Cloning of a novel membrane-linked metalloproteinase from human myeloma cells

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We have isolated a novel cDNA from human myeloma cells encoding a member of the reprolysin family of metalloproteinases. Derived amino acid sequence predicts a protein of approx. 76 kDa. The open reading frame predicts the presence of a leader peptide, a pro-peptide with a 'cysteine switch', a

metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, an epidermal growth factor-like domain and a putative transmembrane sequence. Expression of the mRNA for this metalloproteinase has been demonstrated in human myeloma cells.

INTRODUCTION

Multiple myeloma is a B-cell neoplasm characterized by uncontrolled clonal proliferation of plasma cells within the bone-marrow microenvironment. A recent study suggested that abnormalities in the bone-marrow extracellular matrix (ECM) may be involved in the pathophysiology of B-cell malignancies [1]. Studies in multiple myeloma have revealed abnormalities in the bone-marrow environment, including increased proliferation of stromal cell elements, abnormal expression of adhesion molecules and reductions in and poor organization of components of the ECM [2]. Under normal circumstances the ECM is constantly being remodelled, a process largely performed by members of the matrix metalloproteinase family of enzymes, which include gelatinase A and B, collagenase and stromelysin. These enzymes are regulated by the presence of a family of naturally occurring inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Disruptions in the natural balance of matrix metalloproteinase and TIMPs have been implicated in a variety of pathological situations including tumour metastases and the arthritides [3].

We have undertaken studies designed to characterize the metalloproteinase profile of human myeloma cells with a view to elucidating the possible involvement of these enzymes in the disruption of ECM observed in this disease. Using synthetic oligonucleotide primers designed to amplify members of the matrix metalloproteinase family of enzymes and their inhibitors we have identified a novel metalloproteinase related to the reprolysin family of snake venom metalloproteinases.

EXPERIMENTAL

Cell culture

The human myeloma cell lines, U266-B1, HS-Sultan, ARH-77 and RPMI-8226 were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.). All cell lines were maintained in RPMI 1640 (Life technologies) supplemented with 10 % (v/v) foetal calf serum (Life technologies).

RNA isolation

Total RNA was isolated from cells using a single-step purification method based upon the method of Chomczynski and Sacchi [4].

Briefly, cells (5×10^6) were pelleted by centrifugation, the supernatant removed and the cells lysed with TRIzol (Life technologies). Following addition of chloroform, RNA was separated into aqueous and organic phases by centrifugation at 12000 g. RNA was recovered by precipitation with isopropanol, washed with 75 % ethanol and resuspended in diethyl pyrocarbonate-treated water.

Reverse-transcription PCR (RT-PCR)

Contaminating DNA was removed from RNA by incubating samples with DNase I for 1 h at 37 °C. RNA was reverse transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase primed with random hexamers (Pharmacia). The PCR primers used to amplify TIMP-3 were 5'-ATCA-CCTGGGTCATATGTGCAAGATCAAG-3' and 5'-GATTC-TCAGGGGTCTGTGGCATTGATG-3'. Products of the PCR reaction were electrophoresed on a 1.5 % agarose gel in Tris-Borate-EDTA (TBE). Amplified products were excised and extracted from the gel using the Wizard DNA purification system (Promega). Purified DNA was cloned into a pCRTMII vector (Invitrogen) according to the manufacturers' protocol.

DNA sequencing

DNA sequencing was performed using a Sequenase Kit (USB Biochemicals) using synthetic oligonucleotide primers. Sequenced products were electrophoresed on an 8 % denaturing acrylamide gel.

Northern blot analysis

A sample (15 µg) of RNA was electrophoresed on a 1 % agarose-formaldehyde gel, transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary blotting and fixed by UV cross-linking (Stratalinker, Stratagene). Membranes were prehybridized for 2 h in Easy-Hyb (Boehringer-Mannheim). cDNAs identified from the Expressed Sequence Tag (EST) database were excised, gel purified and [α -³²P]-labelled

Abbreviations used: RT-PCR, reverse-transcription PCR; EST, Expressed Sequence Tag; ECM, extracellular matrix; MCM, myeloma cell metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

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using a random priming method (prime 1, Sigma). Hybridization was performed overnight at 50 °C. Membranes were washed at a final stringency of 0.1 × SSC/0.1 % SDS at 68 °C (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate). Membranes were exposed to autoradiography film at −70 °C. Ethidium bromide staining of the 28 S rRNA served to standardize RNA loading.

Library screening

A human placental cDNA library (Stratagene) was plated according to manufacturers' instructions and screened with cDNAs identified from the EST database. 10⁶ individual clones were screened by filter hybridization on Hybond N (Amersham). Overlapping positive clones were isolated, amplified and used as templates for DNA sequencing analysis.

RESULTS

In experiments designed to amplify the C-terminus of human TIMP-3 two products were amplified by RT-PCR in all cell lines examined, one of approx. 200 bp and the other of 220 bp. Cloning and sequencing identified one product as TIMP-3 (N. McKie, and P. I. Croucher, unpublished work). Translation, in all three reading frames, of the 200 bp fragment revealed the presence of a putative cysteine-rich open reading frame. The inferred protein sequence showed no similarity to any amino acid sequence deposited in the Swiss-Prot database. Screening of the EST database revealed two cDNA clones with 100 % identity to the 200 bp fragment, indicating that this fragment was likely to be expressed in human tissue. The two clones, H11999 and R31348, cloned from an infant brain library and a human placental library, respectively, were obtained from Research Genetics Incorporated (California, U.S.A.). H11999 was used to probe a Northern blot of RNA prepared from human myeloma cell lines and shown to hybridize to RNA of approximately 3.8 kbp (Figure 1).

Figure 1 Northern blot analysis of myeloma cell lines (A) and an ethidium bromide-stained gel (B) shown to illustrate equal loading of RNA samples (A) Positive hybridization of H11999, at approx. 3.8 kb, was shown in all myeloma cell lines examined. Positions of the 28 S and 18 S ribosomal RNA bands are indicated.

Figure 2 Amino acid sequence (A) and domain structure (B) inferred from the DNA sequence of MCMP (A) Highlighted areas identify the putative pro-peptide region¹, cysteine switch², metalloproteinase domain³, epidermal growth factor (EGF)-like domain⁴ and transmembrane domain⁵. The position of the disintegrin-like domain is underlined. (B) The shaded region represents the position of the EGF-like domain and the solid bar shows the position of the putative transmembrane region.

Overlapping cDNAs were isolated from a human placental library by screening with H11999 and the DNA sequenced. A total of 3.5 kbp of cDNA was isolated by this method. The predicted protein sequence for the full-length cDNA is shown in Figure 2(A). The sequence encompasses an open reading frame of 2.2 kbp with an inferred 3' untranslated region of approx. 1.6 kbp. This agrees with the Northern blot analysis (Figure 1), indicating that we are likely to have cloned the full-length cDNA for this protein. Analysis of the amino acid sequence revealed the presence of a number of characteristic domains. These include a signal peptide, a pro-peptide and a zinc-dependent metalloproteinase domain. Furthermore, we were also able to identify a disintegrin-like domain, a cysteine-rich domain, an epidermal growth factor-like region and a putative transmembrane sequence (Figure 2B). These characteristic regions indicate that this protein is a novel metalloproteinase with similarity to the reprotolysin family of snake venom metalloproteinases. We have named this protein myeloma cell metalloproteinase (MCMP).

DISCUSSION

The reprotolysin family of metalloproteinases are a well-characterized group of enzymes present in snake venom. The family has been divided into four classes based upon the characteristic domains present [5]. Class I contains a proteinase domain only, class II a proteinase and disintegrin-like domain, whereas class III contains proteinase, disintegrin-like and cysteine-rich domains. The class IV reprotolysins are similar to the class III molecules with the addition of a lectin-like domain at the C-terminus [6]. Members of this family of enzymes have been shown to degrade basement membranes, having a potent hydrolytic activity against type IV collagen, laminin and fibronectin [7]. These proteinases, particularly the class III reprotolysins, are thought to be the active agents that mediate the potent haemorrhagic activity associated with snake bites.

In the present study we have identified a novel human mRNA whose potential translation products contain a number of characteristics in common with the class III repolysins. MCMP contains the consensus HEXXH motif that defines it as a member of the zinc-dependent metallo-endopeptidase family. Furthermore, MCMP has a further characteristic sequence that distinguishes the pro-region from the metalloproteinase domain that is also found in members of the matrix metalloproteinase family. The motif PRCGV, present in matrix metalloproteinases, and PKMCGV conserved in members of the repolysin family, show considerable similarity with a sequence PLKCGV which is found in MCMP. In the matrix metalloproteinases this region has been implicated in co-ordinating the active-site zinc ion via the cysteine residue, thereby interfering with binding of a water molecule and rendering the proteinase inactive [8]. Cleavage of the pro domain results in a loss of this region and activation of the enzyme. This so called 'cysteine switch' has also been shown to exist in both the matrix metalloproteinases and the repolysins and we would speculate that this motif functions in a similar manner in MCMP. It is therefore likely that this molecule may be activated by cleavage of the pro domain, possibly by members of the matrix metalloproteinase family, to yield an active metalloproteinase on the cell surface. MCMP also has a disintegrin-like domain characterized by the presence of conserved cysteines and the presence of conserved ECD residues, which are present in positions that align adjacent to the RGD motif that is found in the classical disintegrins. There are currently few functional data to suggest that this region can have a disintegrin-like activity; however, Myles et al. [9] have demonstrated that the disintegrin-like domain of PH-30 β , a protein involved in sperm-egg fusion, may possess the ability to bind molecules on the cell membrane. Peptides containing a TDE sequence, which aligns with RGD in the disintegrins, were shown to bind to the egg plasma membrane and inhibit sperm-egg fusion. The corresponding peptide sequence in MCMP is TSE (Figure 3B) and it is therefore possible that this protein may also possess functional binding capabilities. MCMP also contains a cysteine-rich domain of unknown function, an epidermal growth factor-like sequence and a hydrophobic sequence characteristic of a transmembrane domain (Figure 2A).

MCMP has significant amino acid similarity to a number of mammalian proteins with a variety of functions. These include MS2, a cell-surface antigen of approx. 89 kDa, expressed in the murine macrophage lineage [10]. This shows significant identity with MCMP; however, the authors omitted to identify the characteristic zinc-binding motif characteristic of the metalloproteinases (Figure 3A). We have also shown that MCMP has significant identity with both subunits of PH-30, which is an heterodimeric protein implicated in sperm-egg fusion [11,12]. The α subunit contains the consensus zinc-binding motif which aligns with MCMP, whereas the β subunit, although not containing this region, contains a disintegrin-like sequence which also shares considerable identity with MCMP. On the basis of this motif and the demonstration of a putative 'cysteine switch' characteristic of the matrix metalloproteinase family, it was suggested that the α subunit may be catalytically active [13]. The disintegrin-like region of PH-30 β is also thought to play an important role in sperm-egg fusion and MCMP contains a very similar sequence (Figure 3B), suggesting that this MCMP may also play a role in cell-cell and/or cell-matrix interactions. Another similar protein has been identified in the epididymis of a number of mammalian species. This protein, epididymal apical protein 1 (EAP1), has also been proposed to contain a metalloproteinase domain; however, on the basis of the presented sequence it is unlikely to be catalytically active since it lacks the

A METALLOPROTEINASE DOMAIN

MCMP	IVAHELGHNLGMNHDDGRDCSCGAK.....SCIM
ATR-E	TMTHLGHNLGIHHD.TDSCS.GGY.....SCIM
HR1B	IMTHEMGNLGI PHD.GNSCTCGGF.....PCIM
PH-30α	LLVHELGHNLGIRHDHSA.CVCRDK.....HSCLM
PH-30β	ILVQLLSVSMGIAYDNADLCRCRGA.....ICLM
EAP1	RMAHQQLGHNLGMQHD.EFPCTCPS.....GKCVM
MDC	TLAQTLGQNLGMWMMKRRSSAGDCKCPDIWLGCM
MS2	TMAHELGHNLGMSHDEDPGICYPE.PREGGGCIM

B DISINTEGRIN-LIKE DOMAIN

MCMP	NKLVDAGEEC	DCGTPKECEL	DPCCGEGSTCK	LKSFAECAYG
ATR-E	NELLEAGIEC	DCGLEN...	PCCYATTCK	MRPGSQCAEG
HR1B	NELLEAGEEC	DCGSPENCQ.	YQCCDAASC	LHSWVKCESG
PH-30α	NGVVEESEQC	DCGV..NCDT	SECCDQ.ACN	LKGNATCSNE
PH-30β	NNRVEQGEDC	DCGSQEEEC	TDCCDAATC	LKSTSRCAQG
EAP1	NKKLDEGEEC	DCGPPQECT.	NPCDAHTCV	LKPGFTCAEG
MDC	NGFVEAGEEC	DCGSVQECNR	AGGNCKCKCT	LTHDAMCSDG
MS2	NLFVEHGEQC	DCGTPQDCQN	P.CCNATTCT	LKGAECASG
Bit	NKILEQGEDC	DCGSPANCQ.	DQCCNATTCK	LTPGSQCNHG

MCMP	DCCDKCRFLP	GGTLCRGKTS	ECDEVPEYCNG	SSQFCQPDVF
ATR-E	LCCDQCRFMK	KGTVCRVSMV	DRN.DDTCTG	QSADCPRNGL
HR1B	ECCDQCRFRT	AGTECRAAES	ECDIPESTCTG	QSADCPTRDF
PH-30α	LCCSDCQYKN	SGYLCRPSVG	PCDLPEYCTG	QSGKCPDLY
PH-30β	PCCNQCEFKT	KGEVCRESTD	ECDLPEYCNG	SSGACQEDLY
EAP1	ECCESCQIKK	AGSICRAED	ECDFPEMCTG	HSPACPKDQF
MDC	LCCRCKYEP	RGVSCREAVN	ECDIAETCTG	DSSQCPPNLH
MS2	TCCHECKVKP	AGEVCRLSKD	KCDLEEFCDG	RKPTCPEDAF
Bit	.CCDQCKFKK	ARTVCRIARG	DWN.DDYCTG	KSSDCPNWH

Figure 3 Alignment of protein sequences from MCMP, atrolysin-E (ATR-E) (repolysin), HR1B (repolysin), PH-30 α , PH-30 β , EAP1, MDC and MS2

(A) Alignment of metalloproteinase domain. The zinc-binding motif is highlighted and underlined. The glutamic residue within this motif is proposed to be the essential catalytic residue in other families of metalloendopeptidases. (B) Alignment of the disintegrin-like region; conserved cysteine residues and ECD motif are highlighted. Bitistatin is included in this line-up to identify the disintegrin domain. The position of the RGD sequence in bitistatin is underlined.

glutamic residue within the zinc-binding motif [14]. EAP1 also contains a disintegrin-like domain with similarity to MCMP. MDC, a recently described tumour suppressor gene product, shown to be somatically rearranged in primary breast cancer, also shows significant similarities to MCMP [15]. This protein was also suggested to contain a metalloproteinase domain; however, the presented sequence does not appear to contain a characteristic zinc-binding motif, although it does contain a putative 'cysteine switch', and may represent a member of a novel class of zinc-binding metalloproteinases.

The presence of a characteristic metalloproteinase domain and a disintegrin-like domain defines a new family of proteinases of which MCMP is a new member. This family has recently been named the ADAMs (a disintegrin and metalloproteinase) and they are thought to be involved in cell-cell and cell-matrix interactions [16,17]. Wolfsberg et al. have recently reviewed this family and have identified at least 11 members [16]. We would suggest adopting their nomenclature making MCMP the twelfth member of this family (ADAM12). A role for MCMP in multiple myeloma remains unclear; however, it is tempting to speculate a number of potential functions. The presence of a metalloproteinase domain is consistent with a role in the degradation of the ECM observed in multiple myeloma. Furthermore, the

possession of a disintegrin-like domain may implicate this molecule in the loss of adhesion associated with progression from multiple myeloma to plasma-cell leukaemia.

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