

# Metalloendoprotease cleavage triggers gelsolin amyloidogenesis

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**Amyloid diseases like Alzheimer's disease and familial amyloidosis of Finnish type (FAF) stem from endoproteolytic cleavage of a precursor protein to generate amyloidogenic peptides that accumulate as amyloid deposits in a tissue-specific manner. FAF patients deposit both 8 and 5 kDa peptides derived from mutant (D187Y/N) plasma gelsolin in the extracellular matrix (ECM). The first of two aberrant sequential proteolytic events is executed by furin to yield a 68 kDa (C68) secreted fragment. We now identify the metalloprotease MT1-matrix metalloprotease (MMP), an integral membrane protein active in the ECM, as a protease that processes C68 to the amyloidogenic peptides. We further demonstrate that ECM components are capable of accelerating gelsolin amyloidogenesis. Proteolysis by MT1-MMP-like proteases proximal to the unique chemical environment of the ECM offers an explanation for the tissue-specific deposition observed in FAF and provides critical insight into new therapeutic strategies.**

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## Introduction

Proteins involved in human amyloidogenesis have distinct native structures and functions, yet they are believed to form a common cross  $\beta$ -sheet conformation in the misfolded/missembled state that leads to the characteristic fibrillar structures of amyloid deposits. The process of amyloidogenesis is genetically and biochemically linked to

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pathology, irrespective of the type of amyloidogenic protein. Amyloidoses emerge from three classes of proteins, including native folds that undergo amyloidogenic conformational changes, proteins that are normally endoproteolytically cleaved to an amyloidogenic fragment, and proteins where a mutation alters the fold, leading to aberrant endoproteolytic release of an amyloidogenic fragment(s). Plasma gelsolin, a protein of the latter class, when aggregated leads to familial amyloidosis of Finnish type (FAF), which is one of more than 30 different human diseases (Carrell and Gooptu, 1998; Dobson, 2003) in which amyloid is deposited in the extracellular matrix (ECM) of tissues. While Alzheimer's disease (AD) is the result of amyloidogenesis in the brain, FAF is characterized by extensive skin, arterial, neurologic, and ophthalmologic amyloid deposition (Kiuru, 1998). Understanding the basis for tissue-specific amyloid deposition and pathology remains a formidable challenge. Tissue-specific expression differences, influencing the concentration and thus the rate of aggregation, are likely to affect the tissue selectivity of amyloidoses (Sekijima *et al*, 2005). However, there are clearly additional mechanistic factors that contribute to the highly selective deposition of 8 and 5 kDa gelsolin fragments in the ECM.

Unlike many amyloid diseases, there is no sporadic disease associated with wild-type gelsolin deposition. In contrast, inheritance of either the D187N or D187Y mutations leads to FAF with unknown penetrance. Two forms of gelsolin are generated by alternative splicing (Kwiatkowski *et al*, 1986; Yin, 1987; Burtnick *et al*, 1997; Sun *et al*, 1999), intracellular 81 kDa and secreted 83 kDa versions, and, while both splice variants contain the mutation, only the secreted form (plasma gelsolin) is associated with amyloidosis (Kangas *et al*, 1996). Plasma gelsolin variants (D187N/Y) are aberrantly processed by at least two successive proteolytic events to generate the amyloidogenic peptides. The protease ( $\alpha$ -gelsolinase) responsible for the initial intracellular cleavage of plasma gelsolin to generate the 68 kDa fragment was identified as furin (Chen *et al*, 2001), a member of the proprotein convertase family that cycles between the endocytic and exocytic pathways (Molloy *et al*, 1999). D187N/Y mutations abolish  $\text{Ca}^{2+}$  binding in domain 2, destabilizing and rendering the domain accessible to aberrant proteolysis by furin as it transits through the Golgi. However, the identity of  $\beta$ -gelsolinase(s), whose activity results in formation of the 8 and 5 kDa fragments found in amyloid deposits (Ghiso *et al*, 1990; Haltia *et al*, 1990; Levy *et al*, 1990; Maury and Baumann, 1990; Maury *et al*, 1990; Paunio *et al*, 1994; Kangas *et al*, 1999), is unknown, as is the location of proteolysis. Moreover, the physiological basis for the ECM-localized deposition of FAF-associated amyloid fibrils remains unclear.

Herein we show that members of the matrix metalloprotease (MMP) family with MT1-MMP-like activity have a  $\beta$ -gelsolinase activity that generates the 8 and 5 kDa amyloidogenic peptides from the 68 kDa furin-derived gelsolin frag-

ment. While several MMPs generate the 8 kDa amyloidogenic fragment *in vitro*, a fibroblast cell line from a mouse model lacking MT1-MMP and HT1080 cells transfected with siRNA directed to MT1-MMP demonstrates that MT1-MMP activity can be solely responsible for the 8 kDa fragment produced in a cellular context. Given that MT1-MMP specifically and MMPs in general are located proximal to the ECM, the site of amyloid deposition in FAF, we investigated whether the chemical composition of the ECM hastened gelsolin amyloidogenesis. We demonstrate that GAGs, a major component of the ECM, accelerate gelsolin amyloidogenesis *in vitro*. We suggest that the unique enzymological and chemical environment of the ECM region may contribute to selective gelsolin amyloid deposition, emphasizing the role of the ECM in FAF, and likely other amyloidoses.

## Results

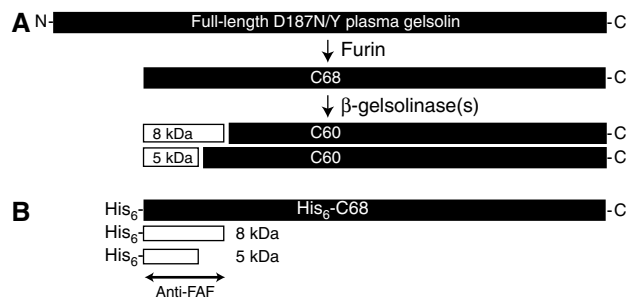
### HT1080 cells have $\beta$ -gelsolinase activity

The fragments found in FAF amyloid deposits result from sequential endoproteolysis of D187N/Y plasma gelsolin (Figure 1A). Initial processing of full-length (83 kDa) variant plasma gelsolin occurs during secretion, at R<sup>172</sup>-A<sup>173</sup>, by the intracellular protease furin in the Golgi (Chen *et al*, 2001). This generates a secreted 68 kDa carboxy (C)-terminal fragment (C68) that contains the amyloidogenic region at its N-terminus. C68 is the substrate for a second protease,  $\beta$ -gelsolinase, whose activity yields the major 8 kDa (70–71 residue) and minor 5 kDa (approximately 53 residue) amyloidogenic fragments. An extensive search for the  $\beta$ -gelsolinase(s) within the exocytic or endocytic pathways, or in blood plasma, did not yield candidate proteases (unpublished results).

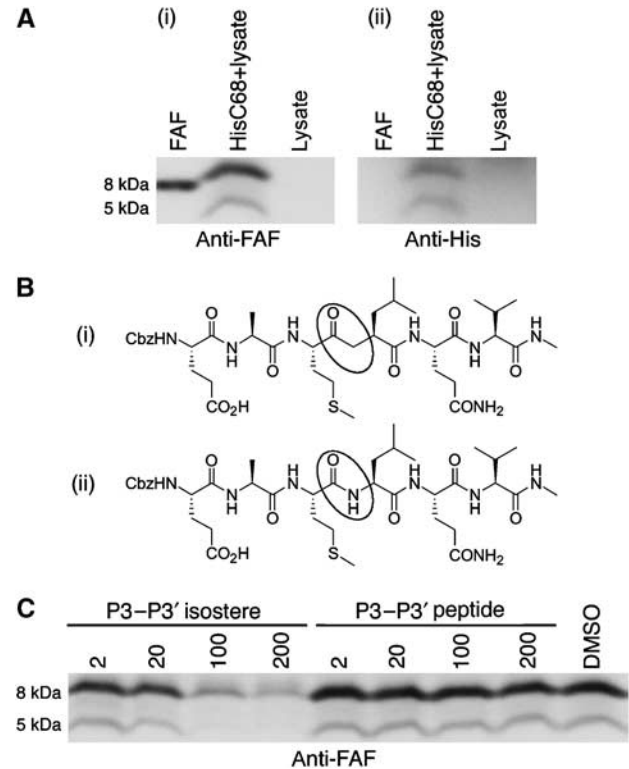
Although the sequences of the  $\beta$ -gelsolinase cleavage sites do not provide substantial insight into the identity of this protease(s), a hint regarding its identity came from the observation that FAF amyloid deposits are localized in the ECM of a variety of tissues (Kiuru, 1998). A number of

proteases function in the formation and remodeling of the ECM. These proteases were investigated as potential  $\beta$ -gelsolinases. A recombinant 68 kDa plasma gelsolin construct corresponding to the furin cleavage product was expressed with a His<sub>6</sub> tag at the N-terminus (His<sub>6</sub>-C68) to facilitate the identification of  $\beta$ -gelsolinase (Figure 1B). The presence of the His<sub>6</sub> tag could be detected using a His-specific antibody. In addition, an antibody that recognizes an epitope in both the 8 and 5 kDa fragments was generated (anti-FAF; Figure 1B).

To provide initial insight into the identity of  $\beta$ -gelsolinase, HT1080 cells, a human fibrosarcoma-derived cell line, were utilized as a known source of numerous ECM proteases. Incubation of His<sub>6</sub>-C68 with medium recovered from HT1080 cells yielded no cleavage into the fragments of interest even when APMA, an MMP activator, was present (data not shown), suggesting that proteases secreted by HT1080 cells do not have  $\beta$ -gelsolinase activity. In contrast, incubation of His<sub>6</sub>-C68 with the HT1080 whole-cell lysate resulted in the generation of multiple fragments, including



**Figure 1** Processing of plasma gelsolin to the 5 and 8 kDa amyloidogenic peptides. (A) Schematic representation of the full-length FAF variant plasma gelsolin (83 kDa) and the cleavage products detected in FAF patients. The first cleavage event, carried out by furin in the TGN, generates a 68 kDa product (C68). C68 is further processed by one or more  $\beta$ -gelsolinases to form the 8 and 5 kDa fragments found in FAF amyloid deposits, as well as a 60 kDa C-terminal fragment (C60). (B) Schematic representation of the recombinant 68 kDa protein generated with a His<sub>6</sub> tag at the N-terminus (His<sub>6</sub>-C68) that is used as a substrate to detect  $\beta$ -gelsolinase activity. In addition, the 8 and 5 kDa products of His<sub>6</sub>-C68 cleavage by  $\beta$ -gelsolinase are shown. Detection on immunoblots was carried out using either an antibody raised against the major 8 kDa product (anti-FAF; indicated) and/or a probe to the His<sub>6</sub> tag.



**Figure 2** Cleavage of His<sub>6</sub>-C68 by HT1080 cell lysate. (A) HT1080 lysate and His<sub>6</sub>-C68 were incubated either alone or together at 37°C for 17 h. The proteolytic products were detected with (i) anti-FAF and (ii) a His probe. The purified recombinant 8 kDa peptide (FAF), used as a standard, runs slightly faster on the gel than the HT1080-derived fragment because it lacks the His<sub>6</sub> tag on the N-terminus. (B) P3–P3′ isostere (i) and control peptide (ii) designed from residues E<sup>241</sup>–V<sup>246</sup> of gelsolin to probe the specificity of the  $\beta$ -gelsolinase activity in HT1080 lysate. The scissile amide moiety in the peptide (ii) and the noncleavable equivalent in the isostere (i) are circled. (C) Analysis of the specificity of cleavage in HT1080 lysate. HT1080 lysate was incubated with either P3–P3′ isostere or P3–P3′ peptide, at the concentrations indicated ( $\mu$ M), for 15 min (37°C) prior to addition of His<sub>6</sub>-C68. The sample labeled DMSO is a control incubated with neither the P3–P3′ isostere nor the P3–P3′ peptide, but with the solvent used. The blots were probed using anti-FAF.

peptides having the approximate molecular weight expected for both the major (8 kDa) and minor (5 kDa) amyloidogenic peptides (Figures 1B and 2A). The observed band at approximately 8 kDa runs slightly higher than does the band for a recombinant 8 kDa FAF fragment, due to the presence of the N-terminal His<sub>6</sub> tag in the cleaved fragment (Figure 2A). A lower band is observed at approximately 5 kDa (Figure 2A). These bands were reactive to the anti-FAF antibody (Figure 2A) and the antibody against the His<sub>6</sub> tag (Figure 2Aii), indicating that the peptides are derived from the N-terminus of His<sub>6</sub>-C68, as expected for  $\beta$ -gelsolinase cleavage products (Figure 1B).

### Proteolysis by the HT1080 lysate is specific for the $\beta$ -gelsolinase cleavage site

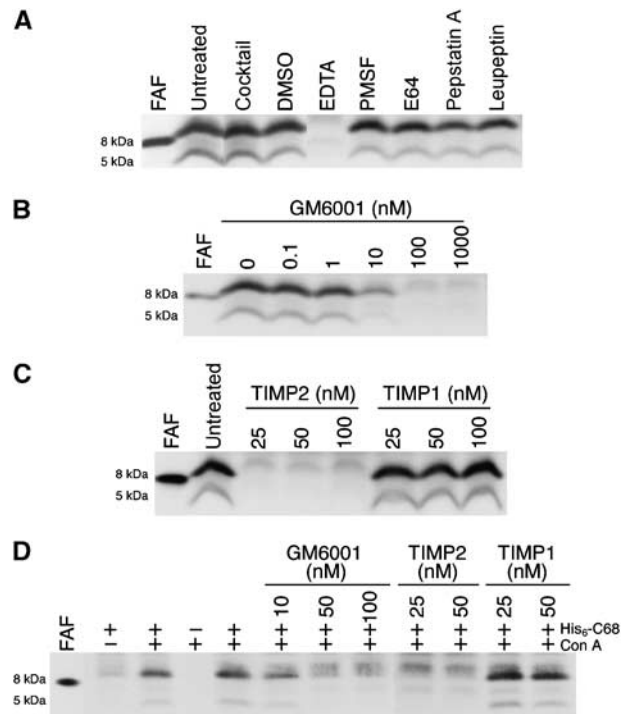
The major 8 kDa fragment of variant gelsolin deposited in FAF patients is thought to result from  $\beta$ -gelsolinase cleavage of C68 at either the A<sup>242</sup>-M<sup>243</sup> or M<sup>243</sup>-L<sup>244</sup> amide bonds, yielding an unknown distribution of 70- and 71-residue peptides that have been isolated from *ex vivo* samples (Maury, 1991).

To determine whether the protease activity in HT1080 lysate cleaves His<sub>6</sub>-C68 in the region defined by the A<sup>242</sup>-M<sup>243</sup>-L<sup>244</sup> sequence to form the 8 kDa fragment observed in FAF deposits, a peptidomimetic inhibitor was designed (supplementary data). This compound mimics the six-residue peptide sequence matching the E<sup>241</sup>-A-M-L-Q-V<sup>246</sup>  $\beta$ -gelsolinase site in gelsolin, with the exception that the amide bond between M<sup>243</sup> and L<sup>244</sup> is replaced by a proteolytically un-cleavable keto-ethylene dipeptide isostere (P3-P3' isostere; Figure 2Bi). Dose-dependent inhibition of the protease activity found in the HT1080 lysate by this isostere would demonstrate that the protease binds and cleaves at or near this amino-acid sequence in gelsolin. The corresponding peptide containing an amide bond at M<sup>243</sup>-L<sup>244</sup> was used as a control (P3-P3' peptide; Figure 2Bii). The isostere or peptide was incubated with HT1080 lysate for 15 min (37°C) before the addition of the His<sub>6</sub>-C68 substrate. The production of the 8 and 5 kDa fragments exhibited concentration-dependent inhibition by the P3-P3' isostere, but not the P3-P3' peptide (Figure 2C), indicating that the active protease in HT1080 lysate inhibited by the P3-P3' isostere was specific for cleavage at or near the site implicated by sequencing studies on the 8 kDa *ex-vivo* amyloid fragment (Maury, 1991). The appearance of the minor 5 kDa fragment also decreased with increasing P3-P3' isostere concentration, suggesting that the two cleavages are carried out either by the same enzyme or by two closely related proteases whose activities are effected by the same inhibitor. Alternatively, generation of the 5 kDa fragment may require initial generation of the 8 kDa fragment.

### Cleavage in HT1080 lysate is carried out by MMPs

The properties of the HT1080 protease were investigated using a panel of protease inhibitors. Inhibitors to serine, cysteine, and aspartic proteases had little effect on C68 processing. However, EDTA inhibited the production of both the 8 and 5 kDa fragments, suggesting the involvement of a metalloprotease (Figure 3A).

An important family of metalloproteases that are highly expressed in tumor cells, such as HT1080 cells, are the MMPs. The potential involvement of MMPs in the HT1080-mediated His<sub>6</sub>-C68 cleavage was assessed using the general MMP



**Figure 3** Inhibition of the HT1080-derived  $\beta$ -gelsolinase. (A) HT1080 lysates were incubated (37°C) with or without the protease inhibitors (EDTA (15 mM), PMSF (1 mM), E64 (100  $\mu$ M), pepstatin A (100  $\mu$ M), leupeptin (100  $\mu$ M)), indicated for 15 min prior to addition of His<sub>6</sub>-C68. The protease inhibitor cocktail contains AEBSF (1.4 mM), aprotinin (0.8  $\mu$ M) leupeptin (21  $\mu$ M), bestatin (36  $\mu$ M), pepstatin A (15  $\mu$ M), and E64 (14  $\mu$ M). (B) GM6001 was added to HT1080 lysate at the concentrations indicated 15 min before addition of His<sub>6</sub>-C68. (C) HT1080 lysate was preincubated for 2 h (37°C) with or without TIMP1 or 2 at the concentrations indicated prior to the addition of His<sub>6</sub>-C68. (D) His<sub>6</sub>-C68 was added to intact HT1080 cells in serum-free DMEM in the presence or absence of Con A (50  $\mu$ g/ml). GM6001, TIMP1, or 2 were added 1 h prior to the addition of His<sub>6</sub>-C68 at the concentrations indicated. After 20 h at 37°C, the media was removed and analyzed for the presence of His<sub>6</sub>-C68 cleavage products. All blots were probed with the anti-FAF antibody and have the recombinant 8 kDa fragment without the His<sub>6</sub> tag for reference.

inhibitor GM6001 (Galary *et al*, 1994a, b). Concentrations of GM6001 as low as 10 nM inhibited the production of both the 8 and 5 kDa fragments from His<sub>6</sub>-C68 in HT1080 lysate, implicating MMPs as potential  $\beta$ -gelsolinases (Figure 3B).

MMPs are not the only metalloproteases in the ECM. A related family of metalloproteases, the ADAM (A Disintegrin And Metalloprotease domain) family, may also be inhibited by hydroxamate inhibitors such as GM6001 (Yong *et al*, 2001). To differentiate between MMP and ADAM activity in the lysate of HT1080 cells, tissue inhibitors of metalloproteases (TIMPs) were utilized. TIMPs are endogenous metalloprotease inhibitors that function to regulate MMP activity *in vivo* (Gomez *et al*, 1997). TIMPs inhibit all MMPs tested so far, with the exception that TIMP1 fails to inhibit transmembrane MMPs (the MT-MMPs, MT1-, MT2-, MT3-, and MT5-MMP) (Will *et al*, 1996; Butler *et al*, 1997; Matsumoto *et al*, 1997; Llano *et al*, 1999; Shimada *et al*, 1999; Lee *et al*, 2003). ADAM family members are generally insensitive to TIMPs, although some examples of TIMP1 and 3 inhibition have been reported (Amour *et al*, 2000; Baker *et al*, 2002). Incubation of TIMP2 with HT1080 lysate prior to addition

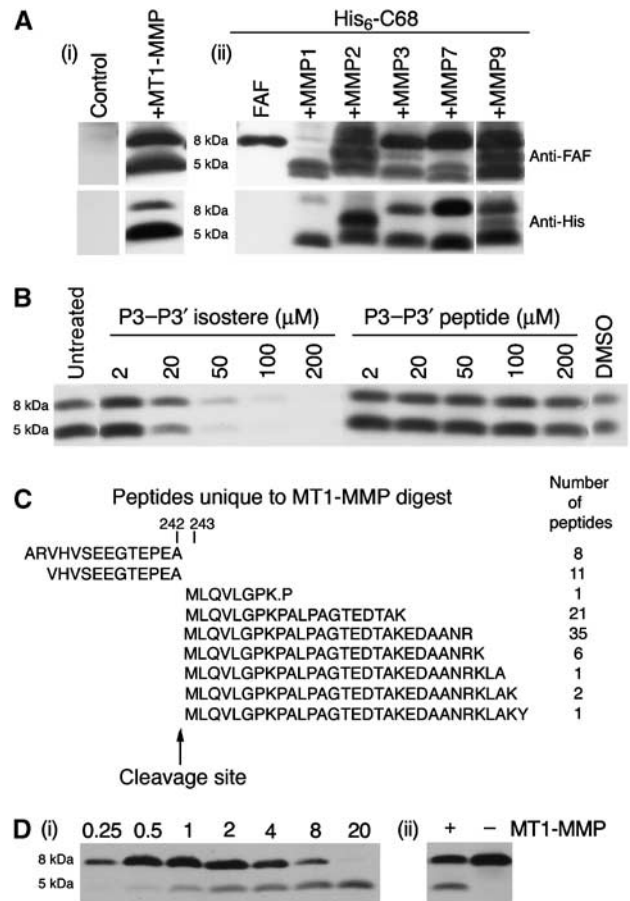
of His<sub>6</sub>-C68 resulted in the inhibition of production of both the 8 and 5 kDa gelsolin fragments at all concentrations tested (Figure 3C). Inhibition of β-gelsolinase activity by TIMP2 indicates that the activity resides in an MMP family member and not in an ADAM family member. In contrast, no inhibition was observed in the case of TIMP1 (Figure 3C). The lack of inhibition of cleavage of His<sub>6</sub>-C68 by TIMP1 implicates an MT-MMP, and more specifically MT1-MMP, an MT-MMP member known to be expressed in HT1080 cells (Strongin *et al*, 1995; Lohi *et al*, 1996), as the primary β-gelsolinase in HT1080 lysate.

Since MT1-MMP is an integral membrane protein with catalytic activity in the extracellular environment, cleavage of C68 by this protease should not require cell lysis. MT1-MMP expression and cell-surface levels have been shown to increase when cells are treated with concanavalin A (Con A) (Lohi *et al*, 1996; Jiang *et al*, 2001). His<sub>6</sub>-C68 was incubated with intact HT1080 cells in the presence or absence of Con A (Figure 3D). In the absence of Con A, production of the 8 kDa fragment is detectable, albeit at very low levels (Figure 3D). Treatment of the cells with Con A prior to His<sub>6</sub>-C68 addition dramatically increases the level of the 8 kDa fragment produced with the 5 kDa fragment becoming detectable, and this production displays sensitivity to the inhibitors GM6001 and TIMP2, but not to TIMP1, consistent with the activity of MT1-MMP (Figure 3D).

#### MT1-MMP is a β-gelsolinase

Incubation of the purified recombinant catalytic domain of MT1-MMP with His<sub>6</sub>-C68 directly demonstrates the ability of MT1-MMP to cleave this substrate to produce 8 and 5 kDa peptides (Figure 4Ai). The specificity of recombinant MT1-MMP for the β-gelsolinase cleavage site in His<sub>6</sub>-C68 was assayed in the absence or presence of the P3–P3' isostere. Incubation of the isostere with MT1-MMP inhibited its ability to cleave His<sub>6</sub>-C68 in a concentration-dependent fashion, while the control peptide had little effect (Figure 4B), demonstrating a similar specificity to the protease present in HT1080 lysate for the β-gelsolinase cleavage site. Although β-gelsolinase generates an 8 kDa amyloidogenic fragment that has either an Ala (70-residue) or Met (71-residue) at its C-terminus, the proportions of these peptides in *ex vivo* amyloid have not been determined quantitatively (Maury, 1991). Inhibition by the P3–P3' isostere indicates that MT1-MMP may be specific for the M<sup>243</sup>–L<sup>244</sup> cleavage site in C68. However, cleavage at the alternative A<sup>242</sup>–M<sup>243</sup> site cannot be ruled out from these experiments, as the isostere could potentially function as a competitive inhibitor for the adjacent cleavage site.

To determine the precise site of cleavage, we analyzed the gelsolin cleavage products generated by the purified soluble proteolytic domain of MT1-MMP using mass spectrometry. Following incubation of the His<sub>6</sub>-C68 substrate in the presence or absence of MT1-MMP, tryptic digests of the resulting peptide(s) were prepared and the distribution of the fragments was determined by multi-dimensional protein identification technology (MudPIT) (Wolters *et al*, 2001; Washburn *et al*, 2002). MudPIT revealed a striking recovery of cleavage products implicating proteolysis between A<sup>242</sup> and M<sup>243</sup> (Figure 4C), affording the 70-residue amyloidogenic peptide identified in patients (Maury, 1991). No evidence was found for cleavage between M<sup>243</sup> and L<sup>244</sup> to yield the 71-residue peptide terminating with M<sup>243</sup> also found in patients (Maury,



**Figure 4** MT1-MMP cleavage of 68 kDa C-terminal gelsolin fragment. **(A)** His<sub>6</sub>-C68 was incubated at 37°C for 17 h either alone, with control, or with the purified recombinant MMPs indicated. The blots were probed with either anti-FAF (top) or His<sub>6</sub>-probe (bottom). The recombinant 8 kDa fragment (FAF) is shown for reference. **(B)** MT1-MMP was preincubated for 2 h with either the P3–P3' isostere or the P3–P3' peptide at the concentrations indicated. His<sub>6</sub>-C68 was added and the incubation continued for 2 h at 37°C. The lane labeled untreated was incubated with buffer only and the lane labeled DMSO was incubated with DMSO only. The blot was incubated with the antibody probe to the His<sub>6</sub> tag. **(C)** MudPIT analyses of MT1-MMP cleavage products of 68 kDa PG performed as described in Materials and methods. The cleavage site of MT1-MMP (between A and M) is indicated by the arrow. **(D)** (i) Recombinant MT1-MMP was incubated with His<sub>6</sub>-C68 for the time periods indicated above each lane (in h). The blot was incubated with the anti-FAF antibody. (ii) The recombinant 8 kDa fragment was incubated in the presence or absence of recombinant MT1-MMP for 4 h. The blot was probed with the anti-FAF antibody.

1991). From the 70-residue peptide, we recovered 86 independent mass spectra of tryptic peptides terminating with A<sup>242</sup> or beginning with the M<sup>243</sup> residue in the MT1-MMP-treated tryptic digest compared to two spectra in the untreated, control sample. This cleavage pattern is consistent with the known preference that MT1-MMP has for the Pro residue being in the P3 position (–P<sup>240</sup>–E–A–M–L–Q–V<sup>246</sup>–) (Zucker *et al*, 2003). Although the 5 kDa fragment from FAF patients has not been carefully characterized to determine the site of cleavage, incubation of purified MT1-MMP for increasing time with His<sub>6</sub>-C68 shows that sequential cleavage events by this protease may result in generation of a 5 kDa fragment from the 8 kDa fragment (Figure 4Di). Indeed, incubation of the recombinant 8 kDa fragment with MT1-MMP results in the

generation of a 5 kDa fragment (Figure 4Dii), providing a potential explanation for the source of this proteolytic fragment.

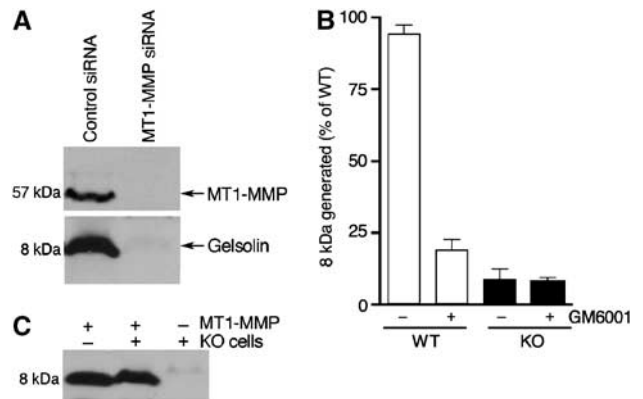
Collectively, these data strongly implicate MT1-MMP activity in generating the 8 and 5 kDa fragments observed in the media of HT1080 cells incubated with His<sub>6</sub>-C68. However, it remains possible that other  $\beta$ -gelolinases could contribute to the generation of the fragments observed in tissues. MMPs are a large family of metalloproteases and, due to similarities in their catalytic activity, it is likely that they have overlapping substrate specificities. Using a panel of purified recombinant MMPs with a range of enzyme concentrations in each case to identify early- and late-cleavage products (data not shown), we determined that, in addition to MT1-MMP, soluble MMPs 3, 7, and 9, but not other MMPs such as MMP2, have an MT1-MMP-like activity that could cleave the substrate to produce fragments of approximately 8 and 5 kDa, which are immunoreactive for both the FAF and His<sub>6</sub> epitopes (Figure 4Aii). These data indicate that multiple MMP family members may contain  $\beta$ -gelolinase activity and contribute to the generation of the amyloidogenic peptides in FAF patients.

#### Cells lacking MT1-MMP fail to generate the 8 kDa FAF fragment

Given the possibility that MMPs besides MT1-MMP could potentially contribute to FAF, we analyzed two distinct cellular systems where MT1-MMP is absent, or greatly reduced, to look at the importance of this family member in regard to  $\beta$ -gelolinase activity in these cells.

The first cell system examined utilized HT1080 cells, where expression levels of MT1-MMP were manipulated with siRNA. HT1080 cells transfected with siRNA directed to MT1-MMP show complete loss of the 57 kDa active MT1-MMP enzyme (Figure 5A, upper panel) compared to cells exposed to control scrambled RNA. When these cells were analyzed for their ability to cleave the His<sub>6</sub>-C68 substrate to the 8 kDa fragment, it was found that the cells lacking MT1-MMP were unable to generate the 8 kDa fragment (Figure 5A, lower panel).

The second system analyzed involved the generation of fibroblast cell lines from both wild-type and MT1-MMP knockout mouse embryos (Holmbeck *et al*, 1999). If MT1-MMP is the predominant or sole source of  $\beta$ -gelolinase in these cells, it is expected that only the wild-type and not the MT1-MMP knockout cells would be capable of generating the 8 kDa peptide from His<sub>6</sub>-C68. Cells prepared from both the wild-type and heterozygous mouse embryos (data not shown), but not two cell lines derived from the two knockout mouse embryos, had  $\beta$ -gelolinase activity. Whereas the lysate of wild-type cells incubated with His<sub>6</sub>-C68 produced an 8 kDa fragment with a protease that is sensitive to the MMP inhibitor GM6001 (Figure 5B), the lysate from the MT1-MMP knockout cells is unable to generate significant levels of the 8 kDa fragment (Figure 5B, dark bars). Transient overexpression of MT1-MMP in the knockout fibroblasts failed to recover processing of His<sub>6</sub>-C68 to 8 kDa—further analyses revealed that the knockout cell line fails to process MT1-MMP from the inactive 60 kDa precursor to the active 57 kDa (data not shown); presumably, the rapid overexpression overwhelms the processing machinery (Lohi *et al*, 1996). Consistent with this interpretation, addition of recombinant MT1-MMP to the knockout cell lysate enables the recovery of the activity that generates the 8 kDa fragment, indicating that



**Figure 5** Cells lacking MT1-MMP do not contain  $\beta$ -gelolinase activity. (A) HT1080 cells were transfected with either control-scrambled RNA or siRNA directed to MT1-MMP. Cell lysates were blotted and probed with an antibody raised against MT1-MMP, shown in the upper panel. Cell lysates were incubated with His<sub>6</sub>-C68 at 37°C for 17 h and the blots probed with the anti-FAF antibody, lower panel. Each assay was executed in triplicate and representative data are shown. (B) Cell lysates (at 4 mg/ml) were generated from either wild type (WT, white columns) or MT1-MMP knockout fibroblasts (KO, black columns) and incubated with His<sub>6</sub>-C68 at 37°C in the presence or absence of GM6001 (200  $\mu$ M) for 20 h. Blots were probed using the anti-FAF antibody and densitometry utilized to quantify levels of 8 kDa produced. Each condition is an average of five replicates from two independent experiments. Data are normalized to the highest level of 8 kDa generated by WT lysate in the absence of the inhibitor. Error bars indicate the standard error of the mean. (C) His<sub>6</sub>-C68 was incubated either alone or with lysate from knockout fibroblasts, in the presence or absence of recombinant MT1-MMP for 4 h at 37°C. The blots were probed with the anti-FAF antibody.

it is the lack of MT1-MMP that is responsible for loss of processing and not the loss or gain of some other factor (Figure 5C). These results provide additional data in an *in vivo* cellular context, suggesting that MT1-MMP can be a critical  $\beta$ -gelolinase in FAF.

#### The ECM environment contributes to FAF by hastening amyloidogenesis

That MT1-MMP generates amyloidogenic gelsolin peptides proximal to the ECM raises the possibility that the chemical environment of the ECM could contribute to selective gelsolin deposition. We have previously shown that amyloidogenesis of the recombinant 8 kDa (71 residue) gelsolin fragment (A<sup>173</sup>-M<sup>243</sup>) is optimal at low pH, leading to speculation that disease-associated fibril formation may occur in an acidic environment (Ratnaswamy *et al*, 1999). However, the identification of the extracellular MT1-MMP as a  $\beta$ -gelolinase now suggests that gelsolin is cleaved and forms fibrils in the neutral pH environment of the ECM. To address whether these peptides could form fibrils at neutral pH, we re-examined the pH dependence of fibril formation *in vitro* using the recombinant 70- and 71-residue FAF 8 kDa fragments, corresponding to those found in FAF patients. Amyloidogenicity was monitored utilizing thioflavin T (TfT), an environmentally sensitive fluorophore that fluoresces upon binding to a cross  $\beta$ -sheet structure. Whereas amyloid production was not detected at pH 7.0 in nonagitated solutions on a laboratory timescale (Ratnaswamy *et al*, 1999), when agitation is used to increase the rate of amyloidogenesis (Cohlberg *et al*, 2002) both the 70- and 71-residue fragments formed TfT-positive

aggregates over the pH range of 4–7. Transmission electron microscopy (TEM) confirmed a well-defined fibrillar morphology for all conditions (Figure 6Aii, inset).

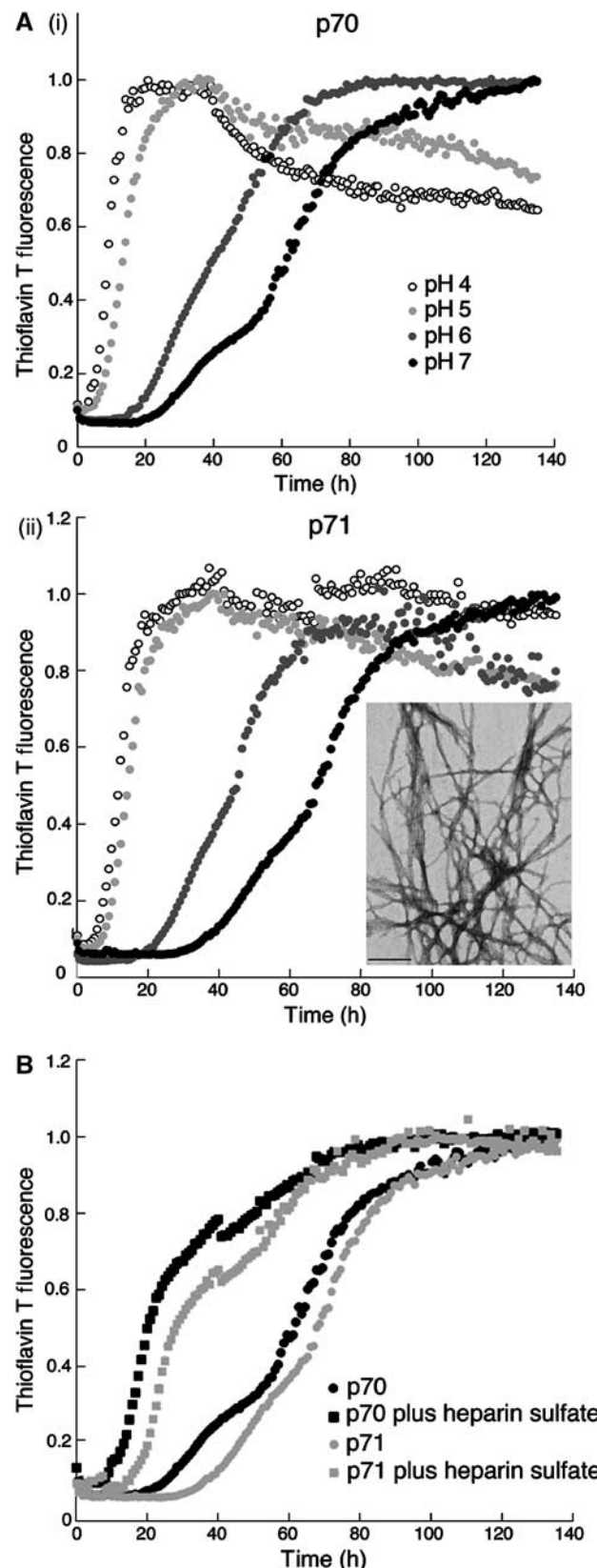
Given that FAF-associated amyloid is predominantly deposited in the ECM (Kiuru, 1998), the role of ECM components in the formation of fibrils was examined. Several ECM components, such as heparan sulfate proteoglycans (HSPGs) and GAGs, including heparan sulfate and heparin, are known to be associated with amyloid fibrils in many amyloidoses (Sipe and Cohen, 2000). To determine whether ECM components may contribute to FAF amyloidogenesis, the rate of fibril formation by the 70- and 71-residue amyloidogenic (8 kDa) fragments was evaluated in response to the addition of heparin. Heparin was found to accelerate the formation of TftT-positive aggregates at pH 4–7 (Figure 6B; pH 7 data shown). TEM analysis of agitated samples in the presence of heparin revealed well-defined fibrillar structures for both fragments (data not shown). These results suggest that GAGs accelerate amyloidogenesis at neutral pH, consistent with previous reports that ECM components stimulate other amyloidogenic polypeptides to form the cross  $\beta$ -sheet structure, rationalizing their presence in the ECM (McLaurin *et al*, 1999; Cohlberg *et al*, 2002; Ancsin, 2003).

## Discussion

MMPs have an established pathological role in diseases such as cancer and arthritis (Knauper and Murphy, 1998; Folgueras *et al*, 2004). We now provide strong evidence that MMPs, specifically MT1-MMP, can fulfill the specific function of  $\beta$ -gelsolinase, cleaving the secreted variant C68 to generate the 8 and 5 kDa amyloidogenic fragments found in FAF patients. While *in vitro* studies show that several MMPs can generate the 8 and 5 kDa amyloidogenic fragments, a fibroblast cell line from a mouse model lacking the membrane-localized MMP, MT1-MMP, and HT1080 cells transfected with siRNA directed to MT1-MMP demonstrates that MT1-MMP activity can be responsible for generation of the 8 kDa amyloidogenic fragment. We also reveal that GAGs, a major component of the ECM, accelerate gelsolin amyloidogenesis *in vitro*. Given the close juxtaposition of the cell membrane and the ECM, a unique chemical and enzymological environment may exist that significantly contributes to deposition of gelsolin amyloid and onset of FAF. A more quantitative examination of the composition of patient deposits in future may reveal further ECM-associated proteolytic events.

**Figure 6** Effects of pH and ECM components on FAF fibril formation. (A) pH dependence of agitated 70-residue (i) and 71-residue (ii) fibril formation monitored by TftT fluorescence. (Aii, inset) EM image of FAF fibrils (70-residue peptide) formed at pH 7 in an agitation assay. The photograph is taken at  $\times 52\,000$  magnification and is a representative of at least three experiments. Scale bar equals 200 nm. (B) Fibril formation in an agitation assay with 20  $\mu$ M 70-residue (black points) and 71-residue (gray points) peptides at pH 7 in either the presence (squares) or absence (circles) of 0.1 mg/ml heparin monitored by TftT fluorescence intensity. Heparin alone did not affect the fluorescence of TftT (data not shown). The fibril formation assays were repeated on at least 5–6 independent occasions and the representative data are shown. TftT fluorescence units were measured every 10 min and plotted against time. The data were then normalized to the TftT fluorescence value at maximal fibril formation to account for the pH dependence of TftT fluorescence intensity.

Circumstantial evidence also exists to suggest that MMPs have a role in fibril formation in AA amyloidosis (Stix *et al*, 2001) and in the most prevalent amyloid disease, AD (Leake *et al*, 2000; Chong *et al*, 2001; Jung *et al*, 2003; Lorenzl *et al*, 2003; Saarela *et al*, 2004). The precise mechanism by which



MMPs effect AD pathology is unknown, though it has been proposed that their action may facilitate deposition through re-modeling of the ECM (Saarela *et al*, 2004).

A potentially important factor influencing the location of FAF deposits is the local concentration of C68. C68 is highly abundant in the serum of patients with FAF (Maury and Rossi, 1993; Maury *et al*, 1997). If transcytosed by the endothelial cells composing the arterial wall to the extravascular space, C68 would be expected to encounter MT1-MMP activity in the area adjacent to the ECM. In this scenario, serum C68 could contribute significantly to the extensive peripheral systemic amyloid deposition associated with FAF pathology. A second possibility is that C68 produced proximal to the site of deposition would have direct access to the local ECM. In this view, the circulating plasma pool may play no role or a reduced role in disease. Though plasma gelsolin is highly expressed by muscle cells, many cell types also synthesize and secrete the protein (Kwiatkowski *et al*, 1988; Paunio *et al*, 1997). Thus, secretion of the C68 fragment into the adjacent ECM by these cells may directly account for the extent and rate of tissue-selective gelsolin amyloid deposition. These possibilities are not mutually exclusive. It remains possible that the extent of tissue-specific systemic deposition reflects the activity of one or both pools.

While C68 levels are likely to play a role in FAF, the distribution, concentration, and level of activity of MT1-MMP-like proteases that have related cleavage specificities are also expected to influence the extent of and site of gelsolin amyloid deposition. MT1-MMP-like proteases are expressed in a variety of adult tissues, with each MMP having distinct expression patterns and levels (Knauper and Murphy, 1998; Pei, 1999). The arterial wall, a site where FAF patients exhibit substantial deposition, is composed of smooth muscle cells. Smooth muscle cells have been shown to express a high level of both gelsolin (Kwiatkowski *et al*, 1988) and MT1-MMP (Su *et al*, 2004). Thus, there is a strong correlation between this major site of deposition and high levels of both substrate and the MT1-MMP enzyme. The potential contribution of other MT1-MMP-like proteases remains to be explored.

Our *in vitro* data suggest that amyloid deposition is also likely to be dependent on components of the ECM in a given tissue. GAGs are known to lower the critical concentration required for misassembly, accelerating the cross  $\beta$ -sheet formation of other amyloidogenic peptides (Snow and Wight, 1989). The colocalization of the GAG accelerators and the MT1-MMP cleavage enzyme provides an explanation for the proclivity of selective gelsolin amyloid deposition in the ECM.

The discovery that furin- and MT1-MMP-like proteases have activities that are potentially responsible for the FAF pathological cascade in humans now makes it possible to target FAF using protease inhibitors. Given the central importance of furin in protein-trafficking pathways (Molloy *et al*, 1999), it seems unlikely that we can inhibit the formation of C68 with furin inhibitors without encountering unwanted side effects. In contrast, numerous potent MMP inhibitors, some of which are quite selective, could prove useful for inhibiting the processing of C68 into the amyloidogenic peptides. A second possibility, revealed by our observation that ECM components accelerate plasma gelsolin amyloidogenesis, is to antagonize the acceleration of gelsolin amyloidogenesis by ECM mimetics. Fibrillex<sup>TM</sup>, a

compound recently approved by the FDA to ameliorate SAA amyloidosis, functions by antagonizing the interaction between the ECM components and fibrils, and could be useful for treating FAF.

The identification of furin ( $\alpha$ -gelsolinase) (Chen *et al*, 2001) and now MT1-MMP-like proteases ( $\beta$ -gelsolinase), in combination with the influence of the ECM on the rate of gelsolin amyloidogenesis, provides important clues for understanding of FAF pathology in humans. The link between the site of origin of precursor substrates and conditions supporting generation and deposition of amyloid remains an elusive feature of many amyloid diseases. The current studies provide important insights into a new general mechanism by which processing at multiple sites and the local environment strongly influence the onset and accumulation of amyloid.

## Materials and methods

### Materials

MMPs and GM6001 were purchased from Oncogene Research products (Boston, MA). General protease inhibitors, Con A and heparin sulfate, were purchased from Sigma-Aldrich (St Louis, MO), and TIMP1 and 2 and the polyclonal rabbit anti-MT1-MMP antibody (#AB815) from Chemicon Inc. (Temecula, CA).

### Design and construction of His<sub>6</sub>-C68

Primers CGTGTGCATATGCCACCGAGGTACCTGTGTCC and GGCCAGCTTGGGTTGGCCGC were used for PCR. Digestion of pET11d with *NdeI/BamHI*, plasma gelsolin pCMV<sup>3</sup> with *PstI/BamHI*, and the PCR product with *NdeI/PstI* followed by a three-part ligation created His<sub>6</sub>-C68 in pET11d. Expression was carried out in BL21 cells and His<sub>6</sub>-C68 purified on Ni-NTA agarose (Qiagen, Valencia, CA).

### Preparation of recombinant FAF fragment and antibodies

The 8 kDa p70 and p71 FAF peptides were recombinantly expressed and purified as described (Ratnaswamy *et al*, 1999). The polyclonal antibodies against GST-8 kDa gelsolin fragment (Ratnaswamy *et al*, 1999) were raised in rabbits. Anti-His probe was obtained from Pierce Biotechnology, Inc. (Rockford, IL).

### Preparation of gelsolin peptides and peptide isosteres

See Supplementary data.

### HT1080 assays

See Supplementary data.

### siRNA

See Supplementary data.

### Generation of mouse fibroblast cell lines

See Supplementary data.

### Fibril formation assay

See Supplementary data.

### Mass spectrometry

See Supplementary data.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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