Cystatin E is a Novel Human Cysteine Proteinase Inhibitor with Structural Resemblance to Family 2 Cystatins*

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A new member of the human cystatin superfamily, called cystatin E, has been found by expressed sequence tag (EST) sequencing in amniotic cell and fetal skin epithelial cell cDNA libraries. The sequence of a full-length amniotic cell cDNA clone contained an open reading frame encoding a putative 28-residue signal peptide and a mature protein of 121 amino acids, including four cysteine residues and motifs of importance for the inhibitory activity of Family 2 cystatins like cystatin C. Recombinant cystatin E was produced in a baculovirus expression system and isolated. An antisera against the recombinant protein could be used for affinity purification of cystatin E from human urine, as confirmed by N-terminal sequencing. The mature recombinant protein processed by insect cells started at amino acid 4 (cystatin C numbering), and displayed reversible inhibition of papain and cathepsin B (Kᵢ values of 0.39 and 32 ns, respectively), in competition with substrate. Cystatin E is thus a functional cysteine proteinase inhibitor despite relatively low amino acid sequence similarities with human cystatins (26–34% identity with sequences for the Family 2 cystatins C, D, S, SN, and SA; <30% with the Family 1 cystatins, A and B, and domains 2 and 3 of the Family 3 cystatin, kininogen). Unlike other human low Mᵦ cystatins, cystatin E is a glycoprotein, carrying an N-linked carbohydrate chain at position 108. Northern blot analysis revealed that the cystatin E gene is expressed in most human tissues, with the highest mRNA amounts found in uterus and liver. A strikingly high incidence of cystatin E clones in cDNA libraries from fetal skin epithelium and amniotic membrane cells (>0.5% of clones sequenced) indicates a protective role of cystatin during fetal development.

Cysteine proteinase inhibitors of the cystatin superfamily are present in a variety of human tissues and body fluids. They seem, therefore, to have an important regulatory role in normal body processes involving cysteine proteinase activity such as bone resorption (1). In addition, numerous pathological conditions have been connected to an increased cysteine proteinase activity, including septic shock (2), cancer progression and metastasis (for review, see Ref. 3), parasitic and viral infections (4), as well as inflammatory reactions in rheumatoid arthritis, purulent bronchiectasis, and periodontitis (5–7). The cystatins are thus implied to function as general regulators of potentially harmful proteinase activities besides being members of the non-immune defense system of the body.

Structurally, the cystatins constitute a single superfamily of evolutionary related proteins (8). The known human members of this superfamily can be grouped into three protein families as defined by Dayhoff et al. (9). Cystatins A and B (Family 1) contain about 100 amino acid residues (Mᵦ = 11,000–12,000) and lack disulfide bridges, and their genes do not encode signal peptides. Family 2 cystatins are secreted proteins of ~120 amino acid residues (Mᵦ = 13,000–14,000) and have two characteristic intrachain disulfide bonds. The human Family 2 cystatins C, D, S, SN, and SA are encoded by genes located in a multigene locus at chromosome 20 together with two pseudogenes (10–12). Family 3 cystatins, represented by human L- and H-kininogen, are more complex members of the cystatin superfamily and contain three Family 2 cystatin-like domains besides being kinin precursors (13). All of the human cystatin superfamily members are tight-binding enzyme inhibitors with specificity against papain-like cysteine proteinases such as the mammalian cathepsins B, H, L, and S (for review, see Ref. 14).

In the present study, we report the discovery of another human cystatin, cystatin E, with sequence similarities too low to agree with any of the three previous cystatin families and with the unusual characteristic of being a glycoprotein. Still, it resembles Family 2 cystatins structurally in containing two putative disulfide bridges and by being a secreted protein. Functional characterization and distribution studies demonstrated that cystatin E is a tight-binding cysteine proteinase inhibitor with unusual tissue expression.

EXPERIMENTAL PROCEDURES

Identification of cDNA for Cystatin E—A data base containing more than one million ESTs1 obtained from over 600 different cDNA libraries has been generated through the combined efforts of Human Genome Science Inc. and the Institute for Genomic Research using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (15, 16). Sequence homology comparisons of each EST were performed against the GenBank data base using the BLAST and BLASTN algorithms (17). ESTs having homology to previously identified sequences (probability equal or less than 0.01) were given a tentative name based on the name of the sequence to which it was

1 The abbreviations used are: EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide-N-glycosidase F; PCR, polymerase chain reaction; Bz, benzoyl.
homologous. A specific homology and motif search using the known amino acid sequence of human cystatin C (M27891) against this human EST data base revealed several ESTs having translated sequences >30% homologous to that of cystatin C. One clone (HAQB6M0) encoding an intact N-terminal signal peptide was identified in a human amniotic or fetal skin epithelium library and was selected for the investigation. This EST and other ESTs from amniotic or fetal skin epithelium libraries were sequenced on both strands to the 3' end, and their homology to the cystatin C cDNA was confirmed.

**Cloning of Cystatin E in Baculovirus**—The entire coding sequence of the cystatin C-related cDNA was amplified using standard PCR technology using 5' (upstream primer, 5'-CGC GGA TCC GCC ATC ATG GCG CGT-3'); downstream primer, 5'-CGC GGT ACC GAA TGG-3'). The amplified fragment was purified and digested with BanHI and Asp718 followed by a second purification. The baculovirus expression vector, pA2, derived from pNR704 (18–20) was ligated with the amplified cystatin coding sequence of the pA2 vector was digested with BanHI and Asp718 followed by agarose gel purification. The purified pA2 vector was ligated with the amplified cystatin coding sequence using T4 DNA ligase (Life Technologies, Inc.).

**Generation of Recombinant Baculovirus and Labeling**—To generate recombinant baculoviruses encoding the novel cystatin sequence, Sf9 cells were co-transfected with 5 μg of transfer vector containing the HAQB6M0 cDNA and 1 μg of BaculoGold (Pharmingen) viral DNA using Lipofectin (Life Technologies, Inc.) in a total volume of 200 μl of serum-free medium. The primary viruses were harvested at 4–7 days postinfection and used in plaque assays. Plaque purified viruses were subsequently amplified and frozen. For pulse-labeling, Sf9 cells were seeded in 12-well dishes with 2 ml of a cell suspension containing 0.5 × 10⁶ cells/ml and allowed to attach for 4 h. Recombinant baculoviruses were used to infect the cells at a multiplicity of infection of 1–2. After 4 h, the media were replaced with 1.0 ml of serum-free medium depleted for methionine and cysteine (−Met/−Cys). At 3 days postinfection, the culture media were replaced with 0.5 ml of −Met/−Cys medium containing 2 μCi each of [35S]Met and [35S]Cys. Cells were labeled for 16 h, after which the culture media was removed and clarified by centrifugation (supernatant). The cells were lysed in the buffer by the addition of 0.2 ml of SDS-PAGE sample buffer (cell extraction, 10 μl of each supernatant and cell extract were resolved by 15% SDS-PAGE. Protein gels were stained, destained, amplified, dried, and autoradiographed. Labeled bands corresponding to the recombinant proteins were visible after 24 h of exposure.

**Purification of Recombinant Cystatin E**—Recombinant cystatin E was purified from baculovirus-infected Sf9 cell supernatants. All purification steps were carried out at 4 °C, utilizing a BioCAD 250 (Perspective Biosystems, Inc.). 500 ml of supernatant was first adjusted to pH 4.5 and then applied at a flow rate of 20 ml/min to a 10-ml Poros HS column pre-equilibrated with 100 mM NaOAc buffer, pH 4.5. The cystatin E was found in the flow-through fraction. After adjusting pH to 8.5, the flow-through fraction was applied at a flow rate of 20 ml/min to a 10-ml Poros HQ column pre-equilibrated in 20 mM Tris-HCl buffer, pH 7.4. The cystatin E was again collected in the flow-through fraction. Finally, cystatin E was captured on a Mimetic Green 1 A6XL Alpha cartridge membranes followed by immunostaining and sequencing identified the major protein band as cystatin E.

**Protein Analyses**—Cystatin E was analyzed for glycosylation by determining the monosaccharide content in two purified preparations of the recombinant protein. About 100 ml of urine was concentrated 10-fold by pressure ultrafiltration using a C-DAK artificial kidney with a retention limit of approximately 1,000 Da (Cordia Dow Corp., Miami, FL) and then incubated for 3 h at room temperature with 3 ml of the immunoaffinity resin described above, containing about 100 mg of covalently coupled IgG.from the anti-cystatin E) serum. The resin was then packed into a column and washed with about 300 ml of a 50 mM Tris buffer, pH 7.4, containing 0.5 mM NaCl, 5 mM benzamidinium chloride, 10 mM EDTA, and 15 mM sodium azide. Immunosorbed material was eluted with 0.2 mM glycine buffer, pH 2.2, containing 0.5 mM NaCl, 5 mM benzamidinium chloride, 10 mM EDTA, and 15 mM sodium azide, and the eluate was immediately neutralized by addition of a 2 mM Tris buffer, pH 8.6, and then concentrated to about 700 μl by ultrafiltration using CentriTec concentrators (Amicon Corp., Danvers, MA). Agarose gel electrophoresis of the eluate revealed the presence of the major protein band, and blotting of the electrophoretically separated proteins on nitrocellulose membranes followed by immunostaining and sequencing identified the major protein band as cystatin E.

**Protein Analyses**—Cystatin E was analyzed for glycosylation by determining the monosaccharide content in two purified preparations of the recombinant protein. About 10 μg of the protein was hydrolyzed with 0.2 M trifluoroacetic acid at 100 °C for 4 h. After drying the hydrolysate in a SpeedVac and reconstituting in 10 μl of deionized water, the resulting monosaccharides were analyzed on a Dionex carbohydrate analyzer (22). A PA-1 column (Dionex) was used to separate the monosaccharides by isocratic elution with 12 mM NaOH. The monosaccharide analyzer (22). A PA-1 column (Dionex) was used to separate the monosaccharides by isocratic elution with 12 mM NaOH. The monosaccharides were detected by integrated amperometry using a pulsed amperometric detector. Glycans analysis was also done by incubation with peptide-N-glycosidase F (EC 3.2.2.18) under conditions.
recommended by the enzyme supplier (Oxford GlycoSystems, Abingdon, UK) followed by SDS-PAGE. Purified preparations of recombinant and natural cystatin E was characterized by SDS-PAGE after reduction in 15 or 16.5% gels with the buffer systems described by Laemmli (23) and Schägger and von Jagow (24), respectively, and by agarose gel electrophoresis at pH 6.5 (25). Automated N-terminal sequencing was carried out after electrophoresis in SDS-polyacrylamide (Novex 4–20% gels), staining with Ponceau S (0.2% in 4% acetic acid), and excision of the band of interest out after electrophoresis in SDS-polyacrylamide (Novex 4–20% gels), blotting onto a ProBlott membrane (Applied Biosystems), staining with 

Enzyme Inhibition Assays—The methods used for active site titration of papain, titration of the molar enzyme inhibitory concentration in complexes between cystatin E and cysteine peptidases have been described in detail earlier (14, 28). The enzymes used for dissociation of 150 $M$ for cathepsins B (31) and 60 $M$ for cathepsin B (30). Corrections for substrate competition were made using Henderson (34, 35) of complexes between cystatin E and cysteine peptidases have been described in detail earlier (14, 28). The enzymes used were papain (EC 3.4.22.2, from Sigma), activable to 70–75% after depolymerization at pH 6.5 (25). Automated N-terminal sequencing was carried out after electrophoresis in SDS-polyacrylamide (Novex 4–20% gels), blotting onto a ProBlott membrane (Applied Biosystems), staining with Ponceau S (0.2% in 4% acetic acid), and excision of the band of interest out after electrophoresis in SDS-polyacrylamide (Novex 4–20% gels), staining with Ponceau S (0.2% in 4% acetic acid), and excision of the band of interest (26), using an Applied Biosystem 477A sequencer and the gas-phase blot cycles. Alternatively, proteins in mixtures were separated by agarose gel electrophoresis and transferred to a polyvinylidene difluoride membrane, and N-terminal sequencing was carried out on the individual protein bands using an Applied Biosystems 477A sequencer (27).

RESULTS AND DISCUSSION

At EST sequencing of human cDNA libraries, several clones with significant homology to the cystatin C sequence (33) were identified. Clones encoding the same unknown protein were identified in libraries from amniotic membrane cells (32 clones) and fetal skin epithelium (27 clones). Complete sequencing of these identified a full-length cDNA clone, designated HAQB60, in an amniotic cell library. The clone contained an open reading frame encoding a 149-residue preprotein (Fig. 1), of which the first 28 likely constitute the signal peptide according to an alignment with human cystatin sequences (Fig. 2) and theoretical considerations (38). This indicated a closer relationship with the secreted Family 2 cystatins than with the intracellular Family 1 cystatins. The open reading frame contained a typical consensus sequence for initiation of translation (39) around the start ATG codon, and was followed by a poly(A) signal, AATAAA, 78 nucleotides downstream from the stop codon, after which a poly(A) sequence was evident a further 20 nucleotides downstream (Fig. 1).

The deduced mature protein sequence was just 34% identical to that of cystatin C, showed lower resemblance (26–30% identity) to the sequences of the other known Family 2 cystatins D, S, SN, and SA (Fig. 2), and even lower similarities of 18 and 23% identical residues when compared with the Family 1 cystatins, A and B (not shown). The resemblance to cystatin domain 2 of human kininogen was similarly low (18% identity), whereas the domain 3 sequence was 29% identical to the cystatin E sequence. However, the sequence contained a Gly residue at exactly the same distance from a central Gln-Xaa-Val-Xaa-Gly motif as the other cystatin sequences and also a Pro-Trp pair toward the C-terminal end of the translation product, like that of the human Family 2 or 3 cystatins. The sequence also contained four Cys residues toward the C-terminal end, alignable with those in Family 2 cystatins. The four Cys residues in cystatin C and the avian analogue, chicken cystatin, form two disulfide bridges stabilizing the cystatin structure (37, 40). The novel protein was thus similar to Family 2 cystatins in parts essential for structure and function and was designated cystatin E. Its evolutionary relationship to the cystatin superfamily seems undisputed, but according to the relatively low sequence similarities, it should be seen as a first member in a new protein family (9). The cystatin E sequence also had some unusual characteristics, including a five-residue insertion between amino acids 76 and 77 and a deletion of residue 91 (cystatin C numbering). These sequence positions correspond to polypeptide parts on the side opposite to the proteinase binding region of chicken cystatin (40) and would likely not affect an inhibitory function of cystatin E. A motif search in addition showed a target Asn-Xaa-Ser/Thr sequence for glycosylation at positions 108–110. On the gene level, a cystatin multigene locus on the short arm of chromosome 20 has been investigated in detail. This locus harbors the genes for the known Family 2 cystatins C, D, S, SN, and SA and in addition two pseudogenes but, according to estimates using cross-hybridizing probes in Southern blotting, likely no additional genes (10–12). Again, this supports that cystatin E is a
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**Table I**

Inhibition of papain and cathepsin B by cystatin E equilibrium constants for dissociation were determined by steady-state kinetics, as described under "Experimental Procedures."

The corresponding values for cystatins C and D, determined by similar methods (41, 43, 44) are given for comparison. The S.D. and number of measurements (n) used to calculate the mean K_i values given are indicated. The K_i values were corrected for substrate competition as described under "Experimental Procedures."

| Cystatin | Papain | Cathepsin B |
|----------|--------|-------------|
|          | K_i | S.D. | n | K_i | S.D. | n |
| E        | 0.39 | ±0.12 | 12 | 32 | ±9.6 | 7 |
| C        | 0.000011 | 0.27 | | | |
| D        | 0.9 | >1000 | | | |

**Fig. 3. Electrophoresis of cystatin E.** A, SDS-PAGE in a 16.5% gel. The gel was silver stained. Lane 3, isolated recombinant cystatin E; lane 4, same as lane 3 but sample treated with PNGase. Recombinant cystatin C (41) and recombinant cystatin D (35) are shown in lanes 1 and 2, respectively, for comparison. Lane M, molecular weight markers, with sizes of relevant bands indicated to the left. B, agarose gel electrophoresis at pH 8.6. The Coomassie-stained gel shows the concentrated eluate from an immunoaffinity column after chromatography of urine from a proteinuria patient (right lane). The major band in the β1-zone had cystatin E immunoreactivity and was subjected to N-terminal sequencing. A human blood plasma sample (left lane) was electrophoresed as a reference. The direction of the electric field is indicated. The point of sample application is marked by an arrowhead.

protein distantly, but significantly, related to the Family 2 cystatins.

Initial attempts to produce cystatin E in E. coli gave negligible amounts of recombinant protein. The cystatin E cDNA was therefore subcloned in a baculovirus expression vector and was expressed in insect Sf9 cells (see "Experimental Procedures"). The recombinant protein was secreted into the cell media of such cultures, with a yield of ~10–20 mg/liter of culture medium. The protein was purified by a combination of ion exchange and dye affinity column chromatographies, resulting in a >95% pure protein preparation according to SDS-PAGE (Fig. 3A), provided that the observed protein band doublet was due to microheterogeneity of the same protein (see below). N-terminal sequence analysis of both protein bands confirmed that this was the case and demonstrated, for both, that a 28-residue segment is proteolytically removed during secretion from the insect cells to yield a mature protein beginning with the sequence Arg-Pro-Glu-Glu-Glu-Glu. The N-terminal Arg residue corresponds to residue 4 in the cystatin C sequence and agrees with a theoretical signal peptidase cleavage site with (small side chains for residues in positions −3 and −1 (38)) (cf. Fig. 1). The M_r of the mature protein bands, calculated from their SDS-PAGE mobility after reduction was ~14,300 and 15,700, in good agreement with a theoretical mass of 13,652 calculated from the sequence.

The recombinant cystatin E remained as a doublet through the above purification procedures (Fig. 3A). As the protein bands shared the same N-terminal sequence, the upper band of the doublet was suspected to be a glycosylated species of cystatin E, given that the theoretical glycosylation site was found at motif analysis of the protein sequence (Asn-Ser-Ser at positions 40, 45), corresponds to an Arg-Met-Val-Gly segment in cystatin C, the N-terminal segment Arg-Leu-Val-Gly at positions 15,700, in good agreement with a theoretical mass of 13,652 calculated from the sequence.

The corresponding N-terminal segment in cystatin D is Thr-Leu-Ala-Gly; the small side chain of the Ala residue might be a partial explanation for the lack of cystatin B inhibition observed for this inhibitor, in contrast to cystatins C and E which both have a hydrophobic (Val or Met) residue that could be interacting with a deep S2 subpocket of cathepsin B (45). An antiserum was raised against the recombinant cystatin E and used in an attempt to find a corresponding protein in human tissues. Placenta was originally investigated as the cystatin E cDNA had been found in amniotic membrane and fetal epithelial cells. Homogenates of placenta contained a cystatin E-immunoreactive protein, with an M_r of ~15,000 according to SDS-PAGE (not shown), but this preparation proved hard to purify for sequencing due to a high content of the similarly charged and sized hemoglobin α-chain in the homogenates. Investigation of urine from a patient with mixed glomerulotubular proteinuria, a body fluid enriched in low M_r proteins originating from blood plasma and previously shown to be a good source of cystatins (21), again identified a protein with cystatin E immunoreactivity. By using the IgG fraction of the
antiserum to produce an immunoaffinity column, the immunoreactive protein could be isolated and characterized by agarose gel electrophoresis, immunoblotting, gel filtration, and N-terminal sequencing. The protein migrated in the β1-zone on agarose gel electrophoresis (Fig. 3B), reacted with the antiserum raised against recombinant cystatin E, cochromatographed with other low Mᵦ cystatins upon gel filtration, and displayed the N-terminal sequence Met-Val-Gly-Glu-Leu-Xaa-Asp-Leu-. Thus, the resulting sequence agreed with that of the postulated mature recombinant cystatin E from residue 6 (residue 9 in cystatin C numbering) onwards for all seven residues that could be identified. These results show that human fluids contain a protein related or identical to recombinant cystatin E. The shorter N-terminal segment of the protein isolated from human urine could be due to proteolytic processing in urine before addition of protease inhibitors and could be analogous with the presence of several N-terminally truncated forms of cystatin C in urine (21). The reasons why cystatin E has been overlooked in several studies aiming at isolation of cystatins from human tissues and body fluids using affinity chromatography with carboxymethyl-papain as the catching ligand (e.g. see Ref. 21) are not clear, but one possibility is that the cystatin E, although with a high affinity for active papain $K_D > 10^{8}$ M$^{-1}$ (1), has significantly lower affinity for the carboxymethylated enzyme. Another explanation might be that cystatin E is present in very low concentrations in body fluids, or that the majority of the inhibitor is complexed to target proteinases in such fluids, preventing binding to the catching proteinase. Estimated from Coomassie staining of cystatin E isolated by immunoblotting with carboxymethyl-papain as the catching ligand, the yield of cystatin C was $\sim$3,000 nmol/100 ml of 20 × concentrated urine, i.e. 1,000-fold higher (21).

To investigate the tissue distribution of cystatin E, Northern blot experiments with the cystatin E cDNA probe were performed. The low nucleotide sequence similarity with Family 2 cystatin cDNAs made it unlikely that the cystatin E probe would cross-hybridize with mRNAs of other cystatins. Indeed, in control experiments, there were no significant cross-reactions with cDNAs for cystatins C and D (not shown). However, given the fact that a previously overlooked cystatin sequence now has been identified, it cannot be ruled out that additional low copy human cystatin gene transcripts with higher similarity to the cystatin E probe could be present in specialized tissues. The indications from the Northern blot results (Fig. 4) are that the cystatin E gene is expressed in most tissues in the body. The distribution pattern of relatively strong mRNA signals in uterus and liver, slightly weaker but significant signals in placenta, pancreas, heart, spleen, small intestine, and peripheral blood leukocytes, and low cystatin E mRNA content in brain, testis, and kidney, is clearly different from the distribution of e.g. cystatin C mRNA. The fact that all of the cDNA clones we identified originated either from amniotic membrane cell or fetal skin epithelium libraries (a total of 59 clones out of 10,000 sequenced, i.e. almost 0.6%) indicates that the cystatin E gene expression is strongly up-regulated in, or specific for, epithelial cells. In addition, the origin of these cells suggests that the inhibitor serves a protective role during fetal development.

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FIG. 4. Expression of the cystatin E gene in human tissues. Blots containing electrophoretically separated samples of 2 μg poly(A)$^+$ RNA from human tissues (Clontech) were hybridized to the full-length cystatin E cDNA probe HAQBM60. The positions of size standards are indicated. PBL, peripheral blood leukocytes.
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