Gelatinase A possesses a β-secretase-like activity in cleaving the amyloid protein precursor of Alzheimer's disease

Rex N. LePage[a,b], Amanda J. Fosang[c], Stephanie J. Fuller[b], Gillian Murphy[d], Geneviève Evin[b], Konrad Beyreuther[e], Colin L. Masters[b], David H. Small[a,b,*]

[a]*Laboratory of Molecular Neurobiology, Melbourne Nerve Growth Research Unit, University of Melbourne, Parkville, Vic. 3052, Australia*
[b]*Department of Pathology, University of Melbourne, Parkville, Vic. 3052, Australia*
[c]*Orthopaedic Molecular Biology Research Unit, Department of Paediatrics, University of Melbourne, Parkville, Vic. 3052, Australia*
[d]*Strangeways Research Laboratory, Cambridge CB1 4RN, UK*
[e]*Centre for Molecular Biology, University of Heidelberg, Heidelberg, Germany*

Received 2 September 1995; revised version received 9 November 1995

Abstract The ability of the 72 kDa gelatinase A to cleave the amyloid protein precursor (APP) was investigated. HeLa cells were transfected with an APP_{695} plasmid. The cells were incubated with gelatinase A, which cleaved the 110 kDa cell-surface APP, releasing a 100 kDa form of the protein. A peptide homologous to the β-secretase site was cleaved by gelatinase A adjacent to a glutamate residue at position −3 (βA4 numbering system). A peptide homologous to the α-secretase site was not cleaved. The results demonstrate that 72 kDa gelatinase A is not an α-secretase, but that it may have a β-secretase activity.

Key words: Alzheimer's disease; Amyloid; Gelatinase; Secretase; Protease

1. Introduction

An early event in the pathogenesis of Alzheimer's disease (AD) is the deposition in the brain of amyloid fibrils assembled from the 4 kDa amyloid protein (βA4) [1]. βA4 is derived from a segment of the β-amyloid protein precursor (APP), a membrane-spanning glycoprotein. βA4 is thought to result from proteolysis of APP by a β-secretase [2,3] which cleaves close to met-596 (APP695 numbering) leading to production of potentially amyloidogenic carboxyl-terminus fragments of APP. An alternative processing route involving an α-secretase which cleaves APP within the amyloid sequence is not thought to contribute to amyloid formation [2,3].

Although the sites of action of the α- and β-secretases within the APP sequence are well documented, the proteases have not been unequivocally identified and characterised [3,4]. The identification and characterisation of APP secretases is important for development of therapeutic strategies to control the build up of amyloid in the brain and the pathological effects of AD [5].

A report by Miyazaki et al. [6] suggested that a 72 kDa metalloproteinase, gelatinase A, may have α-secretase activity. Their studies showed that a synthetic peptide homologous to residues 10–20 in βA4 was cleaved by gelatinase A at a lysine residue known to be the site of cleavage of the α-secretase. However, as gelatinase A was activated by a procedure employing trypsin digestion and stromelysin activation, it was not conclusively demonstrated in that study that the activated gelatinase A was free of trypsin activity. Furthermore, Walsh et al. [7] found that a specific inhibitor of gelatinase A (TIMP-1) failed to inhibit the secretion of APP from PC12 cells and were unable to detect any 72 kDa gelatinase A in the medium conditioned by PC12 cells.

The aim of the present study was to examine the putative secretase activity of gelatinase A. We studied the release of membrane-bound APP from HeLa cells transiently transfected with an APP695 cDNA. Our findings support the view that the 72 kDa gelatinase A is not an α-secretase, and they suggest that the protease may have a β-secretase-like activity.

2. Materials and methods

2.1. Materials

Naphthol AS-MX phosphate, Fast red and 4-aminophenylmercuric acetate (APMA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rainbow molecular weight markers for western blotting were purchased from Amersham Australia Pty. Ltd. (Castle Hill, NSW, Australia). Dulbecco's MEM was obtained from Life Technologies Pty. Ltd. (Mt. Waverley, Victoria, Australia). Keratanase (EC 3.2.1.103) was from Seikagaku (Kogyo, Japan). The primary antibody used in western immunoblotting, which recognizes an epitope near the amino-terminus of APP, was a mouse monoclonal antibody (22C11) raised against a recombinant APP695 bacterial fusion protein [8]. Affinity purified anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was from Promega (Sydney, NSW, Australia). Synthetic C-terminally amidated peptides were synthesised by the Fmoc procedure [9]. The first peptide, designated Pb, was homologous to the β-secretase cleavage site in APP695 between residues 588 and 601 (TEEISEVKMDAEFR-NH_2). The second peptide, designated Pα, was homologous to the a-secretase cleavage site in APP695 between residues 606 and 619 ($\beta A4_{10-23}$, YEVHHQKLVFFAED-NH_2). The aggrecan G1-G2 fragments were prepared as previously described by Fosang and Hardingham [10]. Recombinant human progelatinase A (MMP-2, EC 3.4.24.24) was purified to apparent homogeneity as assessed by SDS polyacrylamide gel electrophoresis [11]. The purified proenzyme, when activated possessed a molecular mass of 72 kDa and possessed a specific activity towards 14C-labelled gelatin of 464 units/nmol [11]. Just prior to its use, the proenzyme was activated by incubation for 2 h at 25°C or 22 h at 37°C with 2 mM 4-aminophenylmercuric acetate (APMA).

2.2. HeLa cell culture and transfection

HeLa cells were transiently transfected by calcium phosphate coprecipitation with a plasmid (pAD-695) encoding the 695-amino acid form of APP [8,12]. The following day, cells were subcultured into 6-well plastic culture dishes at a density of 2×10^5 cells/well in Dulbecco's

*Corresponding author. Dept. of Pathology, University of Melbourne, Parkville, Vic. 3052, Australia. Fax: (61) (3) 9344 4004.
E-mail: david_small@muwayf.unimelb.edu.au

Abbreviations: βA4, amyloid protein; AD, Alzheimer's disease; APP, amyloid protein precursor; APMA, 4-aminophenylmercuric acetate.

SDI 0014-5793(95)01358-X

modified Eagles medium containing 10% fetal calf serum. After 24 h, the medium was removed and the cells in each well were washed with 2 × 1 ml of serum-free medium.

2.3. APP release experiments

HeLa cells were incubated with 200 μl/well of serum-free medium containing 0.1% (w/v) Na-azide for 15 min at 37 °C to inhibit endogenous release of APP [12]. Activated 72 kDa gelatinase A was added at various concentrations in a total volume of 50 μl of buffer (20 mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$) and the cells were incubated for 30 min at 37 °C. The buffer was then removed and concentrated to 40 ml by filtration through Centricon-30 concentrators (Amicon, Beverly, MA) and then analysed by Western blotting as previously described [12].

2.4. Digestion of synthetic peptides

Aliquots of activated 72 kDa gelatinase A (1 μg) were incubated for 18 h at 37 °C with 25 μg of peptide in a total volume of 50 μl of 20 mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$. The digestion was stopped by adding 0.1 volumes of glacial acetic acid. The mixture was centrifuged in a Beckman microfuge at 10,000 × *g* for 15 min at 4 °C and then the digests were analysed by reversed phase high performance liquid chromotography on a Waters C18 Novapak column (0.4 × 150 mm) using an Applied Biosystems 400 solvent delivery system. The absorbance at 214 nm was monitored using an Applied Biosystems 1000S detector. Peptides were eluted with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid/H_2O at a flow rate of 1 ml/min over 60 min [12]. Peptides eluting from the column were identified by amino acid sequencing using an Applied Biosystems model 470A sequencer with an on-line 120A PTH-amino acid analyser, as well as commercially by Chiron Mimotopes Pty. Ltd. (Clayton, Australia) by time-of-flight mass spectrometry using an API Sciex II LC/MS/MS system (Perkin Elmer Sciex, Thornhill, Canada).

2.5. Digestion of aggrecan-G1-G2 fragment

Gelatinase A digestion of aggrecan-G1-G2 fragment was carried out in 50 mM Tris-HCl buffer pH 7.5 containing 10 mM $CaCl_2$ and 100 mM NaCl for 18 h at 37 °C. The digestions were stopped by the addition of 10 mM ethylenediaminetetraacetic acid and 2 mM 1,10-phenanthroline. The digestion of the G1-G2 fragment of aggrecan was analysed by 5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Fairbanks et al. [13]. Gels were stained with silver to visualise the products of digestion [14].

3. Results

Initially, we examined the possibility that gelatinase A could release APP from the surface of intact cells. HeLa cells were transiently transfected with an APP695 cDNA and the cells incubated with various concentrations of gelatinase A. All incubations were performed in triplicate. Similar results were obtained in each incubation. In the absence of gelatinase A, no APP release into the medium was detected (Fig. 1, lane 2). At the lowest concentration of gelatinase A employed (10 ng/ml), no released immunoreactive APP was detected (lane 3). However, at 100 ng/ml gelatinase A, a 100–110 kDa band was seen (lane 4). This band was approximately 10 kDa lower in molecular mass than the major cellular form of APP [8,15,16]. At the highest concentration of gelatinase A (1 μg/ml), a second cleavage product of 70 kDa was detected (lane 5). There was no difference in the APP immunoreactivity in the cells following gelatinase A treatment (data not shown).

These findings indicated that gelatinase A could release APP from the cell surface with a secretase-like action, although they did not indicate the position of the cleavage site in APP. To address this question, synthetic peptides homologous to the α-and β-secretase cleavage sites were incubated with gelatinase A, and the products of digestion were analysed by reversed

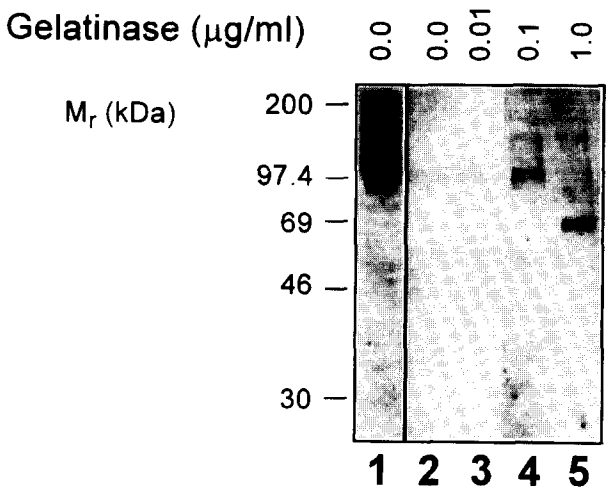

Fig. 1. Release of APP immunoreactivity by 72 kDa gelatinase A from transiently APP695-transfected HeLa cells. HeLa cells were transfected with an APP695 cDNA (pAD-695), treated with Na-azide to inhibit endogenous secretion of APP, and then incubated for 30 min at 37°C with gelatinase A. The APP-immunoreactivity in the HeLa cells (lane 1) and the immunoreactivity released into the medium (lanes 2–5) were analysed by western blotting using a monoclonal antibody (22C11) which recognises APP. The total loading of concentrated medium was 34 μl/lane in lanes 2–5. The amount of cell protein applied in lane 1 was equivalent to 1.1×10^5 cells. The positions of Rainbow molecular weight markers (Amersham Australia Pty. Ltd., North Ryde, Australia) are shown.

phase high performance liquid chromatography. When peptide Pα (YEVHHQKLVFFAED-NH_2) homologous to the α-secretase site on APP was incubated with a high concentration of gelatinase A (ratio of peptide to gelatinase A = 25 μg:1 μg) for 22 h at 37 °C, no breakdown of the peptide was detected (Fig. 2C,D).

A synthetic peptide P_β (TEEISEVKMDAEFR-NH_2) homologous to the region of APP cleaved by β-secretase was cleaved by gelatinase A to yield two major peptide fragments (Fig. 2B). Amino acid sequencing demonstrated that the two products (peaks 1 and 2) eluting at 17.1 min and 20.8 min respectively, were the N and C-terminal fragments of Pβ, respectively, following cleavage of the peptide bond between residues 6 and 7. Peak 3 was identified as the undigested peptide by both N-terminal amino acid sequencing and by time-of-flight mass spectrometry.

To confirm that the gelatinase A was fully active, the same preparation of activated gelatinase A was incubated with the G1-G2 fragment of the proteoglycan aggrecan. Previous studies [17]have shown that this fragment of aggrecan is an excellent substrate for gelatinase A. The G1-G2 digests were analysed by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate according to Fairbanks et al. [13]. Analysis of the digestion pattern of the G1-G2 fragment on silver-stained gels confirmed that gelatinase A cleaved the G1-G2 fragment (Fig. 3, lanes 2,3). Progelatinase A showed a low but significant ability to degrade aggrecan-G1-G2 (Fig. 3, lanes 4,5).

4. Discussion

In this study, the ability of the 72 kDa gelatinase A to cleave

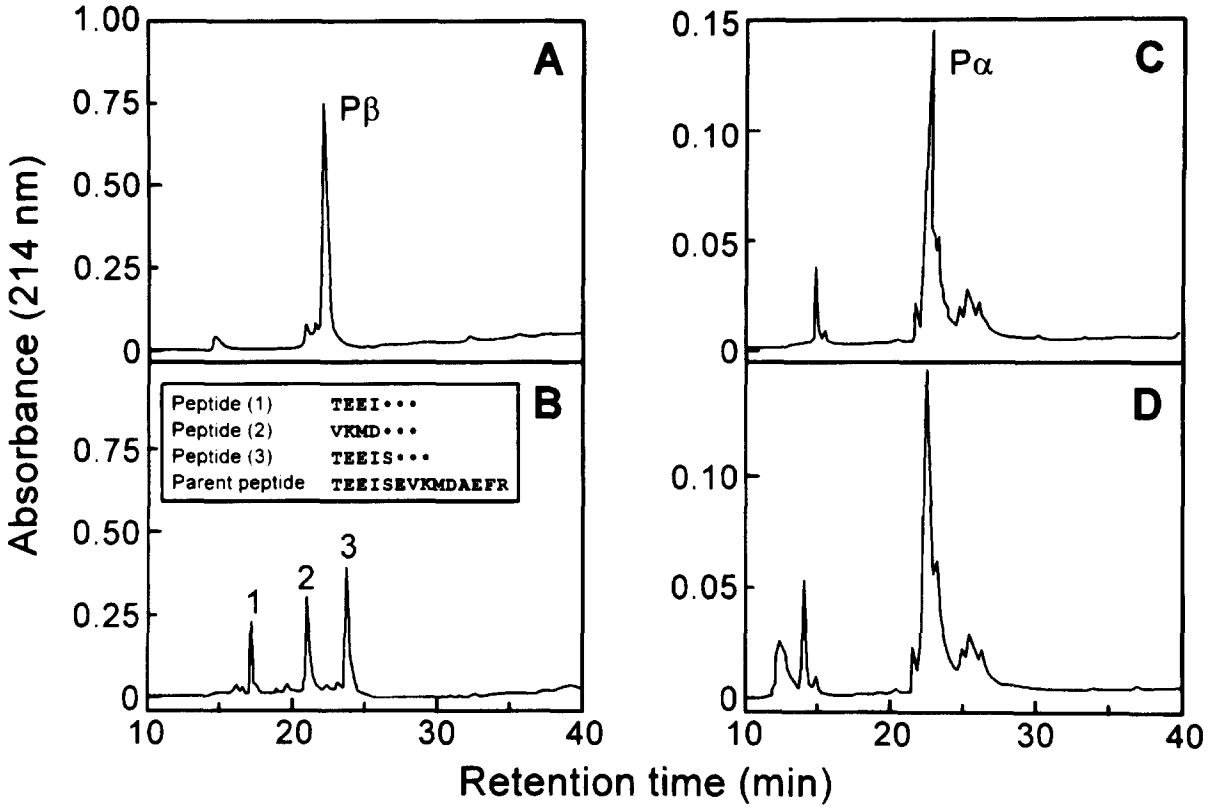

Fig. 2. Elution profile from reversed phase high performance liquid chromatography of peptides after digestions with 72 kDa gelatinase A. Peptide Pβ (A,B) and peptide Pα (C,D) (25 μg) were incubated for 18 h at 37 °C in the absence (A,C) or presence (B,D) of 1 μg of 72 kDa gelatinase in a total volume of 50 μl. The digests were analysed by reversed phase HPLC on a Waters Novopak C18 column (0.4 × 150 mm). The column was eluted with a linear gradient of 0–60% acetonitrile over 60 min in 0.1% (v/v) trifluoroacetic acid:water at a flow rate of 1 ml/min. Peptides were detected by measuring the absorbance at 214 nm. The peptides 1 and 2 were identified by amino acid sequencing and peptide 3 by amino acid sequencing and by time-of-flight mass spectrometry.

APP was investigated. APP was released from the surface of APP695-transfected HeLa cells, indicating that gelatinase A does have some ability to cleave APP specifically at a site near the membrane. Despite this result, gelatinase A did not exhibit any α-secretase activity as shown by the fact that it did not cleave a peptide homologous to the α-secretase cleavage site. However, gelatinase A showed much greater specificity for cleaving a synthetic peptide homologous to the region cleaved by β-secretase. Furthermore, the site of cleavage by gelatinase A (position −3, using the βA4 numbering system) is a known cleavage site by a β-secretase [3].

We were unable to reproduce the finding of Miyazaki et al. [6] that gelatinase A cleaves like an α-secretase. The reason for this result is unclear. However, one major difference between our studies and those of Miyazaki et al. is that we did not use trypsin and stromelysin to activate gelatinase A. In experiments where we attempted to reproduce the activation conditions employed by Miyazaki et al. [6], we were unable to convincingly eliminate the possibility of trypsin contamination in the final preparation. This difficulty is of some importance, as the α-secretase site is adjacent to a lysine residue which can also be cleaved by trypsin. Because we were unable to eliminate the trypsin activity, we employed APMA activation instead. Although it could be argued that gelatinase A shows a completely different pattern of protease cleavage specificity depending

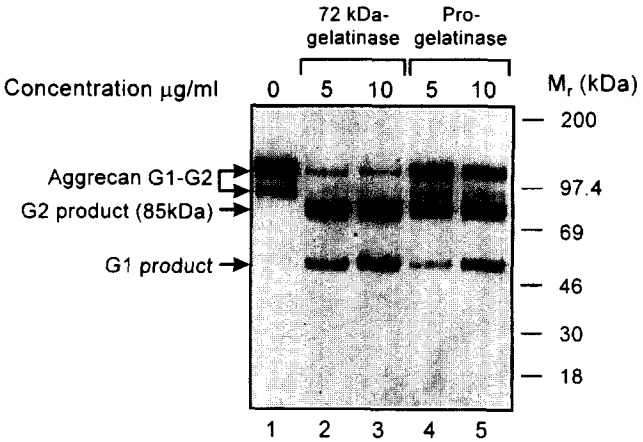

Fig. 3. Analysis of the digestion of purified aggrecan G1-G2 fragment (5 mg/ml) by progelatinase or 72 kDa gelatinase by SDS-polyacrylamide gel electrophoresis. Each lane was loaded with the equivalent of 0.5 μg of the original undigested G1-G2 fragment. All samples were pretreated with 0.025 units/30 μl of keratanase (Pseudomonas sp. from Seikagaku, Japan) for 6 h before electrophoresis. Polypeptides were visualised by staining with silver. Lane 1, G1-G2 fragment; lanes 2 and 4, G1-G2 fragment digested with 5 μg/ml enzyme; lanes 3 and 5, G1-G2 fragment digested with 10 μg/ml enzyme. In lanes 2 and 3 digests were with 72 kDa gelatinase, and in lanes 4 and 5, digests were performed with progelatinase. Progelatinase was activated with 2 mM APMA for 22 h at 37 °C.

upon the method of activation, there is no evidence for this from our studies.

Our results raise the possibility that gelatinase A could have β-secretase activity. However, more evidence is needed to establish this view. Although the main cleavage site for β-secretase is on the N-terminal side of Asp^{1}, other minor sites have been identified [3]. For example, in human kidney 293 cells, APP can also be cleaved between Glu^{-4} and Val^{-3} [18,19]. Studies using COS cells [20] and MDCK cells [21] have also identified a similar cleavage site.

As both APP and gelatinase A are processsed through a secretory pathway, it is possible that they may be co-localised in a subcellular compartment. Alternatively, some release of APP may occur from the cell surface following digestion with an extracellular protease such as gelatinase A. The concept that gelatinase A may be a β-secretase is further supported by the observation that it is produced and secreted from neuronal cells [22].

Our finding that gelatinase A has a β-secretase-like activity adds another enzyme to the growing list of candidate β-secretases (reviewed in [3]). Only further studies will establish whether any of these enzymes acts as a β-secretase in vivo. In view of the fact that several cleavage sites have been identified close to the N-terminal portion of the βA4 sequence [18–21], it is quite possible that more than one protease may act in this capacity.

Acknowledgements: This work was supported by two project grants to DHS from the National Health and Medical Research Council of Australia. GM is supported by the Arthritis & Rheumatism Council, UK. B. Kreunen is thanked for the preparation of figures and G. Reed and K. Last for technical assistance.

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