Molecular Cloning, Expression, and Partial Characterization of Two Novel Members of the Ovalbumin Family of Serine Proteinase Inhibitors*

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A human placental agt11 cDNA library was screened for sequences encoding proteins related to human proteinase inhibitor 6 (PI6), and two plaques were identified that displayed weak hybridization at high stringency. Isolation and characterization of the DNA inserts revealed two novel sequences encoding proteins composed of 376 and 374 amino acids with predicted molecular masses of ~42 kDa. The novel proteins displayed all of the structural features unique to the ovalbumin family of intracellular serpins including the apparent absence of a cleavable N-terminal signal sequence. The degree of amino acid sequence identity between the novel serpins and PI6 (63-68%) significantly exceeds that of any other combination of known intracellular serpins. The two novel serpins encoded by the two novel cDNA sequences have been designated as proteinase inhibitor 8 (PI8) and proteinase inhibitor 9 (PI9). The putative reactive center P1–P1* residues for PI8 and PI9 were identified as Arg339–Cys340 and Glu340–Cys341, respectively. PI9 appears to be unique in that it is the first serpin identified with an acidic residue in the reactive center position. In addition, the reactive center loop of PI9 exhibits 54% identity with residues found in the reactive center loop of the cowpox virus CrmA serpin. Two PI8 transcripts of 1.4 kilobases (kb) and 3.8 kb were detected by Northern analysis in equal and greatest abundance in liver and lung, while the 1.4-kb mRNA was in excess over the 3.8-kb mRNA in skeletal muscle and heart. Two PI9 transcripts of 3.4 and 4.4 kb were detected in equal and greatest abundance in lung and placenta and were weakly detected in all other tissues.

PI8 and PI9 were expressed in baby hamster kidney and yeast cells, respectively. Immunoblot analyses using rabbit anti-PI6 IgG indicated the presence of PI8 in the cytosolic fraction of stably transfected cells that formed an SDS-stable 67-kDa complex with human thrombin. PI9 was purified to homogeneity from the yeast cell lysate by a combination of heparin-agarose chromatography and Mono Q fast protein liquid chromatography and migrated as a single band in SDS-polyacrylamide gel electrophoresis with an apparent molecular mass of 42 kDa. Purified recombinant PI9 failed to inhibit the amidolytic activities of trypsin, papain, thrombin, or Staphylococcus aureus endoproteinase Glu-C and did not form an SDS-stable complex when incubated with thrombin. The cognate intracellular proteinases that interact with PI8 and PI9 are unknown.

The mammalian serine proteinase inhibitors, or serpins, are a superfamily of single chain proteins that typically range between 40 and 60 kDa in molecular mass, resemble α1-proteinase inhibitor in overall structure, and include antithrombin III, plasminogen activator inhibitors 1 and 2, α1-antichymotrypsin, and α2-antiplasmin (1). The majority of serpins participate in the regulation of several proteinase-activated physiological processes including blood coagulation, fibrinolysis, complement activation, extracellular matrix turnover, cell migration, and prohormone activation, to name a few (2). Serpins inhibit proteolytic events by forming a 1:1 stoichiometric complex with the active site of their cognate proteinases, which is resistant to denaturants (3).

In addition to the mammalian serpins, several viral serpins have been identified and implicated as virulent factors. These serpins include the SERP-1 gene product produced by tumorigenic myxoma virus (4) and the CrmA serpin produced by the cowpox virus (5). Insight into the mode of action of these viral serpins has been derived from the findings that the SERP-1 gene product inhibits the serine proteinases of the fibrinolytic system and also inhibits C1 esterase, the first enzyme in the complement cascade (4). Recently, CrmA was found to attenuate the host inflammatory response by acting as a specific inhibitor of the interleukin-1β converting enzyme (ICE) (6), a novel cytosolic cysteine proteinase (5). These findings are significant since they suggest that the proteinase specificity of mammalian serpins may extend beyond serine proteinases and include some cysteine proteinases. In this regard, an α1-antichymotrypsin-like serpin was recently purified from bovine chromaffin granules of adrenal medulla and found to inhibit α
novel cysteine proteinase involved in enkephalin precursor processing (6).

Aside from the serpins that regulate proteinase activity, several members of this superfamily lack a proteinase inhibitory capability and have other physiological roles. These latter serpins were originally identified by data base searching and include thyroxine-binding globulin (7), angiotensinogen (8), and ovalbumin (9). Ovalbumin represents the parent prototype of a unique family of serpins, within the serpin superfamily, that lack a typical amino-terminal cleavable signal sequence but have been found to reside intracellularly, extracellularly, or both (10). Therefore, members of the ovalbumin serpin family may function as dualistic molecules with an intracellular and/or extracellular function. The serpins previously classified as members of the ovalbumin family are plasminogen activator inhibitor-2 (PAI-2) (11), an elastase inhibitor (EI) isolated from monocyte-like cells (12, 13), a squamous cell carcinoma antigen (SCCA) (14), maspin (15), and a novel cytoplasmic serpin isolated in this laboratory and designated as cytoplasmic antiproteinase (16, 17). A functionally inactive form of cytoplasmic antiproteinase was purified earlier from human placenta and designated as placental thrombin inhibitor or PTI (18). Recently, the Genome Database organization has recommended that this serpin be designated as proteinase inhibitor 6, or PI6 (19). Purified PI6 inhibits the amidolytic activities of a broad range of proteinases, including trypsin, urokinase, and factor Xa (16). In addition, trypsin (19). Purified PI6 inhibits the amidolytic activities of a broad spectrum of trypsin-like serine proteinases including thrombin, trypsin, urokinase, and factor Xa (16). In addition, trypsin inhibition by PI6 appears to involve a two-step mechanism that results in the formation of a tight inhibitory complex that is pseudoreversible and behaves similar to the proteinase inhibitor complex formed with α₂-antiplasmin (17). In the present study, we report the molecular cloning, expression, and partial characterization of two novel human PI6 homologs that are divergent within their reactive centers and are predicted to inhibit distinct proteinases. In addition, the reactive center loop of one novel PI6 homolog exhibits a high degree of structural similarity to the serpin encoded by the cDNA gene carried by the cowpox virus. These two novel PI6 homologs have been designated as protease inhibitor 8 (PI8) and proteinase inhibitor 9 (PI9) as recommended by the Genome Database nomenclature committee.

**EXPERIMENTAL PROCEDURES**

Materials—Penicillín-streptomycin-neomycin, methotrexate, ampicillin, dithiothreitol, and N′-benzoyl-ξ-arginine p-nitroanilide were obtained from Sigma. o-Val-Leu-Lys-p-nitroanilide and Phe-Pip-Arg-p-nitroanilide were obtained from Helena Laboratories. Carbobenzoxy-ξ-Phe-Leu-Glu-p-nitroanilide was a product of Bodringer Mannheim. Prestained SDS-PAGE standards (low range) were from Bio-Rad. Natriumcellulose-pamáginas were purchased from Schleicher and Schuell. Tissue culture T-75 flasks were obtained from Corning. Dulbecco's modified Eagle's medium was a product of Mediatech. Fetal bovine serum was obtained from Hyclone Laboratories. 125I-labeled protein A and B. The lysate was centrifuged, and the supernatant was applied to a column (1.6 cm × 8 cm) of Bio-Gel P-100 column buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM isopropyl-1-thio-D-galactopyranoside. Cells containing plasmid pBB/P/PI6 fusion protein were grown to 5 × 10^10 cells/ml at 37°C with shaking in LB/glucomann-aminocillin for 5 h. Isopropylnitroanilide was then added to a final concentration of 0.1 mM, and the culture was incubated with vigorous shaking at 22°C for an additional 24 h. The cells were harvested by low speed centrifugation, resuspended in 20 mM Heps (pH 7.5), containing 200 mM NaCl, 1 mM EDTA, 6 mM mercaptoethanol, 5 mM phenylmethylsulfonyl fluoride and 0.02% NaN₃ (column buffer), and lysed by sonication. Cell debris was removed by high speed centrifugation, and the supernatant was applied directly to a column of amylose resin (1.6 × 8 cm) pre-equilibrated at 4°C with column buffer. Following sample application and wash, the MBB/P/PI6 fusion protein was eluted from the affinity resin in column buffer containing 10 mM maltose. Examination of the maltose eluent by SDS-PAGE indicated a prominent 78-kDa protein that was immunoreactive to commercially available rabbit anti-MBP IgG. Antibodies against the MBB/P/PI6 fusion protein were generated in rabbits (23), and the IgG fraction was purified by protein A-Sepharose column chromatography. This IgG fraction was further purified by passage through a MBB-Affi-Gel 15 column to remove anti-MBP IgG. Cell Expression—The full length cDNA for P18 was directionally cloned into expression vector Zem2292 and expressed in baby hamster kidney (BHK) cells (24). BHK cells transfected with “empty” Zem2292 expression plasmid (P18-free) served as control cells in this study. BHK/PI8 and BHK/Zem 2292 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin-streptomycin-neomycin, and 1 μg/mal neotrexate. PI9 was expressed in yeast as follows. An expression plasmid, P9/pDPOT, was created by cloning the cDNA for P9, along with DNA fragments for the AdH24c promoter and the TP11 terminator, into the yeast shuttle plasmid pDPOT. Spheroplasts from the S. cerevisiae strain ZM118 were transformed separately with either P9/pDPOT or pDPOT (P9-free) and selected for growth on glucose medium. Transformants were grown in SD medium, 1% yeast extract, 1.5% glucose, and 1.5% ethanol at 30°C for 3 days (25).

Preparation of BHK Cell Cytosol—The cytosolic fractions of BHK/PI8 and BHK/Zem 2292 cells were prepared essentially as described (26). Briefly, each cell line in a T-75 flask was washed from the flask by brief trypsinization followed by centrifugation (1500 rpm; 7 min; 25°C). The cells (~10^8 cells) were resuspended in 5 ml of 20 mM Heps (pH 7.5) containing 150 mM NaCl, recrystalized, and resuspended in 1 ml of 20 mM Heps (pH 7.5) containing 1 mM DTT. Cells were then resuspended by six sequential passages through a 25-gauge 1.58-cm needle, and the needle effluent was collected directly into thick walled polycarbonate ultracentrifuge tubes placed on ice. The cytosolic fractions were harvested by low-speed centrifugation and stored at -80°C. The concentration of the cell lysate (100,000 × g for 30 min; 4°C) in a Beckman ultra-centrifuge (model L-75) and subsequently reconstituted with 0.15 mM NaCl.

Preparation of Recombinant P19—Yeasts cells transformed with the P19/pDPOT were suspended in lysis buffer (10 mM Tris-HCl (pH 7.4) containing 2 mM EDTA, 50 mM NaCl, 43 μM phenylmethylsulfonyl fluoride and 1 mM leupeptin) and lysed by glass beads and vortexing. The lysate was centrifuged, and the supernatant was dialyzed at 4°C against 50 mM Heps (pH 7.0) containing 1 mM DTT. The retentate (~30 ml) was applied to a column (1.6 × 20 cm) of heparin-agarose equilibrated at 4°C with 50 mM Heps (pH 7.0) containing 1 mM DTT at a flow rate of 0.5 ml/min. The column was washed with equilibrating buffer, and P19 eluted from the column in a linear NaCl gradient (0–0.5 M) dissolved in equilibrating buffer. P19-containing fractions from the heparin-agarose column were pooled and dialyzed at 4°C against 25 mM Tris-HCl (pH 8.0) containing 1 mM DTT.
The dialyzed sample was concentrated by ultrafiltration to 5 mM lan and applied to a Mono Q HR5/5 column equilibrated at room temperature with 25 mM Tris-HCl (pH 8), 1 mM DTT. PI9 was eluted from the column in a linear gradient of NaCl (0–1 M) in equilibrating buffer. Fractions eluted from the Mono Q column were subjected to SDS-PAGE, and pure fractions were pooled and stored at -20°C.

Northern Analysis—A multiple tissue blot of poly(A)+ mRNA from human tissues (Clontech Laboratories) was probed with a 39-mer oligonucleotide corresponding to the reactive center of PI9, 5'-AGATTCCATGCAGCACTCTGCAACTACAAAGCAGCTG-3' (PI9). The oligonucleotide probe was 5'-labeled with [γ-32P]ATP (Dupont NEN) using T4 polynucleotide kinase (Promega) to yield a specific activity of 1–2 x 10^8 cpm/µg. Hybridization was performed at 55°C in 3×SSPE, 2×Denhardt’s, 0.5% SDS, 100 µg/ml salmon sperm DNA. The blot was washed at 57°C in 2×SSC, 0.1% SDS and exposed to autoradiography. Northern blot analysis of PI8 mRNA was obtained by probing filters with a 1.1-kb DNA fragment generated by polymerase chain reaction using the following primers: 5'-GAGCATCTCCTCTCGCCCCGG-3' and 5'-CAAGCCACTGCACCACTAG-3'. The 1.1-kb fragment was 32P-labeled by random priming, and hybridization was performed at 65°C in ExpressHyb (Clontech Laboratories) solution. The filter was washed at 50°C in 0.1×SSC, 0.1% SDS and exposed to autoradiography. Higher stringency washes (65°C in 0.2×SSC, 0.1% SDS) gave an identical tissue distribution.

Inhibition Assays—Samples of PI8 (cytosolic fraction) and purified preparations of recombinant PI9 were tested for trypsin inhibitory activity essentially as described for tissue factor pathway inhibitor-2 (26). In some experiments, cytosolic fractions were preincubated for 16 h at 37°C with either preimmune rabbit IgG (1 mg/ml final concentration) or rabbit anti-PI6 IgG (1 mg/ml final concentration) prior to incubation with trypsin. PI9 was also tested for its ability to inhibit the amidolytic activities of papain (27), S. aureus endoproteinase Glu-C (28), and human thrombin (26) essentially according to published methods using Nα-benzoyl-DL-arginine p-nitroanilide, Z-Phe-Leu-Glu-p-nitroanilide, and Phe-Pip-Arg-p-nitroanilide (S-2238), respectively, as substrates.

RESULTS

Molecular Cloning and Nucleotide Sequencing of Two Novel Intracellular Serpins—In previous studies, PI6 was identified and subsequently isolated in a functionally active form from the cytosolic fraction of a monkey kidney epithelial cell line, BSC-1 (16), as well as human placenta (17). Cloning and sequencing of the cDNA encoding human placental PI6 revealed that it was a new member of the serpin superfamily and showed greatest sequence identity with other members of the ovalbumin branch of intracellular serpins (17). In an effort to identify additional relatives of PI6, a human λgt11 placental cDNA library was screened with a 209-base pair polymerase chain reaction-generated 32P-labeled probe corresponding to the codons of amino acid residues 67–149 of PI6. Several plaques were identified that showed weak hybridization with the probe at high stringency, suggesting the existence of cDNAs encoding proteins related to PI6. Several of these plaques were isolated and their inserts subjected to DNA sequence analysis. Two of these clones, designated as H2–2-11 and H3–1-11, contained inserts with open reading frames encoding proteins of 376 and 374 amino acids, respectively (Fig. 1A and B). Both proteins have predicted molecular masses of approximately 42 kDa. The 5'-regions of both cDNAs contain a Kozak consensus sequence (29) between nucleotide bases 109–115 of H2–2-11 and 89–95 of H3–1-11 that include an in-frame initiation codon. A second Kozak sequence also exists 117 nucleotide bases downstream of the first initiation codon and...
includesthecodonforMet41ofbothproteins. Theimportanceof
these alternative translational start sites and the extent of
theirutilizationinvisouisunknown. The 3′-untranslatedregion
of the H3–1-11 cDNA contains an AATAAA consensus se-
quence located 99 nucleotide bases downstream of the termi-
nationcodon for nascent mRNA cleavage and polyadenylation
(Fig.1A)(30). However, a polyadenylation consensussequence
was not found in the 3′-untranslated region of the H2–2-11
cDNA after sequencing 151 nucleotides downstream from the
translational termination codon (Fig. 1B), suggesting that the
nucleotide sequence of the H2–2-11 3′-untranslated region is
incomplete.

A computer search of the NBRF protein data base revealed
that the proteins encoded by the H2–2-11 and H3–1-11 cDNAs
were novel but showed considerable amino acid sequence iden-
tity with members of the ovalbumin branch of the serpin su-
perfamily of proteinase inhibitors. The H3–1-11 and H2–2-11-
derived amino acid sequences showed 68 and 63% identity with
PI-6, respectively. We have provisionally designated the pro-
teinsencodedbytheH3–1-11andH2–2-11cDNAsasPI8and
PI9, respectively, according to recommendations provided by
the Genome Database nomenclature committee. Similar to PI6,
PI8andPI9exhibitahighdegreeofaminoacidsequence
identity to other human members of the ovalbumin family of
cytoplasmic antiproteinases including PI1 (PI8, 51% PI9, 49%), PAI-2
(PI8, 46%; PI9, 45%), and SCMA (PI8, 46%; PI9, 45%). In
addition, PI8 showed 63% amino acid sequence identity to PI9. The
two novel cytoplasmic antiproteinases exhibit all the structural
features previously demonstrated to be unique to the
ovalbumin family of serpins that can be summarized as follows
(10): (a) PI8 and PI9 lack an N-terminal extension, and the
open reading frame begins at amino acid residue 23 of
\( a^\)I-proteinase inhibitor; (b) PI8 and PI9 lack a C-terminal exten-
sion terminating at Pro376, the equivalent of Pro391 in
\( a^\)I-proteinase inhibitor; (c) PI8 and PI9 both have a Ser at position
375 of \( a^\)I-proteinase inhibitor in place of a highly conserved
Asn found among serpins distantly related to the ovalbumin
family; and (d) PI8 and PI9 appear to lack a typical N-terminal
deletable signal sequence. The new cytoplasmic antiprotein-
ases also have a potential N-glycosylation consensus sequence
(NX(T/S)) at Asn6 and Asn78 of PI8 and Asn6 and Asn23 of PI9
(Fig. 1, A and B).

Alignment of the deduced primary structure of PI8 and PI9
with the amino acid sequences of PI6 (Fig. 2) and other human
members of the ovalbumin serpin family (data not shown) identified the putative reactive center P_1–P_1^\text{res} residues of PI8 as Arg^{339}, Cys^{340}, respectively, which are identical to PI6. However, the regions flanking this P_1–P_1^\text{res} residues in PI6 and PI8 are highly divergent. The P_2–P_6 residues of PI6 and PI8 show no identity, while Arg^{342} in the P_3^\text{res} position was conserved in both serpins. Since residues in the vicinity of P_1 have been previously shown to influence both protease target specificity and the inhibitory potency of several serpins (31), these findings suggest that PI6 and PI8 interact with the active sites of distinct cognate proteases that have trypsin-like substrate specificity. In contrast, alignment of the PI9 amino acid sequence identified the putative P_1–P_1^\text{res} residues as Glu^{340}, Cys^{341}, respectively. The identification of an acidic P_1^\text{res} residue in the PI9 reactive center is unique to the human serpin superfamily. The only other serpins identified with an acidic P_1^\text{res} residue in their reactive centers are CrmA, EI, SCCA, and PAI-2, ranging from 0 to 7%. A noteworthy feature characteristic of only the cytoplasmic antiproteinase homologs and CrmA is the presence of a conserved Cys residue in the reactive center P_1^\text{res} positions. Since the reactive center has been previously demonstrated to be the most divergent domain of the serpin superfAMILY (34), the cowpox virus may have acquired a mammalian intracellular serpin gene and the reactive center of the viral serpin either converged or remained relatively conserved with the reactive center of PI9.

Expression, Purification, and Partial Characterization of PI8 and PI9—The full-length cDNAs for PI8 and PI9 were ligated into mammalian cell and yeast expression vectors and expressed in BHK cells and yeast cells, respectively, as described above. Using a rabbit anti-PI6 IgG preparation, an immunoreactive doublet that migrated with an apparent M_\text{r} of 42–44 kDa was detected in the BHK/PI8 cell cytosolic fraction by Western blotting (Fig. 4). The immunoreactive doublet was consistently observed in several BHK/PI8 cytosolic fractions with varying intensity of the lower M_\text{r} band ranging from ~20 to 50% of the doublet. Although not investigated further, the duplicity of the putative PI8 may be due to proteolytic cleavage during the preparation of the cytosol. A faint immunoreactive band that migrated at ~42 kDa was also detected in the cytosolic fraction derived from BHK/Zem 229R cells that served as a control (data not shown). The cytosolic fraction of the BHK/PI8 cells inhibited the amidolytic activity of trypsin, thrombin, and S. aureus endoproteinase Glu-C (33).

These observations prompted us to determine the overall structural relatedness of the cytoplasmic antiproteinases and the crmA protein relative to other intracellular serpins. Previous studies have reported ~30% amino acid sequence identity between CrmA and several extracellular serpins including antithrombin III, human and murine α₂-antichymotrypsin, human and murine α₂-proteinase inhibitor, and human heparin cofactor II (32). However, to our knowledge, no study has compared the primary structures of CrmA and recently discovered members of the mammalian intracellular serpins. By employing the NBRF program ALIGN, CrmA was found to share ~39% amino acid sequence identity with PI6, EI, and PAI-2, ~37% identity with PI8 and PI9, and ~35% identity with SCCA (data not shown). As a result, these intracellular serpins appear to represent the closest mammalian relatives of the viral serpin reported to date. These findings are consistent with the previous observation that CrmA lacks a cleavable N-terminal signal sequence and an N-terminal extension common to the ovalbumin family of intracellular serpins (10). In addition, a comparison of the CrmA reactive center loop with the reactive center loops of the mammalian intracellular serpin family revealed a remarkable degree of structural conservation with PI9 (Fig. 3). The amino acid sequence of the PI9 reactive center loop shares ~54% of the structurally conserved residues found in the reactive center loop of CrmA. An insignificant degree of structural conservation is found between the amino acid residues in the reactive centers of CrmA, EI, SCCA, and PAI-2, ranging from 0 to 7%. A noteworthy feature characteristic of only the cytoplasmic antiproteinase homologs and CrmA is the presence of a conserved Cys residue in the reactive center P_1^\text{res} positions. Since the reactive center has been previously demonstrated to be the most divergent domain of the serpin superfamily (34), the cowpox virus may have acquired a mammalian intracellular serpin gene and the reactive center of the viral serpin either converged or remained relatively conserved with the reactive center of PI9.

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Identification of Two Novel Ovalbumin-type Serpins

Fig. 5. Expression and purification of recombinant PI9 from yeast. Aliquots of PI9 fractions from various stages of purification were subjected to 12% SDS-PAGE under reducing conditions. Lane 1, 100 μg of reduced PI9/pDPOT cell lysate; lane 2, 20 μg of reduced heparinagarose PI9 pool; lane 3, 15 μg of reduced PI9 Mono Q pool; lane 4, mixture of reduced standard proteins including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

with the trypsin inhibition data, incubation of the BHK/Zem 229R cytosolic fraction with 125I-labeled thrombin also resulted in a 125I-labeled 67-kDa SDS-stable complex that appeared to be ~10% of that observed in the BHK/PI8 cytosol (data not shown). In addition, incubation of the BHK/PI8 and BHK/Zem 229R cytosolic fractions with anti-PI6 IgG completely blocked formation of the 125I-labeled 67-kDa SDS-stable complex (data not shown). Collectively, the above observations provide evidence for the synthesis of PI8 following transfection of BHK cells with PI8 cDNA based on (a) its cross-reaction with anti-PI6 IgG, (b) its enhanced ability relative to mock-transfected cells to inhibit trypsin amidolytic activity, and (c) its enhanced ability to form an SDS-stable, 67-kDa complex with thrombin. Our data also suggest the low level, constitutive synthesis of hamster PI6 and/or PI8 by the BHK cells that apparently cross-react with rabbit anti-human PI6 IgG.

Recombinant PI9 was purified to homogeneity from transformed yeast cell lysates in a two-step procedure using heparinagarose column chromatography and Mono Q fast protein liquid chromatography. Initial experiments indicated that ~20% of the total protein in the transformed yeast cell lysate migrated in SDS-PAGE with a molecular mass of ~42 kDa (Fig. 5). In contrast, this protein was not observed in the mock-transfected yeast cell lysate by this technique (data not shown), suggesting that the 42-kDa protein was PI9. In the absence of a functional assay for PI9, the presence of high expression levels of the putative PI9 in the yeast cell lysates facilitated identification of this protein in column eluents by SDS-PAGE. As seen in Fig. 5, the PI9 pool from heparin-agarose was ~90% pure, while the PI9 derived from Mono Q fast protein liquid chromatography migrated as a single band in SDS-PAGE with an apparent molecular mass of 42 kDa (Fig. 5). Amino-terminal amino acid sequence analysis of the purified, putative PI9 indicated that the protein, like PI6 (16), was derivitized at the amino terminus. Treatment of the protein with methanolic HCl failed to release a potential N-formyl group, suggesting that the protein was acetylated at the N terminus. In order to demonstrate that the isolated protein was indeed PI9, the purified protein was cleaved with trypsin and the tryptic digest fractionated on a C4 reverse phase HPLC column. One peptide (T28) was isolated and yielded an amino-terminal sequence of LAHVGEV, which is identical to that observed in the PI9 deduced sequence at residues 206–212, thus confirming the identity of the isolated protein as PI9.

The purified recombinant PI9 was then tested for its ability to inhibit the amidolytic activities of trypsin and papain toward S-2251 and N'-benzoyl-ol-arginine p-nitroanilide, respectively. At a 500:1 inhibitor:enzyme molar ratio, PI9 failed to inhibit the amidolytic activities of these proteases under conditions where trypsin and papain amidolytic activities were completely inhibited by a 10-fold molar excess of soybean trypsin inhibitor and egg white cystatin, respectively (data not shown). In addition, purified PI9, at a 500:1 inhibitor:enzyme molar ratio, failed to inhibit the amidolytic activities of human thrombin and S. aureus endoproteinase Glu-C and did not form a high M complex with human thrombin as judged by Western blotting using the rabbit anti-PI6 IgG to detect PI9 (Fig. 4).

Northern Analysis—To identify transcripts that encode PI9 and PI9 and determine their human tissue distribution, radio-labeled probes were used to hybridize immobilized poly(A)+ mRNA by Northern analysis. Northern analysis with a PI8 32P-labeled 1.1-kb probe revealed two transcripts of 1.4 and 3.8 kb (Fig. 6A). Both transcripts were equally abundant in all tissues except in skeletal muscle and heart, where the 1.4-kb transcript exceeded the levels of the 3.8-kb transcript. The transcripts encoding PI9 were detected at the greatest levels in skeletal muscle, liver, lung, and placenta. Two mRNA species of 3.4 and 4.4 kb were detected with a PI9 reactive center oligonucleotide probe (Fig. 6B). Both mRNA species encoding the PI9 reactive center were detected at the highest levels in placenta and lung and were weakly detected in all tissues examined. In addition, two minor PI9 mRNA species of ~7.5–8.0 kb were also detected in placenta. The hybridization of the Northern blots was of sufficient stringency to preclude hybridization of inexact nucleotide matches, eliminating the possibility of nonspecific hybridization. Our previous studies identified a single PI6 transcript of 1.4 kb that was expressed in all tissues except brain and detected in greatest abundance in skeletal muscle and placenta. Therefore, the transcripts encoding the cytoplasmic antiproteinases appear to have an overlap.
ting but differential tissue distribution with P18- and P19-related proteins encoded by multiple transcripts.

DISCUSSION

In the present study, we have cloned and sequenced two human cDNAs encoding novel proteins that exhibit all the structural features characteristic of the ovalbumin branch of the serpin superfamily. In particular, the new serpins showed greatest amino acid sequence identity with a recently discovered member of the ovalbumin serpins that has been designated by the Genome Database organization as proteinase inhibitor 6, or PI6. The extent of amino acid sequence identity between the new serpins and PI6 (63 or 68%) significantly exceeds that reported for any other combination of the ovalbumin serpin family members, which typically range between 45 and 50% (10). The new serpins have been designated at P18 and P19 according to recommendations made by the Genome Database nomenclature committee. Based on primary structure identity and the presence of a unique Cys residue conserved in the reactive center P1 position of the cytoplasmic antiproteinases, these serpins appear to represent a distinct subfamily within the ovalbumin branch of the serpin superfamily. Since serpins are generally classified by the reactive center P1 specificity residue (1, 2), P18 appears to be an Arg-serpin and, like PI6, inhibits trypsin amidolytic activity and forms an SDS-stable complex with human thrombin. On the other hand, P19 was found to be unique in that it is the first human serpin identified with an acidic residue in the reactive center P1 position and has been classified as a Glu-serpin. PI6 has been previously shown to function as a proteinase inhibitor of several prototypical serine proteinases (16, 17), and P18 and P19 both show complete conservation of the PI6 reactive center hinge region that conforms to the structural motif P17EEGTEAAAATP8 recently identified in the majority of inhibitory serpins (35). Serpins that carry unconserved mutations in the reactive center hinge region typically lack serine proteinase inhibitory activity since steric hindrance impedes partial insertion of the hinge peptide into the antiparallel A-B sheet (36, 37), which normally provides a source of favorable interactions that contribute to the overall stability of the proteinase-serpin inhibitory complex. Therefore, since the reactive center hinge region sequence of P19 conforms precisely with the hinge sequences of other inhibitory serpins, this novel cytoplasmic antiproteinase is likely to function as active site-directed proteinase inhibitor.

The novel cytoplasmic antiproteinases displayed all of the structural characteristics common to the mammalian serpins of the ovalbumin family, including the apparent absence of a typical N-terminal cleavable signal sequence (10). These findings suggest that, like PI6 (16, 17, 38), El (12, 13), PAI-2 (39–41), and SCCA (14), the new serpins also reside in the cytoplasm of cells. Nonetheless, similar to other members of the ovalbumin serpin family, P18 and P19 have consensus sites for the potential attachment of an N-linked carbohydrate. Therefore, the possibility that PI8 and P19 are secreted and function in the extracellular milieu under certain conditions cannot be ruled out at present. Previous studies have demonstrated that PAI-2 (40, 41) and SCCA (14) can exist intracellularly and/or extracellularly. For example, PAI-2 has been detected predominately in the cytosolic fraction of resting monocytes as an unglycosylated functionally active inhibitor of urokinase (40, 41). Upon activation of the monocytes with phorbol esters, the majority of intracellular PAI-2 is efficiently glycosylated and secreted (40, 41). Consequently, the degree of glycosylation has no effect on the inhibition kinetics of urokinase by PAI-2 (40). Likewise, SCCA has been reported to be localized in the cytoplasm of normal squamous epithelial cells while the corresponding carcinoma cells secrete a glycosylated form of SCCA (14), apparently in a manner similar to that described for PAI-2. To date, PI6 has been detected only in the cytosolic fraction of all cultured cells examined, and treatment of cells with phorbol esters had no effect on the intracellular localization of functional active PI6 (16, 18, 38). Given the high degree of amino acid sequence identity in regions outside the reactive centers of the cytoplasmic antiproteinases, PAI-8 and P19 may also behave like PI6 and be confined to the cytoplasm of cells.

To date, the only intracellular serpin with a defined intracellular proteinase target is the viral serpin encoded by the crmA gene of the cowpox virus (5). CrmA functions as a specific inhibitor of the ICE (5), which has recently been shown to represent a prototype of a larger family of ICE-like homologs (42–46). The ICE family of cysteine proteinases have been intimately linked to both the negative and positive regulation of apoptosis (44). Mammalian intracellular inhibitors of these cysteine proteinases have not been identified. In the present study, we have made the previously unreported observation that CrmA shows the greatest degree of amino acid sequence identity to the mammalian intracellular serpins of the ovalbumin family, suggesting a possible origin of the crmA gene. In addition, the reactive center of CrmA shows considerable structural similarity to the reactive center of PI9, including a conserved Asp to Glu switch in the P1 specificity site. Moreover, all of the cytoplasmic antiproteinases have a unique Cys residue conserved in the P1 position and found only in the corresponding position of CrmA. CrmA has been shown recently to form a noncovalent tight inhibitory complex with ICE (47), suggesting that the geometric compatibility between serpins and some proteinases is important in determining inhibitory potency. Therefore, since the cytoplasmic antiproteinases are the only mammalian serpins with a Cys residue in the P1 position, they may regulate proteinases by a mechanism analogous to CrmA.

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