptides detected in brains of AD patients (11–13). Although BACE1 acts as the major β-secretase *in vivo* generating most of the Aβ1–40/42 peptides, we suggest that meprin β might act as an additional enzyme responsible for the release of N-terminal truncated Aβ species. However, whether meprin β is important in the pathogenesis of AD remains to be shown.

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