Sequential Amyloid-β Degradation by the Matrix Metalloproteases MMP-2 and MMP-9*

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Background: Abnormal accumulation of Aβ in the brain is associated with neurodegeneration in Alzheimer disease. Matrix metalloproteases (MMPs) MMP-2 and MMP-9 have been implicated in the physiological catabolism of Alzheimer’s amyloid-β (Aβ). Conversely, their association with vascular amyloid deposits, blood-brain barrier disruption, and hemorrhagic transformations after ischemic stroke also highlights their involvement in pathological processes. To better understand this dichotomy, recombinant human (rh) MMP-2 and MMP-9 were incubated with Aβ40 and Aβ42, and the resulting proteolytic fragments were assessed via immunoprecipitation and quantitative mass spectrometry. Both MMPs generated Aβ fragments truncated only at the C terminus, ending at positions 34, 30, and 16. Using deuterated homologues as internal standards, we observed limited and relatively slow degradation of Aβ42 by rhMMP-2, although the enzyme cleaved >80% of Aβ40 during the 1st h of incubation. rhMMP-9 was significantly less effective, particularly in degrading Aβ(1–42), although the targeted peptide bonds were identical. Using Aβ(1–34) and Aβ(1–30), we demonstrated that these peptides are also substrates for both MMPs, cleaving Aβ(1–34) to produce Aβ(1–30) first and Aβ(1–16) subsequently. Consistent with the kinetics observed with full-length Aβ, rhMMP-9 degraded only a minute fraction of Aβ(1–34) and was even less effective in producing Aβ(1–16). Further degradation of Aβ(1–16) by either MMP-2 or MMP-9 was not observed even after prolonged incubation times. Notably, all MMP-generated C-terminally truncated Aβ fragments were highly soluble and did not exhibit fibrilligenic properties or induce cytotoxicity in human cerebral microvascular endothelial or neuronal cells supporting the notion that these truncated Aβ species are associated with clearance mechanisms rather than being key elements in the fibrillogenesis process.

Results: Proteolytic degradation of Aβ by MMP-2/MMP-9 sequentially generates highly soluble, nontoxic, and nonfibrilligenic C-terminally truncated fragments normally present in CSF. Conclusion: The MMP-generated Aβ species likely reflects clearance mechanisms rather than active participation in the amyloidogenesis process. Significance: Matrix metalloproteases are important contributors to Aβ homeostasis.

Metalloproteases are a family of secreted and membrane-bound zinc-dependent endoproteases that are extrinsic regulators of many biological and pathological processes extending from development, morphogenesis, and tissue remodeling to vascularization, wound healing, cell invasion, and tumor metastasis. To achieve these biological functions, metalloproteases proteolytically cleave growth factors, degrade a wide range of extracellular matrix proteins, and regulate cleavage of various receptors. The large family of metalloproteases includes, among others, matrix metalloproteases (MMPs) and a disintegrin-metalloproteases (ADAMs). Twenty six MMPs have been identified so far and are clustered into six major groups as follows: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs according to their domain structure and substrate specificity (1, 2). They are secreted as inactive zymogens, requiring proteolytic removal of a propeptide domain before becoming enzymatically active, and are tightly regulated by specific tissue inhibitors of MMPs, a family of four endogenous protease inhibitors (3). The ADAM family is composed of 21 members identified in the human genome, with only 12 of them exhibiting catalytic activity. They are type I transmembrane proteases that also require the removal of their propeptide N-terminal domain to become enzymatically active. A large number of cell-surface proteins are known to undergo ectodomain shedding by proteolytic cleavage. Although some MMPs can act as “sheddases,”

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¶ The abbreviations used are: MMP, matrix metalloprotease; rh, recombinant human; Aβ, amyloid β; AD, Alzheimer disease; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; Tricine, N(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; APP, amyloid precursor protein; BBB, blood-brain barrier; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IP, immunoprecipitation; CAA, cerebral amyloid angiopathy.
the best known shedding events are mediated by ADAMs, particularly ADAM-9, ADAM-10, and ADAM-17 (4).

Both MMPs and ADAMs have been reported to prevent the synthesis or to facilitate the clearance of Aβ, the 40–42-amino acid peptide composing vascular and parenchymal amyloid deposits in Alzheimer disease (AD). In mammalian cells and throughout life, Aβ is cleaved from its precursor APP by the sequential action of β- and γ-secretases, in a pathway dubbed “amyloidogenic” because the two main products, Aβ40 and Aβ42, are the central components of amyloid lesions in AD brains. APP is also processed by α-secretase in a “nonamyloidogenic” pathway that prevents the release of Aβ and results in the secretion of a soluble APPα fragment, reported to exhibit neuroprotective and memory-enhancing effects (5). Among the various candidates for the α-secretase activity, three members of the ADAM family, ADAM-9, ADAM-10, and ADAM-17, have been proposed, with emerging consensus that ADAM-10 is largely responsible for the cleavage of APP at the Lys687–Leu688 peptide bond (amino acids 16 and 17 of the Aβ sequence) and the generation of the soluble APPα fragment (6, 7).

Members of the MMP family with gelatinase activity, MMP-2 and MMP-9, exhibit the ability to degrade Aβ peptides in vitro (8–12) and have been implicated as central players in the process of Aβ catabolism. A role for the increased expression of MMPs in hemorrhagic complications associated with vascular amyloid deposits in cerebral amyloid angiopathy (CAA) has been suggested by the known association of these proteases with blood-brain barrier (BBB) disruption in multiple vasculopathies and with hemorrhagic transformations after ischemic stroke (13–15). MMP-9 has been shown to degrade fibrillar structures and compact plaques in ex vivo experiments (9) and to co-localize in vivo with neuritic plaques, vascular amyloid deposits, and neurofibrillary tangles. MMP-2 has been demonstrated to be a key element in the disruption of tight junction proteins and induction of BBB permeability alterations. It is secreted by endothelial cells in culture upon stimulation with Aβ peptides, being particularly responsive to vasculotropic Aβ mutants (16).

Through their ability to degrade Aβ, MMP-2 and MMP-9 likely contribute to the brain homeostasis of Aβ and to the maintenance of Aβ levels in biological fluids. Both enzymes are able to generate in vitro Aβ fragments that are found in vivo in cerebrospinal fluid (CSF) (17). However, no systematic studies have been performed identifying the steps through which these truncated species are generated. Using mass spectrometry (MS) approaches, the studies presented herein compare the kinetics of Aβ proteolysis by both MMP-2 and MMP-9 using Aβ40 and Aβ42 homologues as substrates. The data indicate that both enzymes generate sequential intermediates that also serve as self-substrates and identify the end point peptide fragments as molecules of high solubility, lacking fibrillogenic propensity and neurotoxicity. The data support the notion that the proteolytically generated fragments are related to clearance mechanisms rather than being key elements in the amyloidogenesis process.

Experimental Procedures

Aβ Peptides—Synthetic homologues of Aβ42, Aβ40, the C-terminal truncated fragments Aβ(1–34), Aβ(1–30), and Aβ(1–16), as well as the Aβ40 genetic variant containing the L34V substitution were synthesized using N-tet-butylxycarbonyl chemistry by James I. Elliott at Yale University. For quantitative MS, isotopically labeled peptide standards of Aβ42, Aβ40, and the C-terminal truncated fragments Aβ(1–34), Aβ(1–30), and Aβ(1–16) were synthesized with deuterated [3H]phenylalanine residues; labels were introduced at positions 4, 19, and 20 in Aβ42, Aβ40, Aβ34, and Aβ30, whereas Aβ(1–16) contained a single labeled residue at position 4. All synthetic peptides were purified by reverse phase-high-performance liquid chromatography, molecular masses corroborated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS, and concentrations assessed by amino acid analysis, as described previously (16, 18). To remove preformed aggregates, peptides were pretreated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for at least 24 h at room temperature with intermittent vigorous vortexing. After lyophilization, peptides were either reconstituted in deionized water to a 1 μg/μl concentration (stock solution) for immediate use or stored at −80 °C for up to 2 months.

In Vitro Aβ Proteolytic Degradation—The various Aβ homologues were proteolytically cleaved by incubation with activated recombinant human MMP-2 or MMP-9 (R&D Systems, Minneapolis, MN). Prior to the enzymatic assay, and following the supplier’s protocol, both rhMMP-2 and rhMMP-9 were activated at 37 °C incubation with 1 mM para-aminophenyl-mercuric acetate (Sigma) in 50 mM Tris, pH 7.5, containing 10 mM CaCl2, 150 mM NaCl, and 0.05% Brij-35 (TCNB; rhMMP-2 for 1 h and rhMMP-9 for 24 h). After activation, MMP-2- and MMP-9-specific activities were assessed by their ability to cleave the specific fluorogenic peptide substrate Mca-Pro-Leu-7-methoxycoumarin-4-yl acetyl and Dpa is activated by 37 °C incubation with 1 mM para-aminophenyl-mercuric acetate (Sigma) in 50 mM Tris, pH 7.5, containing 10 mM CaCl2, 150 mM NaCl, and 0.05% Brij-35 (TCNB; rhMMP-2 for 1 h and rhMMP-9 for 24 h). After activation, MMP-2- and MMP-9-specific activities were assessed by their ability to cleave the specific fluorogenic peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (R&D Systems; where Mca is 7-methoxycoumarin-4-yl acetyl and Dpa is N-3,2,4-dinitrophenyl-1,2,3-diaminopropionyl (19)) using the manufacturer’s protocol. In all cases both enzymes exhibited comparable specific activity (consistently ranging between 1250 and 1400 pmol/min/μg, depending on the lot).

Proteolytic cleavage of Aβ was performed at 37 °C at an enzyme/peptide ratio of 1:25 (100 nM enzyme, 2.5 μM Aβ in TCNB), as described previously (16). Aliquots were retrieved at different time points for up to 24 h; in the case of Aβ40L34V, incubation with MMP-9 was further extended up to 10 days. In all cases, protease activity was stopped by addition of 5 μl of 15 mM EDTA, pH 8.0, followed by freezing at −80 °C. In all cases, assessment of proteolytic degradation was evaluated by Western blot analysis and by the identification of specific Aβ degradation products via immunoprecipitation (IP) followed by MS analysis, as described below.

Immunoprecipitation/Mass Spectrometry—Intact Aβ peptides and newly generated proteolytic fragments were immunoprecipitated by incubation with paramagnetic beads (Dynabeads M-280, Invitrogen) coated with a combination of 4G8 and 6E10 antibodies (Covance, Princeton, NJ), as described previously (16, 20, 21). After overnight incubation at 4 °C with
the anti-αβ-coated beads, samples were resuspended in PBS containing 0.025% Tween 20 (PBS-T), washed three times with PBS-T and once with 50 mM NH4HCO3, followed by elution with 0.5% formic acid. The eluted IP material was dried down in a Savant SpeedVac concentrator (Thermo Fisher) and further reconstituted in 5 μl of 0.1% formic acid in 50% acetonitrile for MS. One-fifth of the reconstituted sample (1 μl) was combined with an equal volume of α-4-hydroxycinnamic acid matrix (Agilent Technologies) reconstituted in 0.1% trifluoroacetic acid (TFA) and 100% acetonitrile at a concentration of 15 g/liter and 1 μl of the resulting mixture spotted onto a Bruker Daltonics MTP 384 massive target T aluminum plate pre-seeded with 0.5 μl of α-4-hydroxycinnamic acid (1 g/liter). All samples were spotted in duplicate and analyzed at the New York University Mass Spectrometry Core for Neuroscience using a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer (Bremen, Germany) in linear mode using standard instrument settings. For quantitative MS assessments, known amounts of the corresponding deuterated standards were added to the digestion mixture prior to the IP and MS analysis. At least two sets of measurements containing different amounts of the same standard were used for each experiment to estimate the concentration of the truncated peptides generated. In all cases, MS spectra were processed and analyzed by FlexAnalysis, and quantitation was performed by comparison with peak intensities of the pertinent labeled standards.

**Western Blot Analysis**—Detection of αβ by Western blot was performed following previously described protocols (22). Briefly, MMP digestion samples were separated on 16.5% Tris/Tricine SDS-PAGE under reducing conditions and transferred onto 0.45-μm pore size polyvinylidene difluoride membranes (PVDF; Millipore Corp, Billerica, MA) for 45 min at 400 mA using CAPS buffer, pH 11. After blocking for 1 h with 5% nonfat milk in PBS-T, membranes were probed with rabbit polyclonal antibodies anti-αβ40 (1:500, Invitrogen) or anti-αβ42 (1:500, Invitrogen) recognizing specifically the fragments ending at positions 40 or 42, respectively, without immunoreactive with the C-terminal truncated species. This was followed by incubation with HRP-conjugated anti-rabbit IgG (1:3000, BioSource-Invitrogen) for 1 h, development of fluorograms by enhanced chemiluminescence using Supersignal West Pico Luminol/Enhancer and Stable Peroxide solutions (Thermo Scientific), and image analysis with ImageJ software (rsbweb.nih.gov).

**Cell Culture Treatment with the Different αβ Homologues**—Human neuroblastoma cells SH-SY5Y were obtained from the ATCC and grown in DMEM/F-12 (Life Technologies/Gibco) containing 15% (v/v) fetal bovine serum (FBS, Sigma) and 100 units/ml penicillin, 100 g/ml streptomycin (Life Technologies/Gibco). Human cerebral microvascular endothelial cells (hCMEC/D3), provided by B. Weksler, Weill Medical College, Cornell University, New York, NY (23), were grown in EGM-2 medium (Lonza, Allendale, NJ) supplemented with VEGF, IGF, bFGF, hydrocortisone, ascorbate, and 2.5% FBS, as described (18). Both cell types were challenged for 3 days with the different αβ peptides pretreated as above and reconstituted in EGM-2, 1% FBS or DMEM, 1% FBS at 50 μM final concentration.

**Results**

**Aβ(1–40) and Aβ(1–42) Are Substrates of rhMMP-2 and rhMMP-9**—IP/MS analysis of the time course degradation of full-length Aβ40 (average mass M + H = 4,330.86 Da) and Aβ42 (M + H 4,515.10 Da) peptides by rhMMP-2 and rhMMP-9 revealed the generation of three major proteolytic products, all truncated at the C terminus as follows: Aβ(1–34) (M + H 3,788.16 Da); Aβ(1–30) (M + H 3,391.63 Da); and Aβ(1–16) (M + H 1,956.03 Da). Although the resulting degradation fragments were basically the same, Aβ40 and Aβ42 showed different susceptibility to each MMP despite the comparable specific activity of both enzymes. Fig. 1 depicts the time-resolved MALDI-TOF MS spectra and relative ion counts at different time points, illustrating the progression of the enzymatic reaction with the Aβ(1–40) peptide. As illustrated, rhMMP-9 required 30 times longer incubation than rhMMP2 (5 h versus 10 min) to generate Aβ(1–34) and Aβ(1–30) fragments with comparable intensity ratios. The smaller fragment Aβ(1–16) required even longer degradation time to be detected, ~30 min with rhMMP-2 versus more than 5 h with rhMMP-9, suggesting that either the Aβ peptide bond Lys16–Leu17 is less susceptible to proteolysis (e.g. through a steric hin-
drance-like mechanism) or it is generated from a substrate different from the initial intact peptide (e.g. one of the cleavage products) and has, therefore, a delayed appearance. By assessing the relative intensity ratios between the intact peptide and each of the proteolytically generated fragments at all the tested time points, the data indicate that by 30 min of incubation with rhMMP-2, rhMMP-2 reaches a degradation plateau (Fig. 1A), and similar levels of the remaining intact peptide are achieved only after 24 h of incubation with rhMMP-9 (Fig. 1B). Judged by the relative intensity ratios produced by rhMMP-2 at the various time points, it seemed that the smaller fragments were generated at the expense of intermediate degradation products rather than the full-length Aβ40 (Fig. 1A). Notably, the longest incubation (24 h) with MMP-9, but not with MMP2, generated an additional fragment with a mass compatible with the fragment Aβ(1–22) (m/z 2661.87 theoretical; 2661.76 experimental). Further validation for the Aβ(1–40) degradation by rhMMP-2 and rhMMP-9 was obtained via Western blot analysis utilizing an antibody specific to the C terminus of the peptide (anti-Aβ40) that does not react with the proteolytic fragments ending at positions 34, 30, and 16. As shown for selected time points (0, 1, and 24 h), Aβ(1–40) was more efficiently cleaved by rhMMP-2 than by rhMMP-9. Loss of intact Aβ(1–40) as a result of proteolytic degradation was clearly visualized at the 1-h incubation time point with rhMMP-2 (Fig. 1A), whereas rhMMP-9 required a longer incubation time (Fig. 1B). No obvious Aβ oligomerization was visualized in the time frame of the experiments under the current experimental conditions that could account for the different enzymatic susceptibility.

A similar degradation profile illustrated in Fig. 1 for Aβ40 was obtained for Aβ42. As indicated in Fig. 2, the same three fragments ending at positions 34, 30, and 16 were generated with both enzymes, although for rhMMP-9 the degradation process was much slower than with rhMMP-2 (Fig. 2A) despite the comparable specific activities of both enzymes, and the Aβ(1–16) fragment was hardly detected even after the 24-h incubation of Aβ(1–42) with rhMMP-9 (Fig. 2B). Time-resolved MALDI-TOF spectra revealed that the rhMMP-2 degradation products Aβ(1–34), Aβ(1–30), and Aβ(1–16) were generated from Aβ42 within a comparable time frame as those originating from Aβ40; however, the reaction reached a plateau at a higher Aβ42/degradation products relative intensity ratio, a clear indication that more intact Aβ42 than Aβ40 remained undegraded after a 24-h incubation with rhMMP-2. As observed with rhMMP-2 and Aβ40, the smaller fragment Aβ(1–16) seemed to be generated at the expense of intermediate degradation products (e.g. Aβ(1–34) and Aβ(1–30)) rather than the full-length Aβ42 (Fig. 2A). The degradation of Aβ42 was further validated via Western blot using anti-Aβ42, an antibody that specifically recognizes the C terminus of the intact peptide and does not immunoreact with the degradation fragments. Although loss of the intact peptide was clearly detected at the 1- and 24-h time points with rhMMP2, rhMMP-9 was not

**FIGURE 1.** Aβ(1–40) proteolysis by matrix metalloproteases 2 and 9. **A**, time-resolved MALDI-TOF spectra and normalized ion counts at different time points show the progression of the proteolytic degradation induced by rhMMP-2. Western blot probed with anti-Aβ40 illustrates changes in the Aβ40 signal as a function of time when in the presence of rhMMP-2. **B**, time-resolved MALDI-TOF spectra and normalized ion counts at different time points illustrate the progression of rhMMP-9 proteolytic degradation. Western blot probed with anti-Aβ40 highlights changes in the Aβ40 signal as a function of time upon incubation with rhMMP-9. A and B, data are representative of 2–3 independent experiments performed in duplicate.
able to degrade sufficient peptide throughout the length of the experiment to be evident in the Western blot assay, a feature that could not be attributed to peptide aggregation, as the Western blot exhibits only negligible signals for oligomeric A/H9252 species, under the current experimental conditions.

To obtain quantitative information from our MS data, we synthesized peptide homologues to A/H9252 (1–40), A/H9252 (1–42), A/H9252 (1–34), A/H9252 (1–30), and A/H9252 (1–16) isotopically labeled with [2H]phenylalanine residues at positions 4, 19, and 20, and we used them as internal standards, as described under “Experimental Procedures.” Fig. 3 illustrates the position of the labeling (Fig. 3A), the corresponding theoretical masses of the labeled and unlabeled peptides (Fig. 3B), as well as a MALDI-TOF MS spectrum showing the ability of our instrument to resolve the differentially labeled isotope clusters of the endogenous and the labeled counterparts (Fig. 3C). Our shortest peptide A/H9252 (1–16) contains a single [2H]phenylalanine incorporated at position 4-increasing its molecular mass by 8 Da. This mass difference is sufficient to resolve isotopic clusters of peptides up to 2500 Da. For molecular masses in the range up to 4000–4500 Da, where the overlap of isotopic clusters reaches ~16 Da, our labeled peptides A/H9252 (1–40), A/H9252 (1–42), A/H9252 (1–30), and A/H9252 (1–34) incorporated three [2H]phenylalanine residues resulting in a mass increase of 24 Da. Fig. 3C also illustrates the dissimilarities in peak intensity (response factors) obtained for comparable amounts of all five peptides when processed in a single IP/MS sample further emphasizing that, for accuracy of quantitation, each peptide should be paired with its corresponding isotopically labeled internal standard. By adding known amounts of the deuterated standards for A/H9252 (1–40) and A/H9252 (1–42), we performed a kinetic study of the fragments generated by rhMMP-2 and rhMMP-9 at different time points (0, 15, and 30 min and 1, 3, 5, and 24 h) using identical conditions as those illustrated in Figs. 1 and 2. Corroborating the time-resolved mass data, rhMMP-2 was able to degrade more than 80% of the starting A/H9252 (1–40) material during the first 30-min incubation, whereas the efficiency of A/H9252 (1–42) digestion by rhMMP-2, despite the comparable enzymatic activity, was more limited and relatively slower, achieving the 50% degradation plateau in 3 h (Fig. 4, A and C). In both cases, the newly generated A/H9252 (1–34) and A/H9252 (1–30) showed similar concentrations during the first 3 h of incubation, whereas A/H9252 (1–16) steadily accumulated for the duration of the experiment, reaching the highest concentration after 24 h of incubation. Also in agreement with the time-resolved data shown in Figs. 1 and 2, rhMMP-9 showed less efficiency in degrading not only the full-length peptides, especially A/H9252 (1–42), but their newly generated proteolytic fragments ending at positions 34 and 30 (Fig. 4, B and D). As a result, these two fragments, particularly A/H9252 (1–34), were the major proteolytic products that accumulated at the end of the 24-h incubation, and the degradation product A/H9252 (1–16) was poorly generated from A/H9252 (1–40) and remained undetectable from A/H9252 (1–42) throughout the 24-h duration of the experiment.
Sequential Cleavage Pattern for Aβ Degradation by rhMMP-2 and rhMMP-9—The time-resolved qualitative MS data of Aβ degradation illustrated in Figs. 1 and 2 and supported by the quantitative data shown in Fig. 4 suggest a sequential pattern of proteolytic cleavage, indicating that the initial degradation product(s) also served as additional substrates for both enzymes. Visual inspection of the spectra and the corresponding normalized intensity plots indicate an initial cleavage at peptide bond Leu34 Met35 followed by cleavage at position Ala30 Ile31 and later on at peptide bond Lys16 Leu17. Although similar peptide fingerprints were generated by both enzymes, the sequential pattern was more obvious with rhMMP-2. Under the conditions tested for both Aβ40 and Aβ42, there was a time-dependent increase in Aβ(1–34) that reached a maximum between 30 min and 1 h, with a similar situation occurring with Aβ(1–30), although the levels observed were lower than those seen for Aβ(1–34), reaching a maximum at a later time, between 1 and 5 h of incubation. Consistent with the concept of sequential degradation, Aβ(1–16) was initially detectable after 10 min of incubation, and its concentration continued to steadily increase up to the end of the experiment. In the case of rhMMP-9, the sequential degradation was clearly present when Aβ40 was used as a substrate (Aβ(1–34) was initially visible at the 10-min time point whereas Aβ(1–30) appeared at the 30-min time point and Aβ(1–16) at the 1-h time point), although this was not so obvious in the case of Aβ42. Under the conditions tested, it seemed that rhMMP-2 was more active in degrading the newly generated intermediates than rhMMP-9; in other words, Aβ(1–34) and Aβ(1–30) seemed to be better substrates for rhMMP-2 than for rhMMP-9.

To better evaluate whether the intermediate fragments were indeed substrates of both enzymes, Aβ(1–34) and Aβ(1–30) as well as their respective derivatives isotopically labeled with [3H]phenylalanine were custom-synthesized and used in individual experiments with rhMMP-2 and rhMMP-9, and the resulting fragments as well as the starting peptides were quantitated following the same procedure described above for Aβ40 and Aβ42. As indicated in Fig. 5, Aβ(1–34) was an excellent substrate for rhMMP-2, generating Aβ(1–30) and Aβ(1–16) (Fig. 5A). In the 24-h time frame of the experiment, rhMMP-2 was able to degrade 95% of the initial Aβ(1–34), and the fragment Aβ(1–16) became the predominant product in the same time frame. Notably, Aβ(1–34) was not a good substrate for rhMMP-9 despite using enzymes with comparable specific activity. As indicated in Fig. 5B, degradation products were not detected during the first 3 h of the experiment; after 24 h, 83% of the initial peptide remained intact, and Aβ(1–16) represented only 3% of the total signal intensities. As observed before (Fig. 1B), a small signal compatible with the mass of the Aβ(1–22) fragment was observed only at the end of the experiment. When the proteolytic susceptibility of Aβ(1–30) was tested under identical experimental conditions, similar results as those observed for Aβ(1–34) were obtained. Aβ(1–30) was also an

FIGURE 3. Isotopically labeled Aβ species. A, Aβ sequence in 1-letter code indicating the location of the residues that were isotopically labeled with [3H]phenylalanine (boxes). Bold letters highlight the MMP-2 and MMP-9 cleavage sites. B, expected mass differences between labeled and unlabeled Aβ derivatives. Masses shown are weighted average masses for the isotopic envelopes. C, MALDI-TOF spectrum illustrating the potential interference effect of overlapping isotopic distributions and different signal response factors for each peptide in a mixture containing 200 fmol each of unlabeled and isotopically labeled Aβ(1–16), Aβ(1–30), Aβ(1–34), Aβ(1–40), and Aβ(1–42). Inset, zoom image illustrates the capability of the instrument to resolve [3H]phenylalanine-labeled and nonlabeled Aβ(1–16) isotopic clusters. Data are representative of three independent experiments performed in duplicate.
excellent substrate for rhMMP-2 but not for rhMMP-9. As indicated in Fig. 6A, 90% of the starting material generated Aβ(1–16) within 24 h in the presence of rhMMP-2, but 97% remained undegraded when rhMMP-9 was used (Fig. 6B).

Quantitative evaluation of the degradation kinetics of rhMMP-2 indicates that the enzyme was able to degrade 50% of Aβ(1–34) within 1 h (Fig. 5A), while in the same time frame it degraded only 12% of Aβ(1–30) (Fig. 6B).

Aβ(1–16) in the Terminal Component in the Sequential Degradation of Aβ by rhMMP-2 and rhMMP-9—All the above data point to the sequential degradation of Aβ by both matrix metalloproteases following the pattern Aβ40/Aβ42 → Aβ(1–34) → Aβ(1–30) → Aβ(1–16). To further verify whether Aβ(1–16) was indeed the terminal component, equal amounts of synthetic Aβ(1–16) were subjected to proteolytic degradation with both enzymes under identical experimental conditions and comparable specific activities. As indicated in Fig. 7, Aβ(1–16) remained intact after 24 h of incubation with either rhMMP-2 or rhMMP-9. Comparable peak intensities were recovered with both enzymes, and no additional degradation fragments were observed.

Degradation of the Piedmont Aβ(1–40)L34V Mutant by rhMMP-2 and rhMMP-9—In 2005, an autosomal dominant intra-Aβ mutation was reported in a Piedmont family manifesting with recurrent intracerebral hemorrhages. The mutation, a Leu to Val substitution at residue 34, was exclusively associated with CAA with complete absence of parenchymal Aβ deposits of any type, including diffuse and mature plaques. Because the C-terminal truncated Aβ(1–34) is the first and the major intermediate fragment produced by the cleavage of full-length Aβ by MMP-2 and MMP-9, we studied whether the presence of this mutation at the N terminus of peptide bond 34–35 affected the degradation process. Aβ(1–40)L34V and its isotopically labeled ([2H]phenylalanine) derivative were custom-synthesized, purified, and characterized as indicated under “Experimental Procedures” and used in identical degradation experiments to those described above. As illustrated in Fig. 8A, the pattern of degradation of Aβ(1–40)L34V with rh-MMP-2 was the same as that obtained for the wild-type Aβ(1–40) peptide (see Fig. 1A for comparison). Although after 24 h of incubation the final ratios among the different fragments were almost identical for the mutated and wild-type peptides, there were some clear differences in their proteolytic susceptibility at short time points, e.g. at 10 min 76% of the mutated peptide remained intact compared with 38% of the wild-type counterpart. Perhaps the most notable difference at short time points was the amount of Aβ(1–34) retrieved from the reaction mixture, being generated at 2–4-fold higher levels when the wild type was used in the experiments, suggesting that the mutation affects the kinetics of cleavage at peptide bond 34–35 but not the specificity. Quantitative MS using the isotopically labeled Aβ(1–40)L34V peptide corroborated

FIGURE 4. Quantitative assessment of Aβ degradation by matrix metalloproteases 2 and 9 using isotopically labeled standards. Peptide levels (in femtomoles) were measured at various time points and their levels plotted as a function of time. A, Aβ(1–40) degradation by rhMMP-2; B, Aβ(1–40) degradation by rhMMP-9; C, Aβ(1–42) degradation by rhMMP-2; D, Aβ(1–42) degradation by rhMMP-9. In all cases, the data represent mean ± S.D. of quadruplicate experiments.
the data from the time-resolved MALDI-TOF spectra and the relative ion counts.

The mutation had a larger impact on the kinetics of rhMMP-9 degradation. As indicated in Fig. 8B, 84% of the Aβ(1–40)L34V remained intact after 24 h of incubation with the enzyme, and only 17% of the wild-type peptide was left undegraded under the same experimental conditions (see Fig. 1B for comparison). Extended incubation (up to 10 days) clearly shows that the mutation affects the kinetics of the enzymatic reaction; rhMMP-9 required almost 10 days of incubation with Aβ(1–40)L34V to degrade 50% of the initial substrate.

Toxic and Fibrillogenic Properties of Aβ Peptides in Brain Endothelial and Neuroblastoma Cell Cultures—The biological properties of the full-length Aβ(1–42) and Aβ(1–40) peptides as well as of the C-terminal fragments generated by rhMMP-2 and rhMMP-9 activity (the intermediate products Aβ(1–34) and Aβ(1–30) and the final product Aβ(1–16)) were studied on human brain endothelial D3 cells and human neuroblastoma SH-SY5Y cells. Assessment of apoptosis induction and lactate dehydrogenase leakage revealed that only the Aβ(1–42) peptide induced toxic effects in D3 cells (Fig. 9, A1 and A2) and especially in neuroblastoma cells (Fig. 9, B1 and B2), whereas Aβ(1–40), Aβ(1–34), Aβ(1–30), and Aβ(1–16) peptides did not exhibit cytotoxicity after 3 days of treatment. The generation of protofibrillar species and fibrillar components during the incubation time frame was evaluated by fluorescence quantitation of thioflavin T binding in the culture supernatants after the 3-day challenge with the respective Aβ peptides. Consistent with the demonstrated fibrillogenic properties (16, 18), only the supernatants of cells treated with Aβ(1–42), and to a minor extent with Aβ(1–40), showed positive thioflavin T binding; the Aβ(1–34), Aβ(1–30), and Aβ(1–16) fragments generated by the matrix metalloproteases did not form fibrillogenic assemblies in the time frame of the experiments (Fig. 9, A3 and B3).

Consistent with the cleavage properties exhibited by both enzymes and the lack of toxicity of the synthetic truncated fragments illustrated above, preincubation of intact Aβ42 for 24 h with MMP-2 and MMP-9 resulted in a decrease in apoptosis induction in both endothelial and neuronal cells (Fig. 10), further supporting the notion that proteolytic degradation may indeed be associated with physiological clearance mechanisms.
Components of the MMP family of proteases, with few exceptions, feature a common domain structure containing the following: (a) a propeptide domain, which must be removed for the enzyme to become active; (b) a catalytic domain, featuring a Zn\(^{2+}\) ion bound by three histidine residues and a glutamic acid involved in the catalysis; and (c) a C-terminal hemopexin-like domain, believed to be involved in protein-protein interactions and separated from the catalytic domain by a so-called hinge region (1). Of all the MMPs, the closely related gelatinases...
MMP-2 and MMP-9 are structurally unique in that both contain three additional type II fibronectin domains inserted immediately before the Zn$^{2+}$-binding motif within their respective catalytic domains, inserts required to bind and cleave collagen and elastin, while largely differing in the length of their hinge region (3, 25).

The catalytic domains of all MMPs are structurally homologous, a feature that raised questions about redundancy of substrate recognition across this family of proteins. To clarify the issue, substrate phage approaches as well as synthetic peptide libraries and protein degradation strategies have been widely used to construct substrate recognition profiles for the various MMPs. In general terms, these proteases, including MMP-2 and MMP-9, cleave peptide bonds before a residue with a hydrophobic side chain (e.g. Met, Leu, Ile, Phe, or Tyr) (26, 27).

However, the factors that govern substrate recognition and specificity are by far more complex, involving not only the amino acids located at the N- and C-terminal sides of the scissile bond but a number of structural features of the catalytic cleft, including docking points (pockets) to accommodate the substrate as well as the position of the hemopexin-like domain to help with the substrate binding. In this sense, even though MMP-2 and MMP-9 have similar profiles for substrate recognition, differences in the ability of specific amino acids located in those pockets to establish (or not) stabilizing bonds with the selective substrate produce deep changes in catalytic activity (e.g. Glu$^{412}$ in MMP-2 forms a hydrogen bond with the substrate, and Asp$^{410}$ in MMP-9 is shorter and unable to protrude into the cleft to fulfill the same task, resulting in loss of catalytic activity (28)).

Along these lines and under our experimental conditions, MMP-2 and MMP-9 showed specificity for the same peptide bonds in the Aβ molecule (Leu$^{34}$–Met$^{35}$, Ala$^{30}$–Ile$^{31}$, and Lys$^{16}$–Leu$^{17}$, all containing a hydrophobic side chain at the P1 position) while exhibiting significant differences in their catalytic activity. This is clearly exemplified in the degradation of Aβ(1–40); both enzymes showed comparable ability to degrade the same peptide bonds, but they differed in the resulting ratio of the final products in the 24-h time frame of the experiments (Fig. 1). Although MMP-2 efficiently cleaved the full-length peptide as well as the intermediate products Aβ(1–

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**FIGURE 8.** Proteolysis of the Piedmont Aβ(1–40)L34V mutant by matrix metalloproteases 2 and 9. A, time-resolved MALDI-TOF spectra, normalized ion counts, and quantitative data obtained with isotopically labeled Aβ derivatives at different time points show the progression of the proteolytic degradation of the Aβ(1–40)L34V mutant upon incubation with rhMMP-2. B, time-resolved MALDI-TOF spectra, normalized ion counts, and quantitative data obtained with isotopically labeled Aβ derivatives at different time points depict the progression of the proteolytic degradation of the Aβ(1–40)L34V mutant by rhMMP-9. Spectra and normalized ion counts are representative of 2–3 independent experiments performed in duplicate; quantitative data represent mean ± S.D. of quadruplicate experiments.
34) and Aβ(1–30) to generate the fragment Aβ(1–16), the intermediate products accumulated in the case of MMP-9, resulting in a lower yield of Aβ(1–16). The poor efficiency of MMP-9 in degrading Aβ(1–34) and Aβ(1–30) compared with MMP-2, despite both having comparable specific activities, was further evidenced when the purified intermediates were used as substrates instead of the full-length peptide (see Figs. 1, 5, and 6). Both intermediates were almost fully degraded by MMP-2 but barely affected by MMP-9 in the same period of time. A similar effect was observed when the Piedmont Aβ40 mutant (29, 30) was used instead of the full-length wild-type counterpart. The replacement of the hydrophobic Leu residue for the
less bulky hydrophobic Val at the C-terminal side of the scissile bond (position 34) did not significantly affect the ratio of the final Aβ components after 24 h of degradation with MMP-2, although the cleavage kinetics of the full-length Aβ40L34V peptide was somewhat slower than for the wild-type Aβ40 during the 1st h of degradation. In the case of MMP-9, the amino acid change significantly affected substrate recognition, translating in poor degradation of the Aβ40L34V peptide when compared with the wild-type counterpart (Figs. 1 and 8). The decreased susceptibility of the genetic variant to enzymatic degradation could be a potential contributor to the in vivo early onset disease phenotype observed in the Piedmont kindred.

The differences in proteolytic activity between MMP-2 and MMP-9 were accentuated when the longer Aβ2 was used instead of Aβ40. It is known that the structure of these peptides play a critical role in the ability of different enzymes to carry out an effective degradation (7, 31). Compared with its shorter homologue Aβ40, Aβ42 consistently shows poorer solubility, a higher tendency to aggregate, oligomerize, and fibrillize in a shorter time frame, adopting conformations that are highly enriched in β-sheet structures (18). Our data illustrate that although MMP-2 and MMP-9 generated the same cleavage products with both peptides, a significantly larger amount of Aβ42 remained intact after comparable times of incubation (24 h) with both proteases, a rate particularly striking for MMP-9. Because no significant changes in peptide aggregation that could explain these differences in degradation rate were observed during our experimental window (Western blots in Figs. 1 and 2), it is conceivable that more subtle folding variations through time, e.g. the changes in β-sheet content that precede the assembly of aggregates and the formation of fibrils, as we have previously reported (18), may have a profound influence in the degradation outcome of these enzymes in closely related substrates containing the same scissile bonds. Indeed, conditions that favor Aβ fibrillization seem to have a still not fully understood deeper effect in the proteolytic activity of gelsatinases that warrants further studies. Although our experimental conditions, soluble HFIP-treated peptides, suboptimal peptide concentration not ideal for rapid oligomerization/fibrillization, translate in MMP-2- and MMP-9-mediated Aβ cleavage, our previous studies demonstrated the inability of MMP-2 to degrade fibrillar assemblies of the AβE22Q vasculotropic mutant (16). In contrast, MMP-9 has been reported to partially degrade preformed Aβ fibrils (9), generating few identifiable truncated fragments (Aβ(1–20) and Aβ(1–30)) that do not match the sequence of events described in our studies employing soluble Aβ40 and Aβ42 homologues. This is not surprising because most cleavage sites accessible when Aβ is in an unstructured aqueous soluble state, as it is the case of the HFIP-treated Aβ homologues, become enclosed in the β-strand-turn-β-strand motif predominantly formed by residues 18–42 once the peptide adopts more protofibrillar/fibrillar conformations (32) and as a result are less available for proteolytic degradation.

The ability of MMP-2 and MMP-9 to cleave Aβ has been also reported using endogenous enzymes produced by cells in culture (12, 16), thus validating the physiological significance of the results obtained in this work with enzymes from recombinant sources. Some of the peptide bonds targeted by MMP-2 and MMP-9 have been previously identified in Aβ species isolated from the brain (10) and synthetic Aβ40 (8), although no systematic comparative studies have been reported nor a detailed identification of additional intermediate targets of these enzymes have been attempted. Our in vitro data provide support for the sequential degradation pattern Aβ40/Aβ42 → Aβ(1–34) → Aβ(1–30) → Aβ(1–16), thus identifying the generated intermediates as novel substrates for both enzymes while highlighting the fragment ending at position 16 as the terminal product of this degradation chain. Whether this sequential cleavage takes place to the same extent in vivo in the presence of the Aβ-binding protein and lipid molecules (33–35), which could potentially affect the peptide proteolytic processing, remains to be elucidated. Nevertheless and pointing out the physiological relevance of our current findings, previous work by us and by others groups, while not demonstrating the sequential cleavage we are describing, have reported that endogenous MMP-2 and MMP-9 of astrocytic and microvascular endothelial origin exhibit a comparable ability to cleave Aβ (12, 16). Supporting the physiological cleavage potential of MMPs and consistent with our findings, the intermediate and final cleavage fragments identified in our experimental paradigm have also been reported as components of the heterogeneous CSF Aβ profile (17, 36, 37). Our findings on the sequential C-terminal cleavage of Aβ, if reproducible in vivo to a similar extent, give an additional note of caution for the quantitative value of these fragments in biological specimens. Without considering the additional action of a myriad of other proteases known to degrade Aβ, the intermediate products generated by MMP-2 and MMP-9 serve, at least in vivo, as self-substrates of the same enzymes, potentially making their concentration highly variable. In this sense, Aβ(1–16) is likely to have a more stable concentration as the final product in the MMP-degradation cascade.

Accumulation of Aβ in the brain is considered a central element in the pathogenesis of AD with current evidence indicating that peptide buildup reflects an imbalance between its production and clearance. For the majority of AD cases, which are of late-onset and of sporadic origin, the cause of this imbalance is unclear and remains a subject of active investigation. Systemic clearance studies in animal models demonstrated that Aβ peptides have a short life in the circulation, similar to that of insulin or oxytocin, with liver being the main organ and the hepatocytes the main cells responsible for the uptake and degradation/excretion (38). In human healthy individuals, the production and turnover of Aβ are also fast (39), suggesting that small changes in production/clearance are likely to cause abnormal accumulation. Although to date no evidence supports an increase in the overall production of Aβ in sporadic cases, current research suggests an impaired clearance in late onset AD (40). The routes of Aβ clearance are diverse and include perivascular drainage to cervical lymph nodes and CSF, transport across the vessel walls into the circulation mediated by low density lipoprotein receptor-related protein 1 or the P-glycoprotein efflux pump, as well as enzyme-mediated catabolism (7, 31). Enzyme-mediated clearance has received considerable attention during the last decade, and many of the multi-
ple enzymes constituting the brain’s proteolytic machinery have shown potential to participate in Aβ catabolism−converting enzyme, and MMPs (41–43). In some cases the physiological relevance of enzymatic catalytic pathways has been validated in vivo mostly through the use of knock-out animal models and intracerebral gene transfer of some of the candidate enzymes (7). The studies presented herein clearly demonstrate that in the case of MMP-9 and more efficiently in MMP-2, enzymatic degradation of Aβ leads to the generation of smaller, more soluble peptides, with no apparent toxicity, at least within the experimental time frame of our assays, thus highlighting the more soluble peptides, with no apparent toxicity, at least within the experimental time frame of our assays, thus highlighting the potential beneficial role of both enzymes in Aβ catabolism. The broad production of MMP-2/MMP-9 by different brain cell types together with the extracellular localization of both enzymes (44–46) underscore their potential as relevant players for in vivo participation in the clearance of Aβ deposits, validated in vivo by the infusion of a broad spectrum of MMP inhibitors that result in elevated Aβ levels (12).

In contrast to their beneficial ability to degrade Aβ, increased expression and activation of both MMP-2 and MMP-9 have been reported in association with Aβ deposits in vascular and parenchymal lesions in CAA and AD brains, respectively (8, 47), a feature likely reflecting the observed up-regulation of both proteases triggered by Aβ in vitro in human neuronal, glial, and vascular cell line cultures (16, 31). Both enzymes have the capacity to degrade extracellular matrices and basement membranes, and MMP-2 in particular has been linked to the pathogenesis of intracerebral hemorrhage in patients with CAA (47). In experimental models, intracerebral injection of MMP-2 induced opening of the BBB and resulted in cerebral vessel leakage and hemorrhage (48–50), a deleterious effect ameliorated by the MMP-2 inhibitor TIMP-2 (51). MMP-2 activation was also demonstrated as a pivotal component of the disruption of the tight junction proteins occludin, claudin, and zonula occludens-1 occurring concomitantly to BBB permeability alterations in models of ischemia-reperfusion and stroke (52, 53), further highlighting the protease link with cerebral microvascular dysfunction.

Overall, the results presented here highlight the prospective beneficial effect of MMP-2 and MMP-9 for brain Aβ catabolism. Contrastingly, the known capacity of the proteases to degrade extracellular matrices, basement membrane components, and tight junction proteins also indicates that these proteases might undesirably compromise BBB integrity and precipitate a hemorrhagic phenotype, emphasizing the complexity of the cellular pathways and pathogenic mechanisms elicited by Aβ while providing a note of caution in potential therapeutic strategies aimed to enhance the activity of these Aβ-degrading enzymes.

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