Molecular cloning and expression of an intracellular serpin: an elastase inhibitor from horse leucocytes

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Horse blood leucocytes contain an elastase inhibitor (HLEI) belonging to the serpin family. Poly(A)$^+$RNA isolated from these cells was used to construct a cDNA library in λgt10, which was first screened with a synthetic degenerate oligonucleotide probe corresponding to the amino acid sequence of the reactive centre of the inhibitor. Three clones were obtained covering the entire coding region of the protein. Sequencing of these clones showed identity with the amino acid sequence obtained from Edman degradation of the elastase inhibitor. The coding sequence of the HLEI cDNA was cloned into the bacterial expression vector pKK233-2 and expressed in *Escherichia coli* cells. Transformed bacteria expressed significant amounts of the protein, which was immunoprecipitated with a specific anti-HLEI antiserum. Furthermore, HLEI expressed in bacteria inhibited the activity of elastase but not trypsin.

INTRODUCTION

The unrestricted activity of leucocyte proteinases digesting elastin in the lung is believed to be responsible for the occurrence of both chronic obstructive lung disease and resulting emphysema in humans (Janoff, 1985) and horses (Gerber, 1973). The granules of horse leucocytes contain three elastase-like proteinases (Dubin et al., 1976; Koj et al., 1976; Potempa, 1982; Von Fellenberg et al., 1985; Potempa et al., 1986) which are inhibited not only by the horse plasma proteins α_1-proteinase inhibitor (Koj and Kurdowska, 1981; Dubin et al., 1986; Potempa et al., 1991) and α_2-macroglobulin (Dubin et al., 1984), but also by an inhibitor present in the cytosol of horse leucocytes (HLEI) (Dubin, 1977). The latter is a single-chain polypeptide (molecular mass 43 kDa) which inactivates both horse and human leucocyte elastases, and to a lesser extent cathepsin G and chymotrypsin. However, it is ineffective against trypsin (Dubin et al., 1985; Dubin and Koj, 1986). Analysis of the amino acid sequence of the horse inhibitor indicates that it belongs to the serpin superfamily of proteinase inhibitors (Potempa et al., 1988; Dubin et al., 1992). A similar intracellular elastase inhibitor has been found in the human monocytic cell line U-937, and its cDNA sequenced (Remold-O'Donnell et al., 1992).

The high anti-proteinase activity of HLEI indicates a potential application in the treatment of horse lung emphysema. For that purpose, however, large quantities of protein are required which can only be obtained by recombinant DNA technology. Thus the present study was aimed at cloning and expression of HLEI using horse peripheral blood leucocytes and crude bone marrow cells as a source of mRNA.

EXPERIMENTAL

Materials

Restriction enzymes, polynucleotide kinase, calf intestinal phosphatase, *Eco*RI methylase, T4 DNA ligase, T4 DNA polymerase, DNA polymerase I, Klenow enzyme and random priming kit were purchased from Boehringer (Mannheim, Germany). [α-^{32}P]dATP, [γ-^{32}P]ATP, [α-^{35}S]dATP, [^{35}S]methionine and DNA packaging mix were obtained from Amersham International (Amersham, Bucks., U.K.). Both the T7 DNA sequencing kit and reverse transcriptase were from Pharmacia (Uppsala, Sweden). Rabbit reticulocyte lysate was prepared and tested as described earlier (Geiger et al., 1988; Krüttgen et al., 1990).

Isolation of leucocytes and crude bone marrow cells

A total of 70 litres of fresh citrated horse blood was used to isolate horse leucocytes by sedimentation of erythrocytes and centrifugation of plasma at 600 *g* for 15 min (Dubin, 1977). Residual erythrocytes were removed by a short osmotic shock (0.2% NaCl for 30 s). The leucocyte sediment, containing approx. 2×10^9 cells/g and consisting of 80–85% polymorphonuclear cells, was frozen and powdered in liquid nitrogen.

Crude bone marrow cells were isolated from the horse breastbone. Bone marrow was collected by scraping and the material was resuspended in minimal Eagle's medium. After filtering through four layers of cheese cloth, the cells were pelleted by centrifugation at 600 *g* for 15 min. The sediment was frozen and powdered in liquid nitrogen.

RNA preparation and Northern blot analysis

RNA was prepared from powdered frozen cells or samples of frozen horse tissue using guanidinium thiocyanate and centrifugation through a CsCl gradient (Glišin et al., 1974; Ullrich et al., 1977). RNA (5 μg) was heated to 65 °C for 10 min in 50% formamide, 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde prior to gel electrophoresis in 1% agarose containing 2.2 M formaldehyde, 20 mM Mops, 5 mM sodium acetate and 1 mM EDTA. The separated RNA was transferred to Gene Screen Plus membranes (NEN, Dreieich, Germany) according to the supplier's instructions. The filters were prehybridized at 68 °C or 46 °C for a cDNA probe and a degenerate oligonucleotide probe respectively for 3 h in 10% dextran sulphate, 1 M NaCl and 1% SDS. Hybridization was carried out in the same solution with cDNA fragments labelled by random priming (Feinberg and Vogelstein, 1983) or with

Abbreviations used: DTT, dithiothreitol; HLEI, horse leucocyte elastase inhibitor; IPTG, isopropyl thiogalactoside.
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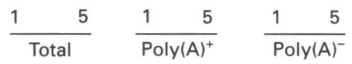

Figure 1 Northern analysis of RNA from horse leucocytes

Total, poly(A)$^+$ and poly(A)$^-$RNA were prepared as described in the Experimental section. The gel load was 1 and 5 μg, as indicated. After separation, RNA was blotted on to nylon membranes and hybridization was carried out with a ^{32}P-labelled degenerate oligonucleotide probe (see the Experimental section).

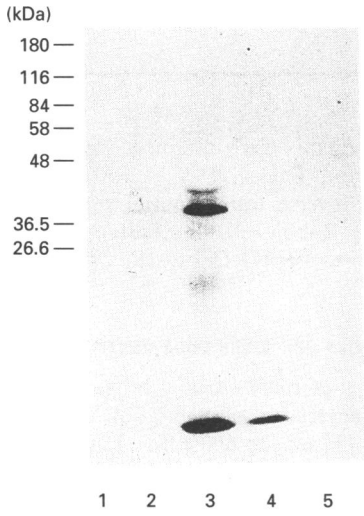

Figure 2 Cell-free translation and immunoprecipitation of HLEI

A 1 μg portion of total RNA (lane 2), poly(A)$^+$RNA (lanes 3 and 4) or poly(A)$^-$RNA (lane 5) was translated in a cell-free system using a reticulocyte lysate in the presence of [^{35}S]methionine. The cell-free-synthesized HLEI was immunoprecipitated with specific antibodies (lanes 1, 2, 3 and 5) or with a preimmune serum (lane 4) and subjected to SDS/PAGE and fluorography (see the Experimental section). As a control, *in vitro* translation was carried out without added RNA (lane 1). The positions of marker proteins are indicated.

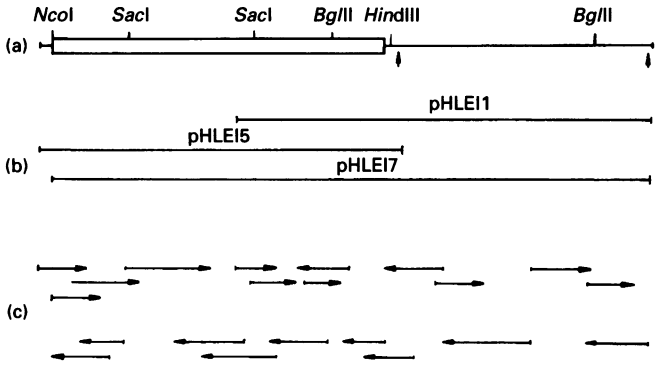

Figure 3 Restriction map of HLEI cDNA

(**a**) Restriction map and organization of HLEI cDNA. The coding region is boxed, and the arrows denote two possible polyadenylation signals. (**b**) Isolated clones coding for HLEI; (**c**) sequencing strategy.

degenerate oligonucleotide probes labelled at the 5′ end using polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989).

Preparation and screening of a cDNA library

A λgt10 library was prepared from poly(A)$^+$RNA isolated from horse leucocytes by affinity chromatography on oligo(dT)–cellulose according to the method of Gubler and Hoffmann (1983), as modified by Rose-John et al. (1988). Briefly, 5 μg of poly(A)$^+$RNA was used for the first-strand synthesis primed with an oligo(dT) in 50 mM Tris/HCl, pH 8.3, 8 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 0.5 mM of each of dATP, dTTP, dCTP and dGTP. The obtained material was treated with RNAase H, the second strand was synthesized using DNA polymerase I and the double-stranded cDNA was blunt-ended using T4 DNA polymerase. The cDNA was ligated into *Eco*RI-cleaved λgt10$^+$DNA and packaged *in vitro*. Screening of the cDNA library was performed with the degenerate oligonucleotide probe (kindly synthesized by Dr. W. J. Stec) covering the region from Met-344 to Ala-351:

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                        A
    A   A   A   T   T   C
5'-CC  TT  AA  TT  TC  TC  GGCAT-3'
    G   G   G   C   C   T
                        G
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The oligonucleotide probe was labelled at the 5′ end using polynucleotide kinase and [γ-^{32}P]ATP. Screening was carried out in 5 × Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.12 M phosphate buffer, pH 6.8 and 3 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate) at 46 °C.

DNA subcloning and nucleotide sequencing

*Eco*RI-digested insert DNA from recombinant phages was subcloned into plasmid pUC19 using standard techniques (Sambrook et al., 1989). Sequencing was performed by standard methods (Sanger et al., 1977) from suitable restriction fragments using the T7 DNA polymerase sequencing kit and plasmid dsDNA (Chen and Seeburg, 1985).

Expression of full-length HLEI cDNA in *Escherichia coli* and Western blotting

The 1.3 kb *Nco*I–*Hin*dIII fragment containing the entire coding sequence of HLEI was cloned into the bacterial expression vector pKK233-2 (obtained from Pharmacia, Freiburg, Germany). Transformed bacteria (JM105) were grown to $A_{600} = 0.3$ and isopropyl thiogalactoside (IPTG) was added to 1 mM. Bacterial cells were collected by centrifugation at 8000 ***g*** and resuspended in PBS (20 mM NaCl, 0.25 mM KCl, 0.8 mM Na_2HPO_4 and 0.15 mM KH_2PO_4). The material obtained after sonication was subjected to SDS/PAGE or used for the estimation of HLEI concentration by rocket immunoelectrophoresis and inhibitory activity as described by Potempa et al. (1988). Western blotting was carried out as described (Schiel et al., 1990).

In vitro translation, immunoprecipitation, SDS/PAGE and fluorography

In vitro translation with [^{35}S]methionine was performed by standard procedures (Krüttgen et al., 1990). The products were immunoprecipitated using specific antiserum to HLEI (Dubin

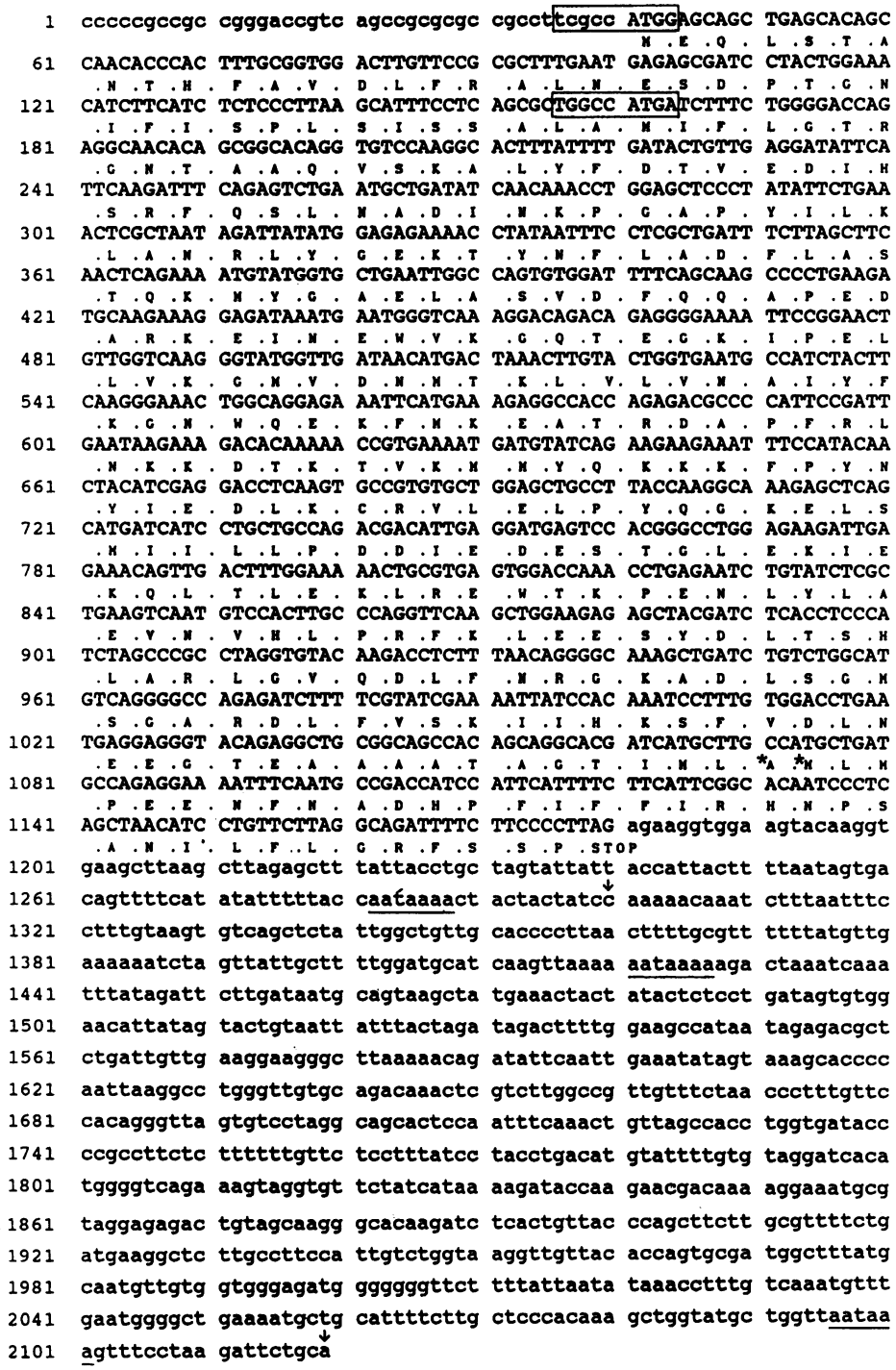

Figure 4 Nucleotide and amino acid sequences of HLEI

The boxed sequences represent the Kozak sequence; the underlined sequences denote polyadenylation signals. The arrows indicate polyadenylation sites, and asterisks indicate the reactive (P_1–P_1') bond in the amino acid sequence.

and Hauck, 1981) in 20 mM Tris/HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 5 mM EDTA and 2 mM methionine for 2 h at room temperature. After adsorption to Protein A–Sepharose for 2 h at 4 °C, the complexes were washed in precipitation buffer, dissolved in 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62 mM Tris/HCl, pH 6.8, and 0.001% Bromophenol Blue, and subjected to SDS/PAGE in a 10% gel (Laemmli, 1970). Fluorography was carried out by standard procedures (Bonner and Laskey, 1974).

Isolation of genomic DNA and Southern blotting

Frozen powdered horse leucocytes were resuspended in 0.5 M EDTA and incubated with 250 μg/ml RNAase A in 0.5% *N*-

laurylosarcosyl for 3 h at 37 °C, and subsequently with 250 μg/ml proteinase K overnight at 37 °C. The sample was extensively extracted with phenol and chloroform (Sambrook et al., 1989) and dialysed overnight against 1000 vol. of 10 mM Tris/HCl, pH 7.5. DNA was digested with restriction endonucleases and separated on a 0.75% agarose gel. Blotting and hybridization were performed using standard methods (Sambrook et al., 1989).

RESULTS

Isolation of mRNA from leucocytes

In previous experiments it has been shown (Dubin and Koj, 1986) that horse leucocytes contain large amounts of HLEI in their cytoplasm. In order to clone the cDNA of HLEI, we designed a degenerate oligonucleotide probe on the basis of a published partial amino acid sequence of HLEI (Potempa et al., 1988), choosing the reactive site, which is variable in all members of the serpin family. The probe was used to analyse total and poly(A)$^+$RNA from horse leucocytes. The Northern blot (Figure 1) shows that HLEI mRNA is present in horse blood leucocytes. The estimated size of the transcript is about 2.3 kb.

To confirm that horse leucocytes indeed express HLEI mRNA, poly(A)$^+$RNA isolated from this source was used for cell-free translation in a rabbit reticulocyte lysate. Using a specific antiserum to HLEI, we immunoprecipitated two polypeptides with apparent molecular masses of 40 and 43 kDa (Figure 2) which are present in the translation products from total and poly(A)$^+$RNA but not from poly(A)$^-$RNA. The band which migrates with the dye front is unspecific background.

Construction and screening of a cDNA library

Having shown that horse leucocytes express mRNA for HLEI, we constructed an oligo(dT)-primed cDNA library from the poly(A)$^+$ RNA of these cells in λgt10. The obtained library contained approx. 2.5×10^5 independent clones with an average insert size of 1.5 kb. Screening of 50000 phages with the degenerate oligonucleotide probe resulted in the detection of 23 positive clones, one of which (pHLEI1; Figure 3b) was analysed in detail. Using a *Sac*I/*Bgl*II fragment at the 5′ end of this clone we rescreened 100000 phages and obtained two additional clones, pHLEI5 and pHLEI7, covering the entire coding region of the protein (Figure 3b).

Nucleotide and amino acid sequence of HLEI

Figure 4 shows the nucleotide and amino acid sequence of HLEI. The translational start was assigned to nucleotide 41, based on the following evidence: (i) the in-frame ATG triplet is found at this position; (ii) nucleotides 36–44 fulfil the criteria of the Kozak sequence (Kozak, 1987); and (iii) complete sequence identity was found when the amino acid sequence deduced from the cDNA was compared with that obtained by Edman degradation (Dubin et al., 1992). Following the translational start signal there is an open reading frame coding for 379 amino acids. This sequence also contains the amino acid sequence selected for the synthesis of the degenerate oligonucleotide probe, used for screening of the cDNA library.

Southern analysis of leucocyte DNA

In order to obtain some information on the genomic organization of HLEI, Southern analysis was carried out. Figure 5 shows the results of Southern analysis of horse leucocyte DNA probed with an HLEI cDNA fragment. The appearance of single bands in different lanes corresponding to different DNA restriction digests indicates that HLEI is encoded by a single-copy gene.

Figure 5 Southern analysis of horse leucocyte DNA

DNA from horse leucocytes was isolated (see the Experimental section) and digested with various restriction enzymes. Portions of 10 μg of DNA digests were subjected to agarose gel electrophoresis, blotted to nitrocellulose and hybridized with a 250 bp ^{32}P-labelled *Sac*I–*Bgl*II fragment of the HLEI cDNA. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

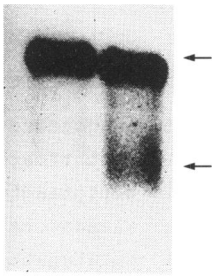

Figure 6 Northern analysis of RNA from horse leucocytes and bone marrow cells

Total RNA (5 μg) from horse leucocytes (lane 1) or horse bone marrow cells (lane 2) was separated by agarose gel electrophoresis, blotted to nylon membranes and hybridized with a ^{32}P-labelled *Sac*I–*Bgl*II fragment of the HLEI cDNA clone as a probe. Two transcripts were detected in the total RNA from stem cells (arrows).

Northern blot analysis of horse leucocyte and bone marrow cell mRNAs

When the mRNA expression pattern of horse leucocyte HLEI was compared by Northern blotting with that in bone marrow stem cells, two mRNA species were detected in the latter case (Figure 6, lane 2). The smaller mRNA species (approx. 1300 bases) might reflect the usage of a second polyadenylation site at position 1299.

Expression of the cloned HLEI cDNA in *E. coli*

Since one of the aims of our study was to demonstrate the feasibility of preparation of HLEI by recombinant technology, we have expressed the HLEI cDNA in *E. coli*. An *Nco*I–*Hin*dIII

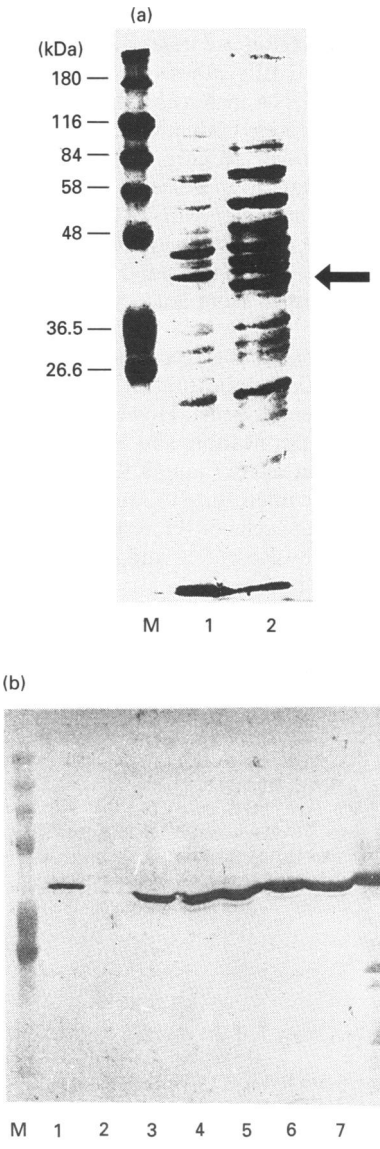

Figure 7 Expression of HLEI cDNA in *E. coli*

E. coli bacteria (strain JM 105) harbouring control plasmid pKK233-2 or plasmid pKK233-2 containing the coding region of HLEI (pExHLEI) were grown to an A_{600} of 0.3 and induced by 1 mM IPTG. After the times indicated below, soluble and insoluble proteins were isolated as described in the Experimental section and subjected to SDS/PAGE. (**a**) The gel was stained with Coomassie Blue G-250. Soluble proteins are from bacteria harbouring plasmid pKK233-2 (lane 1) or plasmid pExHLEI (lane 2) 1 h after induction with 1 mM IPTG. The expected position of HLEI is indicated by an arrow. (**b**) The separated proteins were transferred to a nitrocellulose membrane and Western blot analysis was performed with anti-HLEI antiserum (dilution 1:500). The immune complexes were visualized by the use of a peroxidase-conjugated second goat anti-rabbit antibody. Shown are HLEI isolated from horse leucocytes (lane 1), soluble proteins (lanes 2–7) and insoluble proteins (lanes 8 and 9) isolated from bacteria carrying plasmid pKK233-2 (lane 2) and plasmid pExHLEI (lanes 3–9). Bacteria were isolated after 1, 2, 4, 8 and 24 h after induction (lanes 3, 4, 5, 6 and 7 respectively) or 1 and 24 hours after induction (lanes 8 and 9 respectively). Lanes M contain molecular mass markers.

fragment containing the entire coding sequence of HLEI has been cloned into the pKK233-2 expression vector and transformed into *E. coli* cells. After induction of the transcription of HLEI cDNA by IPTG, soluble and insoluble proteins from the bacteria were isolated and subjected to electrophoresis followed by Western blot analysis. Figure 7(b) shows a time course of HLEI synthesis in transfected bacteria. A single protein species

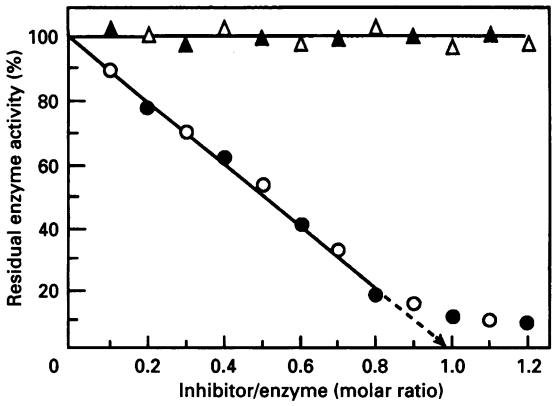

Figure 8 Interaction of HLEI expressed in *E. coli* with human neutrophil elastase and bovine pancreatic trypsin

Mixtures of 0.012 nmol of active enzyme (as determined by active-site titration of the enzyme) and various amounts of HLEI (estimated by rocket immunoelectrophoresis) were incubated in 0.2 M Tris/HCl, pH 8.0, for 5 min at room temperature and then assayed for residual elastase or trypsin activities (Potempa et al., 1988). △, ▲, trypsin; ○, ●, elastase. △, ○, native inhibitor; ▲, ●, recombinant HLEI.

was detected in the soluble protein fraction as well as in the proteins from inclusion bodies. The immunoreactive species were absent from control bacteria (lane 2). The apparent molecular mass of the protein expressed in *E. coli* corresponds to the position of HLEI obtained from leucocytes (lane 1). The amount of inhibitor expressed in *E. coli* 4 h after induction with IPTG was estimated by rocket immunoelectrophoresis and activity was assessed by measuring the inhibition of active-site-titrated human neutrophil elastase or bovine pancreatic trypsin. The obtained material did not contain any anti-trypsin activity, but inhibited elastase with an efficiency identical to that of the native inhibitor isolated from leucocytes (Figure 8). The lysate of the *E. coli* culture which was transformed with control pKK233-2 vector not containing HLEI cDNA exhibited low but detectable anti-elastase activity. This unspecific 'background' inhibitory activity was always subtracted when the inhibition of elastase was measured with the lysate of *E. coli* expressing HLEI, the amount of which was determined by rocket immunoelectrophoresis. With this system, 1 litre of culture (A_{600} = 1.0) contained approx. 500 μg of HLEI in the cytoplasm of the bacteria.

DISCUSSION

During the past few years, several members of the serpin family have been cloned and efficiently expressed by recombinant technology. Human α_1-proteinase inhibitor was expressed both in *E. coli* (Courtney et al., 1984; Bollen et al., 1984; Straus et al., 1985) and in yeast (Cabezon et al., 1984; Rosenberg et al., 1984), yielding either native or variant molecules targeted to specific enzymes (Courtney et al., 1985; Travis et al., 1985; Matheson et al., 1986; George et al., 1989). Moreover, genomic sequence identities between α_1-proteinase inhibitor and human C1 inhibitor (Tosi et al., 1986), α_1-antichymotrypsin (Bao et al., 1987), plasminogen activator inhibitor 1 (Strandberg et al., 1988) and plasminogen activator inhibitor 2 (Ye et al., 1989), as well as between mouse or guinea pig contrapsins (Suzuki et al., 1990, 1991) and rat contrapsin (Ohkubo et al., 1991), have been elucidated. All these serpins are plasma proteins and are synthesized and secreted primarily by hepatocytes (for references, see Koj et al., 1988). HLEI belongs to a new group of intracellular

serpins, and its abundance in circulating horse blood leucocytes suggests an important biological function. The recently cloned equivalent human intracellular inhibitor occurs in much smaller amounts (Remold-O'Donnell et al., 1992).

We have shown that both mature circulating horse blood leucocytes and bone marrow cells contain mRNA encoding HLEI (Figures 1 and 6). The 3′ untranslated region of the mRNA is 939 bases long and shows three potential polyadenylation signals at positions 1282–1288, 1421–1427 and 2096–2101. At least two sites (positions 1282–1288 and 2096–2101) seem to be used, since we found two cDNA clones which are polyadenylated at positions 1300 and 2119. Moreover, when a fragment of a cDNA clone was used as a probe for Northern blot analysis of RNA from horse leucocytes and bone marrow cells, two transcripts were detected in the latter case (Figure 6). The shorter transcript of approx. 1300 bases is detectable in bone marrow cells. This argues for a differential usage of polyadenylation signals. Furthermore, it appears that the 2.3 kb HLEI mRNA from horse leucocytes exhibits a somewhat lower electrophoretic mobility than the respective mRNA from bone marrow cells. A possible explanation would be a different length of the poly(A)$^+$ tail of the two mRNAs. Different mobilities of the principal HLEI mRNA species from leucocytes and bone marrow cells were observed in two independent experiments and thus should not be easily dismissed as electrophoretic artifacts, although the final proof of mRNA polymorphism can only be obtained after removal of the poly(A)$^+$ tails.

Preliminary studies on the tissue distribution of HLEI mRNA indicated that the inhibitor gene is expressed primarily in blood leucocytes and bone marrow cells, and only in trace amounts in some other horse tissues such as lung, liver and kidney (results not shown). Although it is tempting to speculate that HLEI mRNA in the lung has some physiological significance for the protection of this tissue against elastase, this final conclusion may be drawn only after demonstration of actual synthesis of HLEI protein in horse lungs.

Molecular cloning of the HLEI cDNA enabled us to establish its nucleotide sequence. The 5′ untranslated region contains a Kozak-like sequence (Kozak, 1987) at positions 36–44 with a start codon. A second Kozak-like sequence is found about 100 bases downstream, and since it contains an in-frame ATG codon we speculate that both sites can be used as transcription start points. The data from *in vitro* translation (Figure 2, two bands at approx. 40 and 43 kDa) support this idea, but partial proteolytic degradation cannot be excluded.

Analysis of the HLEI cDNA sequence shows the absence of a signal peptide. This is in agreement with the finding that HLEI is a cytosolic protein and cannot be secreted from viable cells (Dubin and Koj, 1986). The encoded protein has 379 amino acids and possesses only one cysteine residue. Thus it is unable to form an intramolecular disulphide bridge, but the formation of intermolecular dimers cannot be ruled out. The predicted amino acid sequence is in complete agreement with the results obtained by Edman degradation (Dubin et al., 1992).

Comparison of the nucleotide sequences of HLEI and human elastase inhibitor (Remold-O'Donnell et al., 1992) indicates 85.2% identity of the amino acid coding sequence, whereas at the level of the amino acid sequence itself, identity is 82.4%. A striking similarity has been found in the C-terminal portion starting from the P_1' (Met) residue: the next 7 amino acid residues are identical in both inhibitors. On the other hand, the residues P_1–P_5 are completely different, and this may influence the specificities of the horse and human inhibitors.

Southern blot analysis of horse genomic DNA led us to the conclusion that HLEI is encoded by a single-copy gene (Figure 5). Further analysis of the intron–exon structure and the organization of the promoter region are necessary in order to understand the regulation of HLEI synthesis.

Expression of HLEI cDNA in *E. coli* led to the accumulation of large amounts of the protein both in the cytoplasm of bacteria and in the inclusion bodies (Figure 7). However, the concentration of HLEI appears to increase only slightly between 1 and 24 h of culture after induction with IPTG. Significantly, the expressed protein acts as an elastase inhibitor but is unable to inhibit trypsin (Figure 8). This is in a good agreement with data obtained with HLEI purified from leucocytes (Potempa et al., 1988).

Cloning and expression of the recombinant HLEI suggests several possibilities for the application of this inhibitor in the treatment of horse lung emphysema. However, this will only be possible after large-scale purification, and may require the use of a more efficient expression vector than pKK233-2. Alternatively, it might prove to be very interesting to introduce HLEI cDNA with a viral vector into the cells of the respiratory tract, as has been suggested recently by Rosenfeld and co-workers (1991) in the case of the α_1-antitrypsin gene.

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