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The Expression and Characterization of Five Recombinant Murine α1-Protease Inhibitor Proteins

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The *Mus musculus* α1-protease inhibitor gene cluster encodes five highly related proteins. The most significant amino acid polymorphisms lie within the reactive-site loop which is important in determining serpin substrate specificity. All five genes are transcribed in *M. mucsculus* adult liver and presumably secreted into plasma. In an attempt to characterize their protein products all five cDNAs were expressed in recombinant mammalian cells and the protease inhibition activity of each determined. Only two of the proteins were efficient inhibitors of neutrophil elastase, the major physiological target of the sole human α1-protease inhibitor (antitrypsin). Four of the proteins were active against chymotrypsin, while no substrate could be identified for the fifth.

α1-Protease inhibitor (α1-PI, also known as α1-antitrypsin) and contrapsin, the major plasma serine protease inhibitors (serpins) in *M. musculus,* are encoded by two neighbouring multigene families on chromosome 12 (1,2). α1-PI inhibits the activity of a number of serine proteases, elastase and trypsin in man (3) and elastase and chymotrypsin in mouse (4). However, its primary physiological role is in the regulation of neutrophil elastase, and in humans α1-PI deficiency leads to abnormal degradation of connective tissue, manifesting as emphysema and other conditions (3). Murine contrapsins are homologous to human α1-antichymotrypsin but have been shown to inhibit trypsin-like enzymes in mouse (4). The diverse physiological roles of α1-antichymotrypsin in man have not been fully elucidated. Multigene α1-PI and α1-antichymotrypsin families seem to have arisen in a number of genera, but not man. Rabbits have at least four α1-PI genes, encoding proteins with different spectra of inhibition (5) whilst goats encode at least two α1-antichymotrypsin genes one of which inhibits elastase whilst the other inhibits trypsin (6). Thus the substrate specificity of these two related families of serpins seems to have evolved in a complex manner, and a major determinant of substrate specificity has been shown to reside in the amino acids of the reactive-site loop of the serpin which interact irreversibly with the protease (3,7).

Five extremely related α1-PI cDNAs have been isolated from adult *M. musculus* liver, with most of the amino acid differences between the translated polypeptides being clustered around the predicted reactive site (8). Indeed the reactive site methionine at the site of protease cleavage, P_1, which is essential for human α1-PI anti-elastase activity is substituted by tyrosine or leucine in three of the five murine α1-PI protein sequences. Thus it could be predicted that the five enzymes differ in their protease substrate specificity.

In order to investigate which of the five cDNAs encode functional anti-elastase activity each has been expressed in mammalian cells and their protease inhibition spectra assayed.

MATERIALS AND METHODS

Construction of plasmids expressing α1-PI genes 1 to 5. The five *M. musculus* α1-PI cDNA clones amplified from C57BL/6 mouse liver were generously supplied by Professor K.Krauter (8). These 5′ truncated cDNAs were initially cloned into pCDNA3 (Invitrogen). The 5′ sequences of each gene were reconstructed by pcr using the intact α1-P14 gene as

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template, so that each gene shares identical mRNA leader and signal peptide sequences and possesses a unique ATG start codon with optimized translation start signals (9) (plasmids pCDNA3PI1 to 5). This common signal peptide sequence varies only in the second residue from native α1-PI4, due to Kozak sequence optimization. In addition residue 13 of the mature protein is altered from Q to P in α1-PIs 1,2,3 and 5 due to the creation of a *Bam*HI site in the cDNA. Following all manipulations the DNA sequences of the 5′ end and exon 5 of each construct were determined to confirm the integrity and identity of each clone (Sequenase kit, Amersham).

In order to maximize expression in transient transfection experiments the adenovirus tripartite translational leader sequence was cloned from the plasmid pWS4 (10) and inserted downstream of the CMV promoter in pCDNA3 (plasmids pWSPI1 to 5).

Expression of recombinant proteins in transfected mammalian cells. Chinese hamster ovary (CHO-K1) cells (ATCC) and SV40 T antigen-transformed Green African monkey kidney COS-7 cells (ECACC) were routinely cultured in DMEM (GIBCO) and 10% Foetal Calf Serum (Globepharm, UK).

Cells were seeded at 10^6 per 25cm^2 flask and transfected 24h later by $CaPO_4$/Hepes precipitation of 5 or 10μg of DNA (11). In transient transfection experiments the medium was stringently washed off with PBS 6h post DNA addition, and the cells refed with protein free hybridoma medium (Sigma) to allow analysis of secreted recombinant protein. CHO cells transfected to produce stably transformed lines were maintained in 10% serum and refed again 48h post transfection with selective medium (1mg/ml Geneticin, GIBCO). Resistant lines were cloned after 2–3 weeks of selection by performing limiting dilution of pooled colonies.

Stably transformed cells were adapted to grow in protein free medium by gradual adaptation, or by selecting viable colonies after seeding cells directly into 0% serum in multiwell plates. Adapted cells were maintained on fibronectin coated flasks and dissociated for passaging with 0.01% trypsin (Sigma) which was inactivated with an equal molar amount of soybean trypsin inhibitor (Sigma).

Transgene expression in CHO cells was chemically induced by replacing the supernatant of barely confluent cell monolayers with medium containing 2.5mM sodium butyrate (12).

Analysis of recombinant proteins. Secreted recombinant α1-PI produced by stably transformed CHO cells and transiently transfected COS-7 cells were analysed in the culture supernatant by SDS PAGE and enzyme inhibition assay. Recombinant α1-PIs were identified after electrophoresis and western transfer by reaction to a polyclonal rabbit anti-mouse α1-antitrypsin serum (1:10000, Nordic) visualized using an alkaline phosphatase conjugated secondary antibody (Promega). Supernatants were occasionally concentrated 8–15 fold using Microsep centrifugal concentrators (Flowgen, UK) before assay.

Protease inhibition assays. Protease inhibition assays were based on Klumpp and Bieth (13) with modifications by O.Drummond (National Science Laboratory). Test samples were mixed with an equal volume of test protease in 0.2M TrisHCl pH8/0.1% BSA in microtitre plates for 10 minutes at 37C before addition of chromogenic substrate (peptide p-nitroanilide). Cleavage of substrate to generate p-nitroaniline was followed spectrophotometrically at 450nm over time. Inhibited protease activity was compared to a standard curve prepared using dilutions of purified human α1-PI (Sigma) mixed with the test protease. Protease and substrate concentrations were as follows (supplied by Sigma except where noted): porcine pancreatic elastase (0.1μM) with Suc-A-A-A-pNA (1.5mM); human neutrophil elastase (0.01μM) with MeOSuc-A-A-P-V-pNA (0.5mM, Calbiochem); bovine pancreatic chymotrypsin (0.04μM) with N-Suc-A-A-P-F-pNA (1.5mM); bovine pancreatic trypsin (0.16μM), porcine plasmin (0.05u/ml), bovine thrombin (1u/ml), human urokinase (0.2u/ml), human plasma kallikrein (0.025u/ml) all with S2288 (1mM, Chromogenix); bovine factor Xa (1u/ml) with S2222 (1mM, Chromogenix).

Protease binding assays. 30μl aliquots of cell culture supernatants were incubated with various dilutions of pancreatic elastase, trypsin or chymotrypsin (0.02–0.5μM) before loading on standard SDS 10% PAGE (without boiling) and analysed by western blotting.

RESULTS AND DISCUSSION

Expression of recombinant murine α1-PI genes in tissue culture. Each of the five recombinant proteins was initially cloned in the expression vector pCDNA3 which allows expression in a wide host range using the CMV IE promoter/enhancer, and also allows for plasmid amplification in COS-7 cells. This plasmid was improved by the addition of adenovirus translational signals from the vector pWS4 (10) which was found to dramatically increase transient gene expression. Whilst addition of an intron upstream of the cDNAs in pCDNA3 did increase expression somewhat, its effect was deleterious in pWSPI clones (results not shown).

As detailed in ‘Methods’ each construct shares identical transcription, translation and protein secretion signals and should therefore be expressed to similar levels. The supernatants from COS-7 cells transfected with each of these five WSPI plasmids were analyzed by SDS PAGE and western blotting using a polyclonal serum raised against mouse α1-PI and each of the five secreted recombinant proteins were detected, with the same mobility as the proteins in *M. musculus* plasma

(52kDa for the glycosylated protein) (Figure 1A). However, whilst proteins α1-PI1–4 were detected at levels similar to each other, a far lower level of α1-PI5 was detected. This result was reproducible both in transient and stable transfections of COS-7 and CHO cells also and may reflect either a real difference in the expression or stability of this protein, or a difference in the recognition of this protein by the polyclonal serum. In an attempt to address this concentrated supernatants were run on the Phastgel system (Pharmacia) and total proteins silver-stained. No quantitative differences between the five transfections were obvious, however, the band corresponding to α1-PI could not be unambiguously distinguished (results not shown).

CHO cells with stably integrated copies of the pCDNA3PI constructs were selected and cloned. In order to improve poor initial yields of α1-PI5, CHO cells were also transformed with pWSPI5. These cloned α1-PI secreting lines were adapted to grow permanently in serum free conditions to allow production and assay of recombinant α1-PI without interference from bovine α1-antitrypsin (Figure 1B).

Protease inhibition activity of recombinant murine α1-PI genes. The five recombinant α1-PIs were tested for ability to inhibit a number of potential target extracellular proteases. Culture supernatants from stably transformed CHO cells or transiently transfected COS-7 cells were preincubated with target protease prior to addition of chromogenic substrate, and inhibitory activity compared with that of human α1-PI (Tables 1 and 2). The results could not be quantitated because the level of recombinant protein present in the culture medium could not be directly measured. Results obtained from transiently transfected COS-7 cells were reasonably reproducible and the western blot analysis implied that at least α1-PIs1–4 were expressed at similar levels. The physiological target of human α1-PI, neutrophil elastase, is clearly inhibited by α1-PI1 and even more effectively by α1-PI2, as is the related enzyme, pancreatic elastase. α1-PI3 and α1-PI4 did not appear to inhibit neutrophil elastase, and had only a minor effect on the pancreatic elastase. These results would seem to confirm the importance for anti-elastase activity of the active site P_1

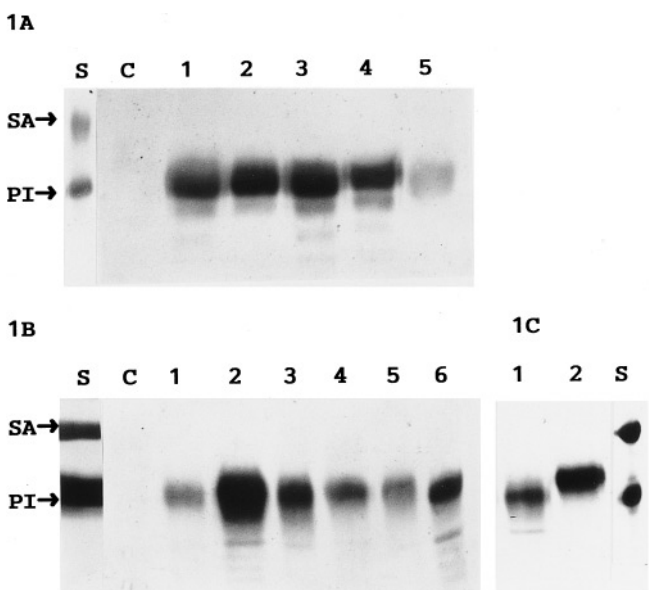

FIG. 1. Recombinant murine α1-PIs 1–5. Western blots of supernatants from transiently and stably transfected cell lines. (A) COS-7 cells transiently transfected with lane C, pWS4; 1–5, pWSPI1–5. (B) CHO cells stably transformed with C, nil; 1–5, pCDNA3PI1–5; 6, pWSP15. (C) Higher resolution comparison of adapted cell lines expressing PI5 1, 5W9 (pWSPI5); 2, 5ABc9 (pCDNA3PI5); S, mouse serum; SA, serum albumin (the leading edge of which is recognized by this antiserum); PI, α1-protease inhibitor.

TABLE 1
Protease Inhibition Activity of Transiently Expressed Recombinant Murine α1-PIs

	Pancreatic elastase		Neutrophil elastase		Chymotrypsin		Trypsin	
Plasmid	α1-PI (ng)	%	α1-PI (ng)	%	α1-PI (ng)	%	α1-PI (ng)	%
pWS4	135	100	445	100	185	100	315	100
pWSPI1	936	693	1283	288	819	441	589	189
pWSPI2	5818	4310	2458	552	1841	995	604	192
pWSPI3	302	223	340	76	1270	687	763	242
pWSPI4	187	139	343	77	1169	632	453	143
pWSPI5	102	76	403	91	149	81	485	154
n =	5		3		5		4	

The supernatants from transfected COS-7 cells were harvested and spun clear four days post transfection. The inhibition activity of each extract was determined relative to dilutions of pure human α1-PI and expressed as the equivalent amount of human α1-PI. Percentage values are calculated relative to background vector alone transfection, pWS4. While the absolute levels of inhibition activity varied somewhat between experiments, the relative activities of the 5 recombinant α1-PIs were reproducible. The average of ''n'' determinations is recorded from at least two independent transfections.

methionine present only in α1-PI1 and 2. On the other hand all four proteins, α1-PI1–4, seem to inhibit chymotrypsin. The assay using trypsin as the target did not give any reproducible positive results, even when a variety of substrates were tested. Earlier studies have shown that plasma purified murine α1-PI inhibits murine chymotrypsin but not trypsin although some activity against bovine trypsin was observed (4). α1-PI5 lacked any measurable inhibitory activity against these four proteases, which could reflect the apparently lower level of detectable α1-PI5 protein on western blot, or may reflect a true difference in the target affinity of α1-PI which has a different P_1 residue (leu) to either α1-PI1 and 2 (met), or 3 and 4 (tyr).

The level of each of the five recombinant α1-PIs expressed by the stable CHO lines in protein free medium clearly varied when visualized by western blot (Figure 1B). Furthermore, expression of α1-PI by a particular cell line varied between experiments, therefore, the relative results obtained

TABLE 2
Protease Inhibition Activity of Stably Expressed Recombinant Murine α1-PIs

Cell line	Plasmid	Pancreatic elastase α1-PI (ng)	Chymotrypsin α1-PI (ng)	Trypsin α1-PI (ng)
CHOpfm	None	80	205	960
1.3Dpfm	pCDNA3PI1	540	800	1250
2.23Fpfm	pCDNA3PI2	375	5000	2050
3.4Dpfm	pCDNA3PI3	60	1200	1190
4.4Bpfm	pCDNA3PI4	140	600	1040
5W9pfm	pWSPI5	220	215	1200
5ABc9pfm	pCDNA3PI5	1550	1000	1175
5W9	pWSPI5	200	250	930
5ABc9	pCDNA3PI5	165	215	960

Cell lines adapted to protein free medium (pfm) were grown in 25 cm^2 flasks to near confluence and refined with 5 ml 2.5 mM butyrate/medium. After seven days supernatants were harvested and spun clear. Each value is the average of two independent experiments. Results in the bottom two rows (5W9 and 5ABc9) are from non-adapted parent cell lines growing in 10%FCS, washed and then switched into pfm. Neither the absolute nor relative inhibition activities were quantitatively reproducible between experiments.

from one set of cell supernatants could not be averaged between experiments. However, given these reservations the results appear to confirm that α1-PI1 and 2 inhibit elastase, whilst α1-PIs 1–4 all inhibit chymotrypsin. Again the results against trypsin are poor. A cell line expressing readily detectable levels of α1-PI5 made with pWSPI5 (5W9) again seems to produce virtually no inhibitory activity. Surprizingly a cell line (5ABc9) made using the plasmid pCDNA3PI5 did produce both anti-elastase and anti-chymotrypsin activity in some supernatants. However, on further investigation it appeared that the protein expressed by this cell line had been altered during adaptation of the cell line to serum free growth. The active recombinant protein has slightly slower mobility on SDS PAGE after adaptation (Figure 1C) and the protein expressed by cells prior to prolonged adaptation has no inhibitory activity. The nature of the change in the protein expressed has not been investigated, but could be due to a mutation, an alteration in secondary modification, or even a recombination between the hamster chromosomal α1-PI gene and the mouse transgene.

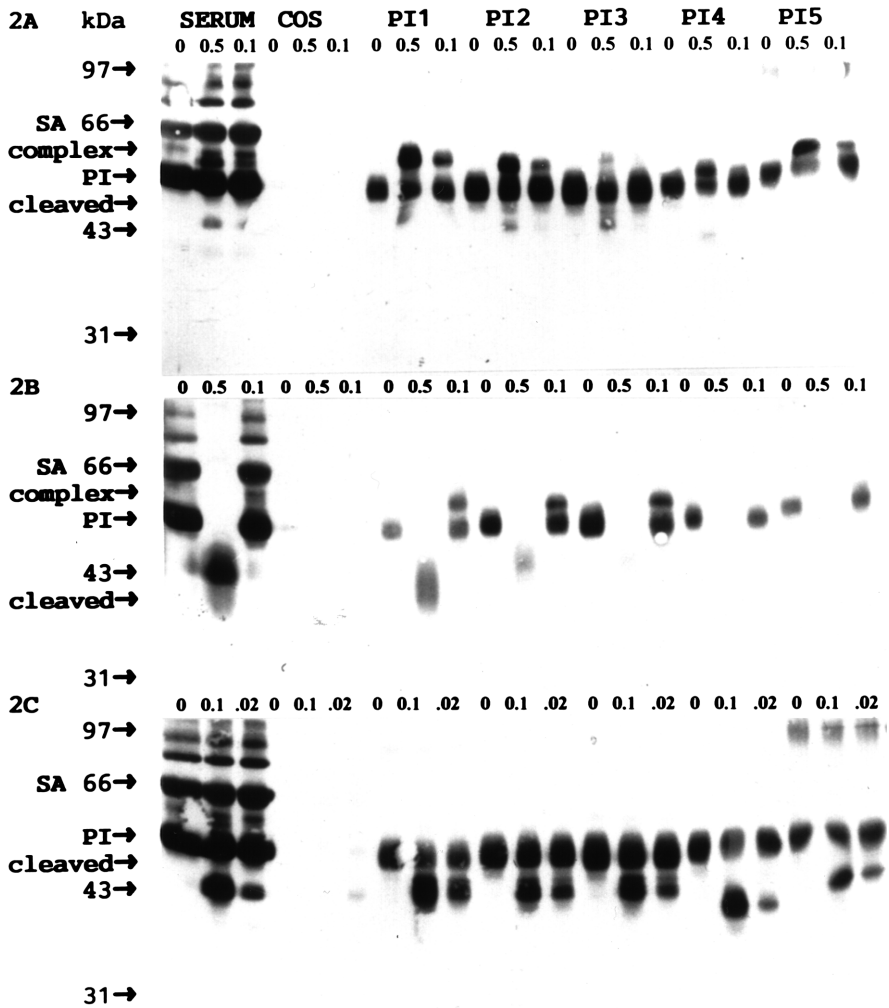

FIG. 2. Interaction of recombinant murine α1-PIs 1–5 with elastase, trypsin, and chymotrypsin. Samples (30μl) were incubated with an equal volume of varying concentrations of protease before electrophoresis then western blotted. SERUM, 1μl 1/10 mouse serum; COS, untransfected cells; PI1–4, cells transfected with pWSPI1–4; PI5, stable cell line 5ABc9; SA, serum albumin. Protease concentrations were (A) pancreactic elastase 0, 0.5, and 0.1μM; (B) trypsin 0, 0.5, and 0.1μM; (C) chymotrypsin 0, 0.1, and 0.02μM.

Attempts were made to identify other potential targets for the murine α1-PI proteins by attempting to inhibit proteolytic activity of a small number of commercially available plasma proteases. Under the conditions used none of the five recombinant α1-PIs, nor the pure human α1-PI, significantly inhibited porcine plasmin, bovine thrombin, human urokinase, human plasma kallikrein or bovine factor Xa.

Initial experiments were performed to investigate whether differences in the binding of the five recombinant α1-PIs and target proteases could be discerned by SDS PAGE resolution of complexed proteins, visualized by western blot (Figure 2). Above a certain concentration each of the proteases cleave the α1-PI giving a band of 35–45kDa, but at lower concentrations it is possible to see an SDS stable complex of about 60kDa formed between protease and serpin. At least four of the five proteins formed similar complexes with elastase even though they differed markedly in enzyme inhibition activity. Three of the proteins formed complexes with trypsin, although the ability of the murine PIs to inhibit trypsin is poor. Interestingly chymotrypsin did not form a stable complex with the PI proteins under these conditions, even though it is inhibited effectively by them. There does not seem to be a simple correlation between protease binding and inhibition for the α1-PIs, an observation already demonstrated with the rabbit α1-PI-E which binds but does not inhibit trypsin (5). It appears that binding is a different activity to inhibition and may not be determined by the same amino acids in the reactive loop. More detailed investigations will probably require purification and quantitation of the recombinant α1-PIs.

In conclusion, we have constructed cell lines which secrete each of the five *M. musculus* α1-PIs. The two proteins most similar to human α1-PI in the reactive loop are inhibitors of elastase, whilst four of the family seem to have antichymotrypsin activity, and do not strongly inhibit trypsin. No activity has yet been shown for α1-PI5, but the availability of these cell lines should allow further characterization of these proteins.

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