FEBS Letters 443 (1999) 8–10 FEBS 21403

Identification of a splice variant of neutrophil collagenase (MMP-8)

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Received 28 October 1998

Abstract We have identified a splice variant of human neutrophil collagenase (MMP-8) transcript (MMP-8alt) that has a 91 bp insertion between codons for amino acid residues 34 and 35 of MMP-8 cDNA. This splice variant encodes an open reading frame for a 444 residue protein, lacking a secretory signal sequence. Our data suggested that, as opposed to the original MMP-8, the translation product of MMP-8alt is not a secreted protein; nevertheless, it is enzymatically active. Further studies aimed at identifying the physiological substrates of MMP-8alt protein may lead to uncover novel roles it plays in cellular physiology.

Key words: Neutrophil collagenase; MMP-8; Alternative splicing; In vitro translation; Co-translational processing; Autoactivation

1. Introduction

Pre-mRNA splicing is a widely used biological mechanism in higher eukaryotes for generating mature mRNA. More recently, it has become apparent that pre-mRNAs of many genes are capable of undergoing alternative splicing and generate multiple species of mature mRNA. Some of the splice variants occur at the non-coding region of the mRNA and do not influence the amino acid sequence of the translation products but may somehow affect translation efficiency [1]. On the other hand, alternative splicings can also occur at the coding region of the mRNA, resulting in translation products with different tissue distribution or subcellular localization [2–4]. The ability to detect such splicing variants for a given mRNA has greatly increased due to the use of polymerase chain reaction (PCR) coupled with the reverse transcription (RT) of mRNA.

Neutrophil collagenase (MMP-8) is a member of the matrix metalloproteinase (MMP) family. It is capable of cleaving all three α-chains of types I, II, and III collagen. It is a secreted glycoprotein which is synthesized as a latent enzyme. The activation of this enzyme requires autolytic removal of 80 amino acids from the N-terminus [5,6]. MMP-8 was previously thought to be expressed exclusively by neutrophils, but recently its expression has also been detected in chondrocytes and was found to be capable of cleaving aggrecan in cartilage [7–9]. In the process of analyzing the expression of MMP-8 in different cell types, we identified a splice variant of human MMP-8. Here we report the initial characterization of this splice variant.

2. Materials and methods

2.1. Cell culture

Two monocytic cell lines, U937 and THP-1 cells, were maintained in RPMI 1640 medium with 10% fetal bovine serum (Life Technologies, Inc.). Human chondrocytes were prepared from cartilage taken from patients undergoing joint replacement as described [10] and cultured in Dulbecco's modified medium with 10% fetal bovine serum.

2.2. cDNA and genomic DNA cloning

Total RNA isolation from THP-1 cells, U937 cells and human chondrocytes was accomplished using TriZol reagent (Life Technologies, Inc.). The RNA was reverse transcribed using the 1st Strand cDNA Synthesis Kit (Life Technologies, Inc.) and oligo-dT primer. PCR amplification of MMP-8 cDNA fragment corresponding to nucleotides 68–850 of the published sequence (GenBank accession number J05556) was performed with Pfu polymerase (Stratagene) for 30 cycles (95°C, 1 min; 55°C, 2 min; 72°C, 3 min) using primer pair A/B (A, sense: AATGG TACCG ATCAT GTTCT CCCTG AAGA; B, antisense: ATGGC CTGAA TTCCA TCGAT GTCA). A genomic DNA fragment containing the nucleotide sequence between 101 and 250 of the MMP-8 cDNA was generated by PCR with Taq polymerase (PE Applied Biosystems) for 30 cycles (95°C, 1 min; 54°C, 2 min; 72°C, 3 min with 3 s extension after each cycle) using primer pair C/D (C, sense: GCTCT TACTC CATGT GCA; D, antisense: ACGAT CACAT TAGTG CCA). The relative position of the 91 bp exon generated by alternative splicing within the intron was determined by PCR using primer pair D/E (E, sense: CTACA GTAGT GAAGA GGA). cDNAs containing the entire protein coding region of MMP-8 and MMP-8 alternatively spliced form, MMP-8alt, were generated by RT-PCR and cloned into the *Asp*718/*Bam*HI sites of pcDNA3+ vector (Invitrogen). DNA was sequenced using the dideoxy-mediated chain termination method of Sanger et al. with a Sequenase 2.0 kit (United States Biochemical).

2.3. In vitro translation of MMP-8 and MMP-8 activation

In vitro translation of MMP-8 cDNA subcloned into pCDNA3+ vector was accomplished using TNT T7 Coupled Reticulocyte Lysate System (Promega) with [^{35}S]methionine (Amersham). In certain cases, canine pancreatic microsomal membranes (Promega) were also included in the in vitro translation reaction to detect co-translational processing and glycosylation. For studying MMP-8 autoactivation, samples of in vitro translated proteins (50 μl) were diluted to 1 ml with 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 10 mM $CaCl_2$, and 50 μM $ZnCl_2$, and concentrated with a Centricon 10 (Amicon) to a final volume of 50 μl. Samples were then diluted 10-fold with the same buffer containing 0.05% Brij-35 and were activated by treatment with 2 mM *p*-aminophenylmercuric acetate for 90 min at 37°C. In vitro translated, ^{35}S-labeled proteins were subjected to SDS-PAGE and autoradiography.

3. Results

The nucleotide sequence of MMP-8 cDNA (GenBank accession number J05556) encodes a protein of 467 amino acids, with a secretory signal sequence of 20 residues followed by the prodomain of 80 residues. In the course of obtaining the N-terminal fragment of MMP-8 cDNA from different cell types,

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PII: S0014-5793(98)01654-8

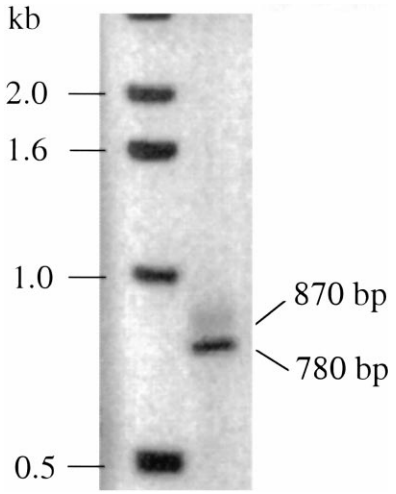

Fig. 1. Analysis of the RT-PCR products generated from U937 cells using primer pair A/B.

we identified two distinct DNA fragments from human U937 cells by RT-PCR using primers A and B. The sizes of these two fragments are ~780 bp and ~870 bp, respectively (Fig. 1). These two PCR products were recovered and sequenced. The ~780 bp fragment matches exactly the corresponding MMP-8 cDNA sequence in GenBank, whereas the ~870 bp fragment contains an additional 91 bp between residues 173 and 174 of the published sequence, as shown in Fig. 2A. This additional segment of the transcript may be the result of an alternative splicing of the MMP-8 pre-mRNA.

To identify the potential intronic sequence of the genomic DNA which corresponds to the cDNA insert of MMP-8, we utilized primers C and D to amplify the genomic DNA. These two primers are located at the 5′ side and the 3′ side of the 91 bp insert, respectively. A 2.5 kb DNA fragment was generated from the reaction. This 2.5 kb fragment contains an intron about 2.3 kb in length which separates residues 173 and 174 of the published cDNA sequence. The 91 bp sequence we identified was within this intronic sequence. In addition, the 91 bp insert was immediately preceded by a consensus splice acceptor sequence, AG, at the 5′ junction and immediately followed by a consensus splice donor sequence, GT, at the 3′ junction (Fig. 2B). Therefore, this result clearly showed that the 91 bp insertion was the result of alternative splicing of the pre-mRNA to include an additional exon [11]. The two new introns 5′ and 3′ of this 91 bp alternative exon are 1.9 kb and 360 bp, respectively.

This 91 bp insert interrupts amino acid residues 34 and 35 in the prodomain of the MMP-8 protein. All three possible reading frames of this insert contain stop codons. One of them has three stop codons followed by an ATG that is in frame with the rest of MMP-8 coding sequence. The next potential initiation codon is found at nucleotide 323, Met-85 of MMP-8. To analyze the potential translation products of this alternatively spliced MMP-8 (MMP-8alt), cDNA encoding MMP-8 and MMP-8alt were subjected to in vitro transcription and translation. In vitro translated MMP-8 generated two major products of approximately 53 kDa and 43 kDa (Fig. 3A). In vitro translated MMP-8alt yielded two major products of approximately 49 kDa and 43 kDa (Fig. 3A). The 43 kDa product in both cases is most likely a translation product starting

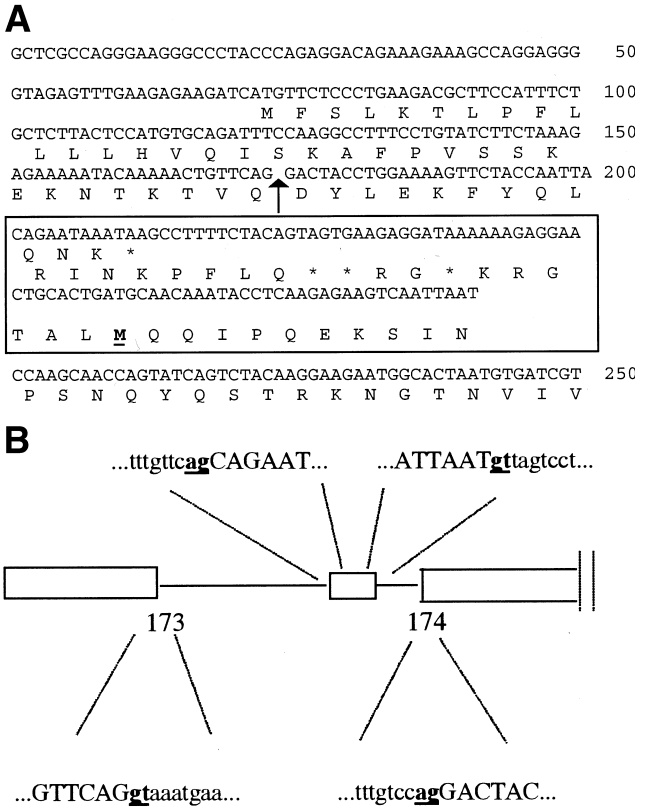

Fig. 2. Nucleotide sequence and genomic structure of MMP-8 around the 91 bp insert. A: Nucleotide sequence of the 5′ 250 bp of MMP-8 cDNA and the sequence of the 91 bp insert. The nucleotide sequence of the 91 bp inset is shown in the box. Numeration follows the published sequence. The putative new methionine initiation codon for MMP-8alt is underlined and in boldface. B: Genomic organization of MMP-8 gene encodes two alternatively spliced transcripts. Drawing shows splicing patterns that generate MMP-8 and MMP-8alt transcripts. Open boxes represent exons, and lines represent introns. Numbers indicate the postions in the MMP-8 cDNA. Consensus splice donor/acceptor sites are underlined and in boldface.

at Met-85 of MMP-8 and the corresponding methionine in MMP-8alt. The 49 kDa protein would be the result of translation from the new methionine within the 91 bp insert.

Since the MMP-8 signal peptide is not part of the longest open reading frame of the MMP-8alt transcript, MMP-8alt should not be processed for secretion in the same way as MMP-8. To verify this, the translation products were further analyzed for co-translational processing and core glycosylation by the addition of canine pancreatic microsomal membranes to the translation reaction. In vitro translation of MMP-8 but not MMP-8alt in the presence of canine pancreatic microsomal membranes generated higher molecular weight products (Fig. 3A). This result is in agreement with the fact that MMP-8 but not MMP-8alt possesses a signal sequence to translocate the protein into the microsomal membranes for glycosylation.

Activation of MMPs requires the removal of the prodomain from the protein by autolytic cleavage via a cysteine switch activation mechanism [12]. This activation can also be achieved in a test tube by treatment of MMPs with *p*-aminophenylmercuric acetate, which stimulates autolysis. The critical cysteine residue of MMP-8 is cysteine 91. This cysteine residue is present in all three translation products mentioned

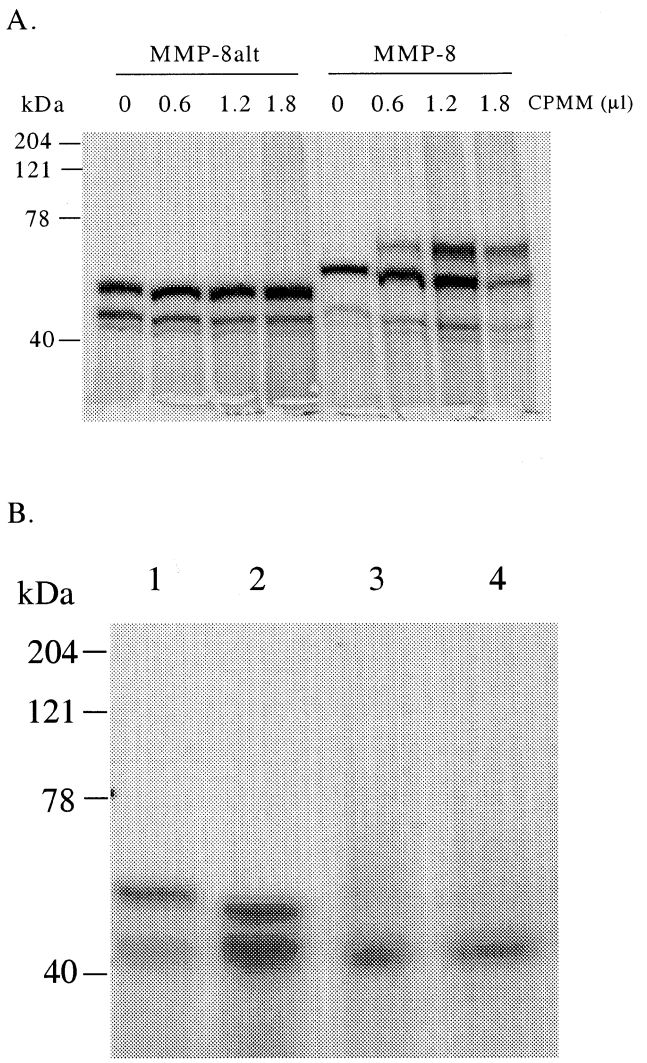

Fig. 3. Analysis of in vitro translated MMP-8 and MMP-8alt. A: In vitro translation of MMP-8 and MMP-8alt was carried out in the absence or presence of indicated amounts of canine pancreatic microsomal membranes (CPMM). B: In vitro translated MMP-8 and MMP-8alt were activated by treatment with *p*-aminophenylmercuric acetate (APMA). Lane 1, MMP-8; lane 2, MMP-8alt; lane 3, MMP-8+2 mM APMA; lane 4, MMP-8alt+2 mM APMA.

above. We found that upon treatment with *p*-aminophenylmercuric acetate both in vitro translated MMP-8 and MMP-8alt are converted to a 41 kDa form (Fig. 3B). This size change is consistent with the size conversion of proenzyme to the active enzyme. The 43 kDa internal translation product, presumably amino acids 85–467 of MMP-8, is also converted to the 41 kDa form (Fig. 3B).

4. Discussion

In the present study, we have identified an alternatively spliced transcript of MMP-8, MMP-8alt. This version of the MMP-8 transcript is the result of alternative splicing of a 2.3 kb intron that interrupts the codons for amino acids 34 and 35 of MMP-8 and encodes a potential protein of 444 residues that lacks a secretory signal peptide. As a result, and unlike MMP-8, MMP-8alt was not co-translationally processed in the presence of canine microsomal membrane, even though all the potential *N*-glycosylation sites are maintained in MMP-8alt. It would thus be expected to be an intracellular protein. Nevertheless, the protein generated from MMP-8alt appears to be enzymatically active since autoactivation experiments showed that protein generated from the MMP-8alt transcript underwent autolysis to generate a protein with a size consistent with the active form of MMP-8.

Recent studies have shown that MMP-8 is not a unique gene product of neutrophils since it is also expressed by chondrocytes in human articular cartilage [8,9]. It is capable of cleaving not only collagen but also aggrecan. In our study, the alternatively spliced transcript of MMP-8 that we identified was also not unique to U937 cells. In addition, we detected it in human chondrocytes and THP-1 cells by RT-PCR. Since the protein product of this MMP-8alt transcript is most likely located inside cells, it may use still other proteins as substrates. Thus, the physiological role of MMP-8alt protein remains to be determined.

Whether this alternatively spliced form of MMP is unique for MMP-8 or occurs elsewhere in the MMP family is not known at this time. Several members of MMP family whose genomic structure have been analyzed all contain an intron at the similar position as MMP-8 [13–15]. These include both collagenase-1 (MMP-1) and collagenase-3 (MMP-13). In the case of membrane-type MMPs, an extended family of MMP, an alternatively spliced MT-MMP-3 was identified recently [16]. This alternative splicing occurs near the transmembrane region of MT-MMP-3, which results in soluble instead of membrane-anchored MT-MMP-3. With the use of RT-PCR, it is possible that more alternative spliced form of MMPs will be identified. This new information may uncover novel roles for MMPs in cellular physiology.

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