Communication

Molecular Cloning of Bomapin (Protease Inhibitor 10), a Novel Human Serpin That Is Expressed Specifically in the Bone Marrow*

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Serine proteinase inhibitors or serpins are a superfamily of homologous proteins that are for the most part involved in the regulation of proteolytic processes in a variety of biological systems. Utilizing a polymerase chain reaction-based strategy we have cloned a novel member of the ovalbumin family of serpins from a human bone marrow cDNA library. The new gene encodes a 397-amino acid protein, designated bomapin, with a calculated molecular mass of 45 kDa and 48% amino acid identity with plasminogen activator inhibitor-2, human leukocyte elastase inhibitor, and cytoplasmic antiprotease. A single 2.3-kilobase bomapin transcript is highly expressed in human bone marrow cells but was undetectable in all other analyzed human tissues. In vitro transcription and translation of the bomapin cDNA revealed the synthesis of an appropriately sized protein that was able to form SDS-stable complexes with thrombin and trypsin. The restricted expression of bomapin to the bone marrow raises the possibility that this serpin may play a role in the regulation of protease activities during hematopoiesis.

Serine proteinase inhibitors or serpins are a ubiquitous superfamily of homologous proteins that resemble a\(a\)-proteinase inhibitor (\(a\)-PI) in overall structure (1). In general, serpins contain a highly exposed reactive site loop near the C terminus of the molecule that interacts as a pseudosubstrate for the target protease. The inhibitory specificity of the serpin is mainly defined by the P\(_1\)-P\(_2\) amino acid residues in the reactive site loop. Interaction of these amino acids with the active site of the target protease triggers a dramatic conformational change in the serpin that results in a stoichiometric 1:1 inhibitor complex with the protease, which is typically stable to treatment with denaturants such as SDS (2). In addition to the serpins that regulate protease activity, several members of this superfamily lack a protease inhibitory capability, e.g. angiotensinogen and ovalbumin (1, 3). Ovalbumin represents the parent prototype of a unique family of proteins within the serpin superfamily, which have several structural features in common, including the absence of a typical cleavable signal sequence (4). Previously identified members of this ovalbumin family of serpin proteins (ov-serpins) include plasminogen activator inhibitor-2 (PAI-2) (5), human leukocyte elastase inhibitor (6), and squamous cell carcinoma antigen (7). More recently, a tumor suppressor called maspin (8), which is presumably not a protease inhibitor (9, 10), and cytoplasmic antiprotease or CAP (11), also known as placental thrombin inhibitor (12) or as protease inhibitor 6 (13), have been identified as ov-serpins. Although cytoplasmic proteases play a key role in a variety of cellular functions (14, 15), little is known about the physiological targets of the ov-serpins that are expressed predominantly or exclusively as intracellular proteins (i.e. PAI-2, human leukocyte elastase inhibitor, and CAP). Kumar and Baglioni (16) have shown that overexpression of PAI-2 has a moderate protective effect on tumor necrosis factor-induced cytosis in a fibrosarcoma cell line, and these authors have speculated that this serpin may neutralize a protease that is involved in mediating the cytolytic activity of tumor necrosis factor (16). Presently, the role of proteases (e.g. interleukin-1\(\beta\)-converting enzyme (ICE)-like proteases) in programmed cell death or apoptosis is an emerging concept in biology (15). Current information indicates that proteases of the ICE family cleave substrate proteins specifically after aspartic residues (15, 17, 18), which are absent in the reactive site loops of the known human intracellular serpins including PAI-2. Based upon the observation that the cowpox virus encodes a serpin with aspartate in the P\(_1\) position that is capable of inhibiting ICE-related proteases, i.e. the crmA gene product (19, 20), and that apoptosis in tissue culture cell lines can be inhibited by the crmA gene product (21, 22), we hypothesized that an endogenous cytoplasmic serpin with a similar function may be expressed during specific stages of cellular differentiation. A polymerase chain reaction (PCR)-based homology cloning study was initiated in an attempt to identify novel ov-serpins, and we decided to utilize bone marrow as a source of mRNA from hematopoietic cells in all stages of cellular differentiation. Here we report the cloning of a novel human ov-serpin, designated bomapin for bone marrow-associated serpin, that is expressed highly specifically in the bone marrow. As suggested by the nomenclature committee of the Genome Database collaboration, the systematic title proteinase inhibitor 10 was assigned for bomapin to facilitate data base searches.

EXPERIMENTAL PROCEDURES

Cloning of Bomapin—A human bone marrow cDNA library in the vector pGAD10 was purchased from Clontech (Palo Alto, CA). This library had been generated from pooled sternal bone marrow from 24 Caucasians (age 16-70). The mRNA had been reverse transcribed by oligo(dT) and random priming. The library was amplified according to the manufacturer's instructions, and 2 \(\mu\)g of DNA were used as template for PCR reactions. DNA samples were adjusted to PCR buffer conditions in a total volume of 50 \(\mu\)l with 30 pmol of each PCR primer and 1.25 units of Taq polymerase (Perkin-Elmer). PCR was performed for 30 cycles in an automated thermocycler (Perkin-Elmer) with cycle times of 1 min at 94 \(^\circ\)C, 1 min at 55 \(^\circ\)C, and 2 min at 72 \(^\circ\)C. The primers used in the initial PCR amplifications included the following: 1) corresponding to amino acids 216-221 (sense) in \(a\)-PI: AAG CCT GTG CAA

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ATG ATG, AAA CCT GTA CAG ATG ATG, and AAA CCT GTG CAG ATG ATG; 2) corresponding to amino acids 324–318 (antisense) in \( \alpha_1 \)-PI: GT TCT GGA CAT TCC AGA GA and CT CCC TGA CAT GCC TGA GA; 3) corresponding to amino acids 372–367 (antisense) in \( \alpha_1 \)-PI: GAA AAG GAA GGG ATG GTC and GAA AAG AAA GGG ATG ATC. The primer pair that was used in the PCR that led to the amplification of bomapin sequence is underlined (corresponding to nucleotides 643–660 in Fig. 1, respectively). PCR products were sequenced into the vector pCRII utilizing a T/A cloning kit (Invitrogen, San Diego, CA) and analyzed by restriction digestion and DNA sequencing using M13 forward and reverse primers. The pGAD10-based primers for anchored PCR to obtain the 5’- and 3’-sequence of bomapin were ATT CGA TGA TGA CCC ACC (sense) and TGG CGG GGT TTT TCA TGA TCT (antisense, base position 180–199) and TA 12-nucleotide 5’tail of the CAP cDNA were based on the published sequence (11), had the complete coding region of bomapin. Primers for control amplification by PCR was carried out as described above using the primers to amplify bone marrow, placenta, and brain cDNA libraries in pGAD10 (Clontech). (XAR-5, Kodak). Templates for PCR amplifications were human bone marrow cDNA. Blots were exposed for up to 10 days to autoradiography film (Kodak). The positions of the primers used in the initial PCR that led to the isolation of bomapin cDNA are indicated by arrows above the nucleotide sequence.

RESULTS AND DISCUSSION

Cloning of Bomapin—Based upon three relatively conserved regions in the nucleotide sequences of the known human ov-serpins (5–8, 11), one set of three sense and two sets of two antisense primers were designed (referto Figs. 1 and 2 for the primer positions). Primers were designed to differ in up to 6 positions from the corresponding individual nucleotide sequences of the known ov-serpins to allow amplification of related sequences. Utilizing a human bone marrow cDNA library in the plasmid vector pGAD10 as the template, PCR amplifications with the 12 different possible combinations of forward and reverse primers were carried out. Products of the expected sizes for ov-serpins were detected in 11 reactions, and 2 reactions (positions of the primers used in the initial PCR that led to the isolation of bomapin cDNA are indicated by arrows above the nucleotide sequence.)

In vitro Transcription/Translation of the Bomapin cDNA—The complete bomapin coding region was subcloned into the vector pBluescript SK(+) (Stratagene, La Jolla, CA) using BamHI and XhoI restriction sites. The cDNA insert in this vector was constructed in this way by in vitro transcription and translation in the presence of [\( ^{35} \)S]methionine (Amersham Corp.) for 15 h at 65°C, and the blot was washed to a final stringency of 1 SSC, 0.5% SDS at 65°C. The experiment was performed twice with similar results. Human adult multiple tissue Northern blots I and II containing 2\( ^{32} \)P mRNA probes (Clontech), labeled as described above, was used as a positive control to confirm approximately equal mRNA loading in all lanes. Blots were exposed for up to 10 days to autoradiography film (XAR-5, Kodak). Templates for PCR amplifications were human bone marrow, placenta, and brain DNA libraries in pGAD10 (Clontech). PCR was carried out as described above using the primers to amplify the complete coding region of bomapin. Primers for control amplifications of the CAP cDNA were based on the published sequence (11), had 12-nucleotide 5’-extensions, and included GTA CCG GAA TTC TCT GGA CAT TCC AGA GA and CT CCC TGA CAT GCC TGA GA (5’ and 3’ primer sequences of the known ov-serpins to allow amplification of related sequences). Based upon three relatively conserved regions in the nucleotide sequences of the known human ov-serpins (5–8, 11), one set of three sense and two sets of two antisense primers were designed (referto Figs. 1 and 2 for the primer positions). Primers were designed to differ in up to 6 positions from the corresponding individual nucleotide sequences of the known ov-serpins to allow amplification of related sequences. Utilizing a human bone marrow cDNA library in the plasmid vector pGAD10 as the template, PCR amplifications with the 12 different possible combinations of forward and reverse primers were carried out. Products of the expected sizes for ov-serpins were detected in 11 reactions, and 2 reactions (positions of the primers used in the initial PCR that led to the isolation of bomapin cDNA are indicated by arrows above the nucleotide sequence.)

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Furthermore, the first ATG codon in this sequence is at the 5'-end, denoted bomapin, with a calculated molecular mass of 45.4 kDa (Fig. 1). The large open reading frame that encodes a 397-amino acid protein was subsequently used for cDNA amplification. Both strands of cloned PCR products from three independent amplifications were completely sequenced, confirming the sequence shown in Fig. 1. The isolated cDNA contains a single open reading frame beginning with this ATG codon leads to the synthesis of a functional protein (refer to Fig. 4). Determination of the authentic translation start site will ultimately require the purification of this protein followed by N-terminal sequencing of the native protein. The deduced amino acid sequence contains 22 residues including a cluster of charged amino acids that might be important for interactions between bomapin and other molecules. This domain might be important for interactions between bomapin and other molecules.

Sequence Analysis—The isolated cDNA contains a single large open reading frame that encodes a 397-amino acid protein, denoted bomapin, with a calculated molecular mass of 45.4 kDa (Fig. 1). The first ATG codon in this sequence is at the 5'-sense) and 3'-antisense) ends of the 1191-nucleotide open reading frame were subsequently used for cDNA amplification. Both strands of cloned PCR products from three independent amplifications were completely sequenced, confirming the sequence shown in Fig. 1.

Alignment of the bomapin amino acid sequence with other serpins. Pairwise alignments between α1-PI, bomapin, PAI-2, human elastase inhibitor (E1), and CAP were generated using the GAP routine of the Genetics Computer Group (Madison, WI) software package (gap weight, 3.0; length weight, 0.1) and adjusted manually to reduce the number of gaps in the final alignment. Amino acids are numbered according to the sequence of mature α1-PI (1). The great similarity of the bomapin primary structure with proteins (4) all of the structural features that distinguish bomapin is a new member of the ovalbumin family of serpin (26). All of the structural features that distinguish bomapin is a new member of the ovalbumin family of serpin (26).
proteases have been described in the serpin
rate reactive sites for inhibitory interactions with different
stable complexes with trypsin-like proteases. However, the
strate that this novel ov-serpin has the ability to form SDS-
and especially trypsin, the bomapin complexes were progres-
proteases. In the presence of higher concentrations of thrombin
capability of bomapin to form SDS-stable complexes with these
thrombin (32 kDa) and trypsin (24 kDa), demonstrating the
to the combined molecular masses of bomapin and reduced
bands were detected with an apparent molecular mass of 74
(27), and it is possible that Asp-360 (P3 position in the align-
ment in Fig. 2) acts as the P1 residue in an interaction between
bomapin and an ICE-related protease.

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Fig. 3. Detection of the bomapin transcript in bone marrow. Panel A, a Northern blot of 2 μg of poly(A)⁺ RNA isolated from human bone marrow was hybridized to a random decamer-primed ³²P-labeled cDNA probe corresponding to nucleotides 643–977 of the bomapin sequence and exposed for 24 h to autoradiography film. Panel B, human bone marrow, placenta, and brain cDNA libraries in the vector pGAD10 (Clontech) were used at the indicated DNA quantities as a template for 50-μl PCR amplifications utilizing specific primers to amplify the coding regions of bomapin (upper panel) and CAP as a control (lower panel). 10 μl of the PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Fig. 4. Bomapin forms SDS-stable complexes with thrombin and trypsin. The complete bomapin coding region was expressed by in vitro transcription/translation in the presence of [³⁵S]methionine. Samples (2.5 μl) of reaction mixtures generated in the absence (lanes 1 and 2) and in the presence (lanes 3–10) of the bomapin expression construct were diluted with 5 μl of Tris-buffered saline and incubated (15 min, 37 °C) without protease (lanes 1 and 3) and in the presence of thrombin (0.2 pmol, lane 4; 1 pmol, lane 5; 4 pmol, lanes 2 and 6; 20 pmol, lane 7) or trypsin (0.2 pmol, lane 8; 1 pmol, lane 9; 4 pmol, lane 10). Samples were heated to 100 °C for 3 min in the presence of 2% SDS and 100 mM dithiothreitol, proteins were resolved by SDS-polyacrylamide gel electrophoresis (9% separating slab gel), and detected by fluorography.

Bomapin Forms SDS-stable Complexes with Proteases—Sequence analysis indicated that bomapin is a potentially pro-
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