Murine serpin 2A is a redox-sensitive intracellular protein

Emma C. MORRIS*, Timothy R. DAFFORN†, Sharon L. FORSYTH‡, Melinda A. MISSEN‡, Anita J. HORVATH‡, Lynne HAMPSON§, Ian N. HAMPSON§, Graeme CURRIE‖, Robin W. CARRELL† and Paul B. COUGHLIN‡[1]

*Department of Haematology, University College Hospital, Grafton Way, London WC1E 6AU, U.K., †Department of Haematology, Wellcome Trust Centre for Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, U.K., ‡Monash University Department of Medicine, Australian Centre for Blood Diseases, Level 5, Clive Ward Centre, Arnold Street, Box Hill 3128, Australia, §Obstetrics and Gynaecology and Reproductive Health Care, St Mary's Hospital, University of Manchester, Oxford Road, Manchester, U.K., and ‖School of Botany, University of Melbourne, Parkeville 3010, Australia

Murine serpin 2A is expressed at high levels in haemopoietic progenitors and down-regulated on differentiation. When it is constitutively expressed in the multipotent haemopoietic cell line, FDCP-Mix, it causes a delay in differentiation and increased clonogenic potential. The serpin is also dramatically up-regulated on T-cell activation. It has an unusual reactive site Cys-Cys sequence, a unique C-terminal extension and lacks a typical cleavable N-terminal signal sequence. In spite of these features, the protein is not a member of the ovalbumin–serpin family, but is instead most closely related to human antichymotrypsin. We have shown that the serpin is intracellular with prominent nuclear localization. Transverse urea gradient gels and CD studies show that the protein undergoes the stressed–relaxed conformational change typical of inhibitory serpins. However, we have not detected complex-forming activity with a set of proteases. Thermal denaturation studies also show that the protein has decreased structural stability under reducing conditions, although it lacks disulphide bonds within the core of the molecule. Our results show that serpin 2A is an intracellular protein with the potential to mediate its biological effects via interaction with non-protease intracellular targets. Furthermore, the results presented suggest a model whereby the serpin interactions could be modulated by redox conditions or conformational change induced by cleavage of the reactive-site loop.

Key words: haemopoiesis, protease, Serpina3g, stem cell.

INTRODUCTION

The serpin family of protease inhibitors have a well-established place in the control of extracellular proteolytic cascades. Deficiency or dysfunction of antithrombin, antitrypsin and C1 inhibitor has been associated with thrombosis, emphysema and angio-oedema [1]. Recently, there has been interest in the cellular effects of serpins with reports of antithrombin, pigment-epithelium-derived factor and maspin exhibiting angiostatic activity [2–4]. Attention has also focused on the role of serpins which are retained, either wholly or in part, within the cell [5]. This group includes human protease inhibitor 6 (PI-6) and PI-9, which are proposed to regulate the intracellular proteases cathepsin G and granzyme B respectively [6–8]. Other intracellular serpins, including monocyte–neutrophil elastase inhibitor (MNEI) and squamous cell carcinoma antigens 1 and 2, appear to have similar roles [9–11]. The viral serpins poxvirus serine protease inhibitors 1 and 2 [SPI-1 and SPI-2 (*crmA*)] are also intracellular and are important modulators of the virus–host interaction. SPI-1 expression affects host range restriction, whereas SPI-2 (*crmA*) suppresses host production of interleukin-1β by virtue of its inhibition of caspase 1 [12–15]. So far, all of the mammalian intracellular serpins are members of the ovalbumin–serpin group which is characterized by (1) lack of a conventional secretion signal sequence, (2) a tendency to have oxidizable residues in the vicinity of the reactive-site loop, (3) similar intron–exon structures and (4) clustering of the human members at two chromosomal loci at 6p25 and 18q21.3 [5,16,17]. The function of intracellular serpins has largely been inferred from their *in vitro* interactions with target proteases but there is little to tell us whether their activity is regulated or to explain the significance of the redox-sensitive residues around the reactive site.

We have been interested in murine serpin 2A which has some features of the intracellular serpins, although it is most closely related to human antichymotrypsin with overall acid sequence identity of 60%. Serpin 2A was originally described by Inglis et al. [18] in the teratocarcinoma cell line EB22. Curiously, the original cDNA was truncated at the 5′-end as a result of an alternative splicing event. It was subsequently shown that this serpin was part of a multigene cluster of at least nine serpins on murine chromosome 12 at a locus syntenic with human chromosome 14q32.1 [19]. The human locus contains the genes encoding antitrypsin, antichymotrypsin, protein C inhibitor and cortisol-binding globulin. After its original description by Inglis et al. [18], serpin 2A was identified by Hampson et al. [20] as a gene expressed in the pluripotent haemopoietic cell line FDCP-Mix A4, which was dramatically down-regulated on differentiation. Similarly, when granulocyte macrophage-colony-forming cells were isolated from murine bone marrow and induced to differentiate, down-regulation of expression could be shown. When FDCP-Mix A4 cells were stably transfected with serpin 2A, they showed delayed differentiation and increased clonogenic potential suggesting that the serpin played an active role [20]. Northern-blot studies showed serpin 2A message in lymphoid

Abbreviations used: DTT, dithiothreitol; MENT, myeloid and erythroid nuclear termination stage-specific protein; MNEI, monocyte–neutrophil elastase inhibitor; NLS, nuclear localization sequence; PI, human protease inhibitor; SPI, poxvirus serine protease inhibitor; TUG, transverse urea gradient.

[1] To whom correspondence should be addressed (e-mail Paul.Coughlin@med.monash.edu.au).

tissues and expression was markedly up-regulated in primary splenocyte cultures on T-cell activation. Furthermore, a dramatic and rapid up-regulation of serpin 2A expression was seen after T-cell activation in a transgenic mouse model with a defined clonal T-cell population (D. Kioussis, personal communication).

The amino acid sequence of serpin 2A [20] is similar to human antichymotrypsin; however, it has several distinctive features including (1) a unique Cys-Cys sequence at the predicted P_1–P_1', (2) an unusual C-terminal extension of 30 amino acids containing two additional cysteine residues (the C-terminal extension is also present in independent serpin 2A entries in murine expressed sequence tag databases) and (3) it lacks a conventional secretion signal sequence suggesting an intracellular location. We have studied the subcellular localization of serpin 2A and analysed the biochemical properties of recombinant protein. Our results show that serpin 2A is localized both in the cytoplasm and the nucleus of the cell and suggest that the biological activities of the protein will be modulated by cellular redox conditions.

MATERIALS AND METHODS

Cell culture

Sf9 cells were grown in SF900 II serum-free medium (Life Technologies, Melbourne, Australia), supplemented with foetal calf serum (5 %, v/v), penicillin (10 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (Sigma–Aldrich, Poole, Dorset, U.K.) at 27 °C. FDCP-Mix A4 cells were grown as described previously [20].

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum in 5 % CO_2. COS-7 cells were transfected using Superfect or the DEAE-dextran/chloroquine method as described previously [21].

Preparation of antibody to serpin 2A

Bacterial recombinant serpin 2A was made as described previously [20]. The recombinant serpin was found in inclusion bodies which were purified by standard techniques and then suspended in Freund's adjuvant and used to immunize rabbits. Immune serum recognized a single band on Western-blotted lysates of FDCP-Mix cells. To decrease non-specific, diffuse background on Western blots, the serum was adsorbed on *Escherichia coli* BL21 lysate coupled with Affigel-10 beads (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The antibody was purified by binding to an affinity column coupled with solubilized recombinant serpin 2A and subsequently eluted with 0.1 M glycine (pH 2.5).

Subcellular localization of serpin 2A

COS-7 cells transfected with pCIneo serpin 2A were allowed to adhere to 10-well microscope slides (ICN, Seven Hills, NSW, Australia) overnight and then washed with PBS before fixation. FDCP-Mix A4 cells were cytospun on to polylysine-coated microscope slides. The cells were fixed with 3 % (w/v) paraformaldehyde in PBS for 15 min and then permeabilized in 0.1 % (v/v) Triton X-100/PBS for 15 min. Cells were blocked with 3 % (w/v) BSA and 1 % (v/v) goat serum in PBS. Primary antibodies (anti-serpin 2A serum or pre-immune serum) were diluted 1:100 in blocking buffer. The secondary antibody was that of FITC-conjugated goat anti-rabbit (Sigma–Aldrich), diluted 1:100 in blocking buffer. Propidium iodide (5 μg/ml) was used as a nuclear counterstain. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). Prepared cells were visualized by either UV light microscopy (Nikon, E800) or laser-scanning confocal microscopy (Bio-Rad MRC600).

Western blotting

Native PAGE, SDS/PAGE and transverse urea gradient (TUG) gel electrophoresis were performed using standard techniques [22–25]. Electrophoretic gels were transferred on to nitrocellulose using a Mini Trans-Blot module (Bio-Rad Laboratories) by standard techniques. Membranes were blocked with 5 % (w/v) skimmed milk in PBST [PBS/0.1 % (v/v) Tween 20]. Primary antibody was diluted in 0.5 % milk/PBST. Horseradish peroxidase-conjugated secondary antibody was detected using ECL® reagent (Amersham, Little Chalfont, U.K.).

Production of recombinant His-tagged serpin 2A

The plasmid pUC19 with the serpin 2A open reading frame inserted into the *Bam*H1 site was modified by insertion of a double-stranded oligonucleotide cassette to encode an N-terminal His_6 tag. The His_6-serpin 2A fragment was excised from pUC19 and inserted into the multiple cloning site of the pBacgus-1 transfer plasmid (Novagen, Nottingham, U.K.). Correct insertion of His_6-serpin 2A was confirmed by nucleotide sequencing using the Sequenase™ Version 2.0 sequencing kit.

Co-transfection of pBacgus His_6-serpin 2A with linearized baculoviral DNA into Sf9 cells was performed using both the liquid overlay and direct plaquing techniques as described in the BacVector System Manual (Novagen). Sf9 cells growing in exponential phase (cell density 10^6 cells/ml) were infected on day 0 with recombinant viral master stock 1.5 plaque-forming units (pfu)/cell. The cells were harvested after 72 h, washed three times with PBS and then resuspended in 5 pellet-volumes of hypo-osmotic lysis buffer [50 mM Tris/HCl (pH 8.0)/5 mM $MgCl_2$/1 mM dithiothreitol (DTT)/2 μg/ml of leupeptin/0.1 μM 4-(2-aminoethyl)benzenesulphonyl fluoride], incubated on ice for 30 min, passed three times via a 26G needle and then treated with three freeze–thaw cycles. The cell lysate was centrifuged at 13000 ***g*** for 15 min at 4 °C and the supernatant loaded on to a washed 3 ml (bed volume) Ni^{2+}-nitrilotriacetate agarose (Qiagen, Clifton Hill, Victoria, Australia) column at 0.5 ml/min. After completion of the load, the column was washed with 5 column volumes of degassed lysis buffer containing 20 mM imidazole and then eluted using a 40 ml of linear gradient (20–250 mM imidazole) in the same buffer. Fractions containing His_6-serpin 2A of 90 % purity, as judged by SDS/PAGE and Coomassie Blue R-250 stain, were pooled and dialysed against 50 mM Tris/HCl (pH 8), 1 mM DTT and 0.1 mM EDTA.

Interaction of His_6-serpin 2A with proteases

Interaction between His_6-serpin 2A and a set of proteases

Trypsin, chymotrypsin, plasmin, thrombin, factor Xa, leucocyte elastase, cathepsin G, prostate-specific antigen and papain were obtained from Sigma. Equimolar concentrations of His_6-serpin 2A and enzyme were incubated together at 37 °C for 2 h in 50 mM Tris/HCl (pH 8), 1 mM DTT and 0.1 mM EDTA, after which an equal volume of reducing SDS/PAGE sample loading buffer was added and the samples heat-denatured at 100 °C for 2 min. Reactions were run on 12.5 % (w/v) reducing SDS/PAGE and transferred on to nitrocellulose.

Interaction between His_6-serpin 2A and granzyme B

His_6-serpin 2A (0.8 μM) was incubated with recombinant granzyme B (0.7–14 μM) for 60 min at 37 °C in 20 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM DTT and 0.05 % (v/v) Nonidet P40. To demonstrate that the granzyme B was functionally active, a parallel reaction was performed in which recombinant

His_6-tagged protease inhibitor-9 (12 μM) was incubated with enzyme (0.7–14 μM). At the completion of the reactions, the samples were mixed with equal volumes of SDS/PAGE loading buffer. Reactions were run on 12.5 % reducing SDS/PAGE and transferred on to nitrocellulose. PI-9 was detected using monoclonal antibody to the His tag (Qiagen). Recombinant PI-9 and granzyme B were a gift from Dr Phillip Bird (Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia).

Interaction of His_6-serpin 2A with caspase 3

Inhibition of caspase 3 by His_6-serpin 2A was tested using the Biomol Quantizyme Assay System (Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.) according to the manufacturer's instructions. Briefly, His_6-serpin 2A was dialysed against 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 10 % (v/v) glycerol. The enzyme (3.6 nM) and His_6-serpin 2A (3.6–360 nM) were incubated for 30 min at 37 °C after which residual enzyme activity was measured by adding the chromogenic substrate Ac-DEVD-pNA (final concentration, 200 μM) and recording the increase in absorbance at 405 nm using a microtitre plate reader (BioRad Benchmark, Bio-Rad Laboratories).

Mass spectroscopy of His_6-serpin 2A

Recombinant protein was incubated with sequencing grade trypsin (Promega, Anandale, NSW, Australia) in the ratio 100:1 for 2 h at 37 °C in 20 mM Tris (pH 8), 0.1 mM EDTA, 2.5 mM $CaCl_2$ and 5 mM 2-mercaptoethanol. Matrix-assisted laser-desorption ionization–time-of-flight-MS was performed using an Applied Biosystems Voyager DE-STR.

Conformational stability of His_6-serpin 2A

A serpin-to-enzyme ratio of 20:1 (w/w) was used to examine the effect of loop cleavage on conformational stability. His_6-serpin 2A (2 μg) was incubated with 0.05 μg of protease (trypsin, chymotrypsin or plasmin) in a total volume of 80 μl for 2 h at 37 °C. Cleavage reactions were terminated by placing the reactions on ice and adding 20 μl of 5 × TUG sample loading buffer. The reactions were analysed by TUG gel electrophoresis. The gels were transferred to nitrocellulose and Western-blot analysis was performed.

CD analysis

Changes of protein secondary structure were measured by monitoring the CD signal at 222 nm using a 0.05 mm path-length quartz cuvette. When assessing the thermal stability, His_6-serpin 2A (0.25–0.5 mg/ml) was incubated with or without enzyme (trypsin or chymotrypsin in the ratio 20:1) for 2 h at 37 °C. The reaction was stopped by adding 4-(2-aminoethyl)benzenesulphonyl fluoride (1 mM final concentration) and dialysed in 50 mM phosphate buffer (pH 7.4) at 4 °C for a minimum of 8 h, to ensure minimal contamination with chloride ions. Thermal unfolding experiments were performed with a heating rate of 60 °C/h. To assess redox effects on structural stability, DTT (1–25 mM) or diamide (1 mM) was added to the His_6-serpin 2A before analysis. The data were fitted to a two-state protein unfolding model by the method of Lawrence et al. [26,27] using the least-squares method within Grafit version 3.0 (Erithracus Software Ltd., 1992). Each spectrum and temperature melt represents the average of two scans.

Homology model building

A homology model of serpin 2A was constructed using the MODELLER program [28]. A basis set of homologous structures was constructed with reference to alignment of the serpin 2A sequence against a database of known structures (ovalbumin 1OVA, human plasminogen activator inhibitor II 1BY7 and human α_1-antitrypsin 1QLP). These structures were combined with multiple-sequence alignment data obtained using ClustalW [29] in conjunction with the primary amino acid sequence of serpin 2A. These data were then used by the MODELLER routines to produce a homology model.

RESULTS

Subcellular localization of serpin 2A

As serpin 2A lacks a typical, cleavable secretion signal we predicted that the protein would be localized within the cell. We therefore examined the distribution of the protein within endogenously expressing FDCP-Mix A4 cells using laser-scanning confocal microscopy (Figures 1A and 1B). In keeping with our prediction, most of these cells showed prominent nuclear and cytosolic serpin 2A. There was considerable variation in the distribution of serpin 2A with some cells showing predominant nuclear localization, whereas others demonstrate prominent cytosolic as well as nuclear serpin. This variability may reflect the morphological heterogeneity of the FDCP-Mix A4 cell line which, although it is pluripotent and self-renewing, contains cells at various levels of spontaneous differentiation. Another, as yet unexplored, possibility is that the subcellular distribution of the serpin is related to the cell-cycle stage of individual cells.

We anticipated that other members of the *Spi-2* serpin family may be expressed in the FDCP-Mix cells and that they may cross-react with the antibody used in the present study. Because of this possibility, and also to exclude any other non-specific binding, we examined the subcellular distribution of the protein in COS-7 cells transiently transfected with a full-length serpin 2A construct. Figures 1(C) and 1(D) show cytosolic as well as prominent serpin 2A. The nature of the transient transfection process is such that cells expressing the construct are intermingled with non-expressing cells. In Figure 1(C), the faint outline of several non-expressing cells can be seen indicating that there is minimal non-specific antibody binding.

Interaction of serpin 2A with proteases

The target protease specificity of serpins is largely determined by the amino acid sequence of the reactive-site loop and, in particular, the nature of the residues adjacent to the predicted scissile bond. For serpin 2A, the predicted P_1–P_1' residues are Cys-Cys which do not correspond to the preferred cleavage site of any known protease. Inspection of the proximal hinge region of the reactive-site loop shows it to be similar to other inhibitory serpins, suggesting the possibility of functional protease inhibitory activity. We therefore tested the ability of serpin 2A to form SDS-stable complexes with a set of proteases. None of the proteases tested formed stable complexes with the serpin. However, reaction with trypsin, chymotrypsin and cathepsin G caused the protein to migrate with a relative molecular mass of 40 kDa, consistent with cleavage in the reactive-site loop (Figure 2). Treatment of serpin 2A with elastase yielded a lower molecular mass product indicating a different, or additional, site of cleavage. Matrix-assisted laser-desorption ionization–time-of-flight-MS analysis of serpin 2A digested with trypsin consistently yielded a peak with mass 2.869 kDa matching the peptide sequence

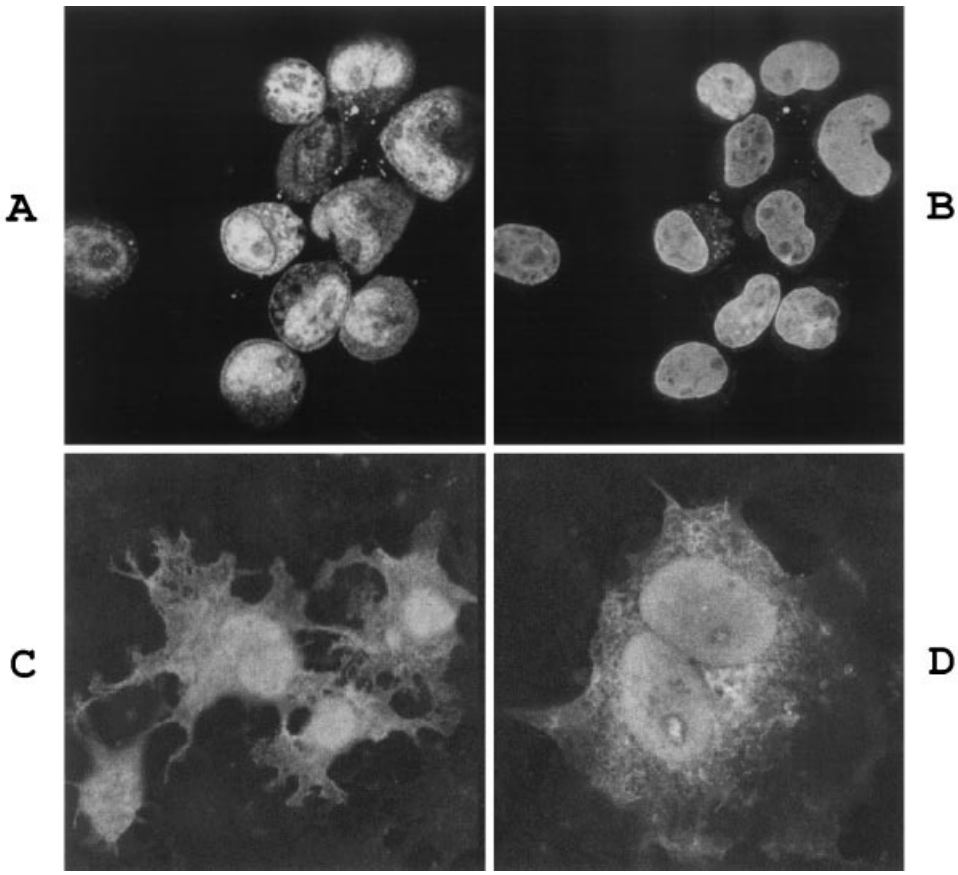

Figure 1 Localization of serpin 2A in transfected COS-7 cells and FDCP-Mix A4 cells by immunofluorescence staining

(**A**) Laser-scanning confocal microscopy images of FDCP-Mix A4 cells showing the pattern of distribution of serpin 2A, (**B**) the same cells stained with propidium iodide, (**C**) low- and (**D**) high-power laser-scanning confocal microscopy of COS-7 cells transfected with pCIneo serpin 2A. Cells stained with pre-immune serum (results not shown) demonstrated minimal background staining on immunofluorescence.

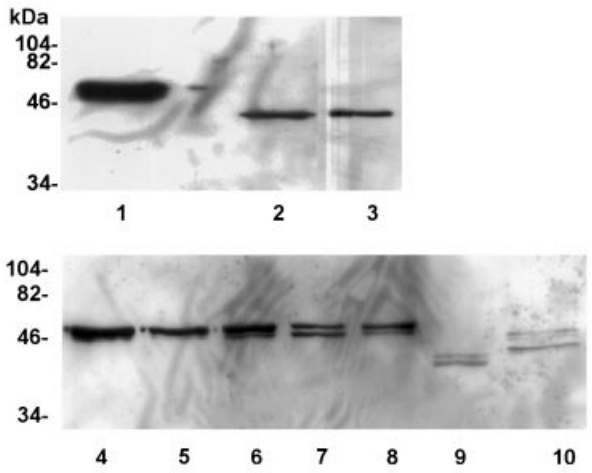

Figure 2 Interaction of His_6-serpin 2A with proteases

Recombinant His_6-serpin 2A (1 μM) was incubated with equimolar concentrations of proteases for 2 h at 37 °C, submitted to reduced SDS/PAGE, Western-blotted and detected with antibody to serpin 2A. Lane 1, no protease; lane 2, trypsin; lane 3, chymotrypsin; lane 4, no protease; lane 5, thrombin; lane 6, factor Xa; lane 7, plasmin; lane 8, prostate-specific antigen; lane 9, elastase; and lane 10, cathepsin G.

CCAVFD ... SDTK, demonstrating cleavage of the protein in the reactive-site loop at the predicted P_2–P_1 bond.

In view of the intracellular localization of serpin 2A and the known expression of granzyme B in FDCP-Mix cells and activated T-cells, we tested for interaction between the proteins [30]. Incubation of recombinant granzyme B with His_6-serpin 2A did not result in any detectable SDS-stable complex formation (Figure 3A). In contrast, when granzyme B was incubated with recombinant PI-9 the expected complex was evident on SDS/PAGE (Figure 3B). Serpin 2A was also tested for inhibitory activity against the pro-apoptotic cysteine protease and caspase 3. As serpins do not normally form SDS-stable complexes with caspases [14], we used a functional assay in which serpin 2A was incubated with caspase 3 and then residual enzyme activity measured. No evidence of inhibition was demonstrable even when serpin 2A was in 100-fold molar excess over caspase 3 (Figure 4). The presence of 100 nM Ac-DEVD-CHO resulted in complete inhibition of enzyme activity. In summary, at the end of testing serpin 2A against a range of proteases we were not able to demonstrate inhibitory activity.

Structural stability of serpin 2A

Treatment of recombinant serpin 2A with proteases produced a pattern on SDS/PAGE consistent with cleavage of the reactive-

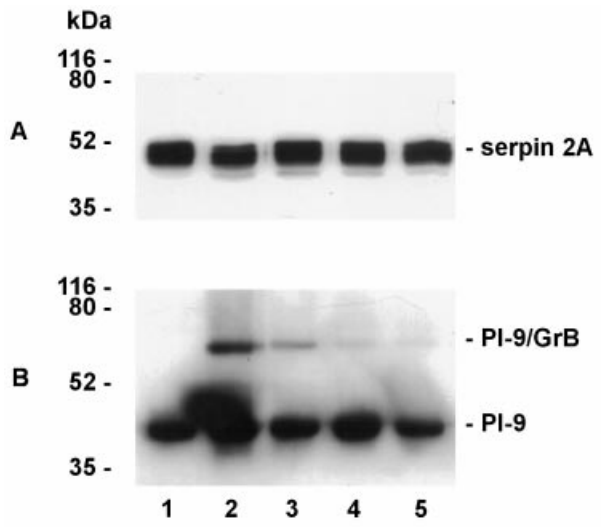

Figure 3 Interaction of His_6-serpin 2A with granzyme B

Western blot showing the interaction between serpin 2A and granzyme B (**A**) or PI-9 and granzyme B (**B**). (**A**) Serpin 2A (0.8 μM) was incubated with granzyme B: 14 μM (lane 2), 2.8 μM (lane 3), 1.4 μM (lane 4) and 0.7 μM (lane 5). Lane 1 contains serpin 2A incubated in the absence of granzyme B. (**B**) PI-9 (12 μM) was incubated with granzyme B: 14 μM (lane 2), 2.8 μM (lane 3), 1.4 μM (lane 4) and 0.7 μM (lane 5). Lane 1 contains PI-9 incubated in the absence of granzyme B. PI-9 was detected using monoclonal antibody to the PI-9 His tag.

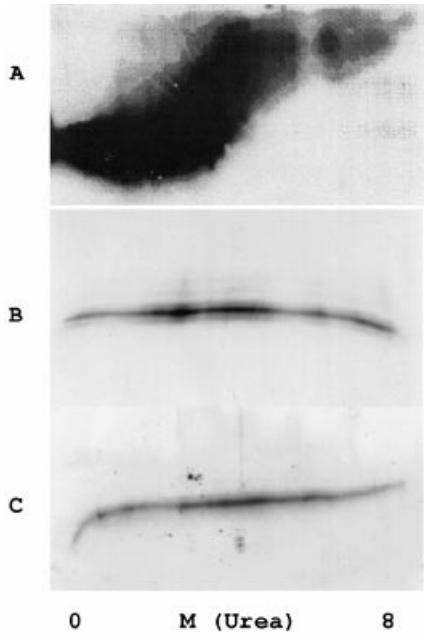

Figure 5 TUG gel electrophoresis of native and cleaved His_6-serpin 2A

Purified His_6-serpin 2A, either native (**A**); cleaved with chymotrypsin (**B**); or cleaved with trypsin (**C**) was submitted to TUG gel electrophoresis, Western-blotted and detected with antibody to serpin 2A.

site loop. We therefore examined the structural stability of the protein in its native and cleaved forms to determine whether it would undergo the stressed to relaxed conformational change. To address this issue, we used TUG gel electrophoresis. Figure 5(A) shows the appearance of non-cleaved serpin 2A with a sigmoid shape across the urea gradient, typical of the transition from stressed to relaxed conformation. The band corresponding to serpin 2A was diffuse, but this was consistent with the behaviour of the recombinant protein on native PAGE (Figure 6, lane 6). Although it is common for recombinant proteins to migrate as diffuse bands on native PAGE, serpin 2A derived from endogenously expressing FDCP-Mix A4 cells demonstrated a similar appearance (Figure 6, lane 5), suggesting that this is a property of the protein rather than an artifact of the recombinant. By contrast, serpin 2A which had been cleaved by chymotrypsin or trypsin produced a strikingly different appearance on TUG gel electrophoresis (Figures 5B and 5C) in which the serpin

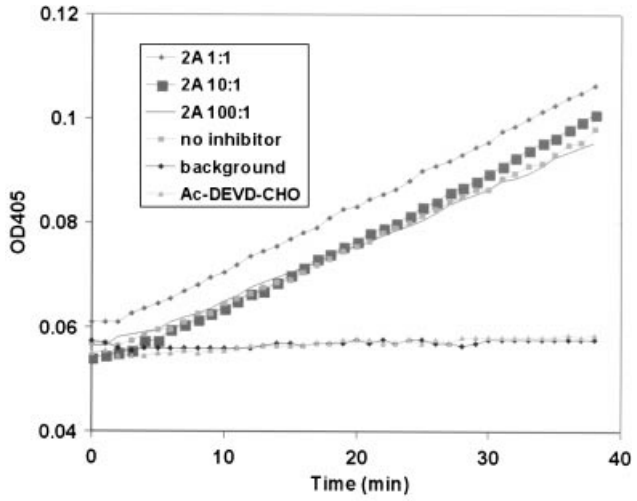

Figure 4 Interaction of His_6-serpin 2A with caspase 3

Caspase 3 (3.6 nM) was incubated with His_6-serpin 2A (3.6–360 nM) for 30 min at 37 °C and residual activity measured by adding the chromogenic substrate Ac-DEVD-pNA and recording the increase in absorbance at 405 nm (OD405). The Figure also shows the activity of caspase 3 after incubation either with buffer or with a molar excess of the caspase 3 inhibitor Ac-DEVD-CHO.

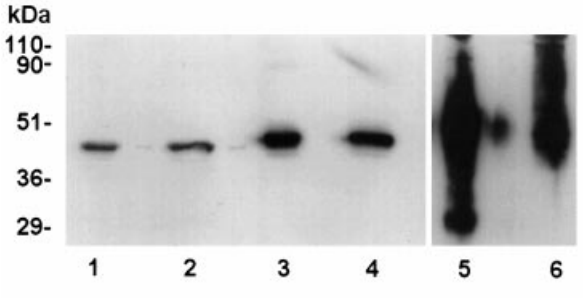

Figure 6 Native and denaturing gel electrophoresis of FDCP-Mix A4 cell lysate and recombinant His_6-serpin 2A

FDCP-Mix A4 cells were washed and resuspended in 50 mM Tris/HCl (pH 8), 1 mM DTT, 0.1 mM EDTA, containing 0.1% Triton X-100 and protease inhibitors. After 15 min incubation on ice, the lysate was centrifuged at 10000 ***g*** to remove cellular debris. Lysate (40 μg) was submitted to non-reducing (lane 1) or reducing (lane 2) SDS/PAGE. His_6-serpin 2A (100 ng) was submitted to non-reducing (lane 3) or reducing (lane 4) SDS/PAGE. Lanes 5 and 6 show native PAGE of FDCP-Mix A4 cell lysate (50 μg) and His_6-serpin 2A (500 ng) respectively.

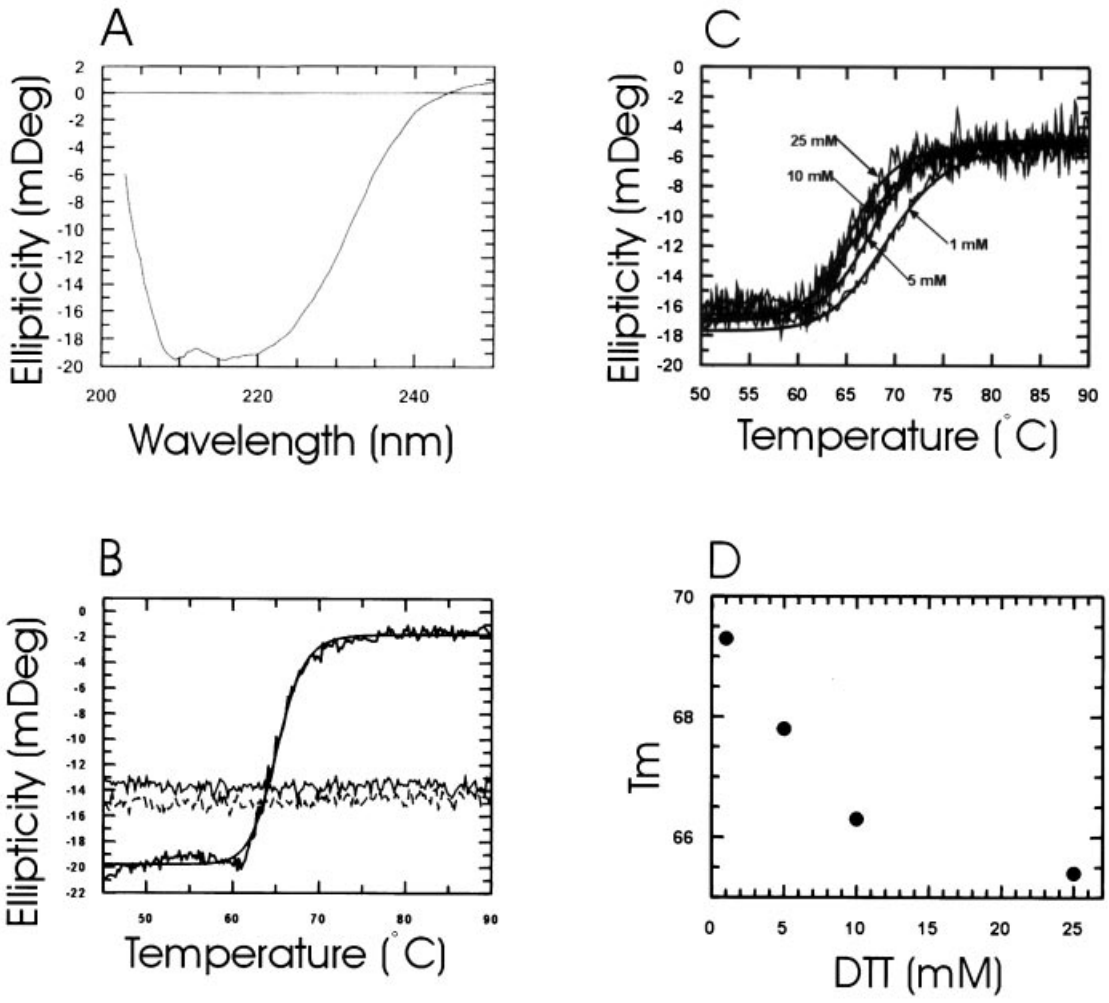

Figure 7 Thermal stability of serpin 2A assessed by CD

(**A**) CD spectrum of native recombinant His_6-serpin 2A. (**B**) Thermal stability of native (thick line), trypsin-cleaved (continuous thin line) and chymotrypsin-cleaved (broken line) His_6-serpin 2A obtained by measuring the change in CD signal at 222 nm with respect to temperature. (**C**) Thermal stability of His_6-serpin 2A measured in the presence of 1, 5, 10 and 25 mM DTT. (**D**) The relationship between thermal stability of His_6-serpin 2A, as indicated by melting temperature T_m, in relation to concentration of the reducing agent DTT.

appears as a well-resolved band, with loss of the sigmoid-shaped transition typical of the relaxed conformation.

In view of the unusual appearance of serpin 2A on native PAGE and to corroborate our findings, we employed CD to characterize further the conformational changes in the protein. The CD spectrum of the purified recombinant serpin was typical of a protein containing mixed α-helical and β-sheet elements indicating that the protein was correctly folded (Figure 7A). To investigate the structural stability of the protein, it was subjected to heating while measuring ellipticity at 222 nm. The native recombinant serpin 2A showed a sigmoidal decrease in ellipticity with a melting temperature T_m of 69.3 °C (Figure 7B). By contrast, serpin 2A which had been treated with trypsin or chymotrypsin demonstrated no significant change in ellipticity up to a temperature of 90 °C (Figure 7B). This pattern of change in stability is indicative of the stressed to relaxed conformational transition, typical of inhibitory serpins which occur upon insertion of the reactive-site loop into the A β-sheet. Therefore while we have been unable to demonstrate an inhibitory complex between serpin 2A and the proteases tested, it appears that the major structural prerequisite for inhibitory activity is present.

Redox sensitivity of serpin 2A

Examination of the deduced amino acid sequence of serpin 2A shows the presence of two cysteine residues in the reactive-site loop, two additional cysteine residues in the C-terminal extension and another near the N-terminus. All of these cysteines are in sites which are likely to be exposed on the surface of the molecule and would be predicted to take part in redox reactions. Two other cysteine residues lie within the body of the molecule and molecular modelling shows that the side chains of these residues would not take part in internal disulphide bonds nor be solvent-exposed (Figure 8). Under the conditions which we have used to purify and store recombinant serpin 2A there is no evidence of disulphide-linked multimerization on non-reduced SDS/PAGE (Figure 6, lanes 3 and 4). However, molecular modelling indicates

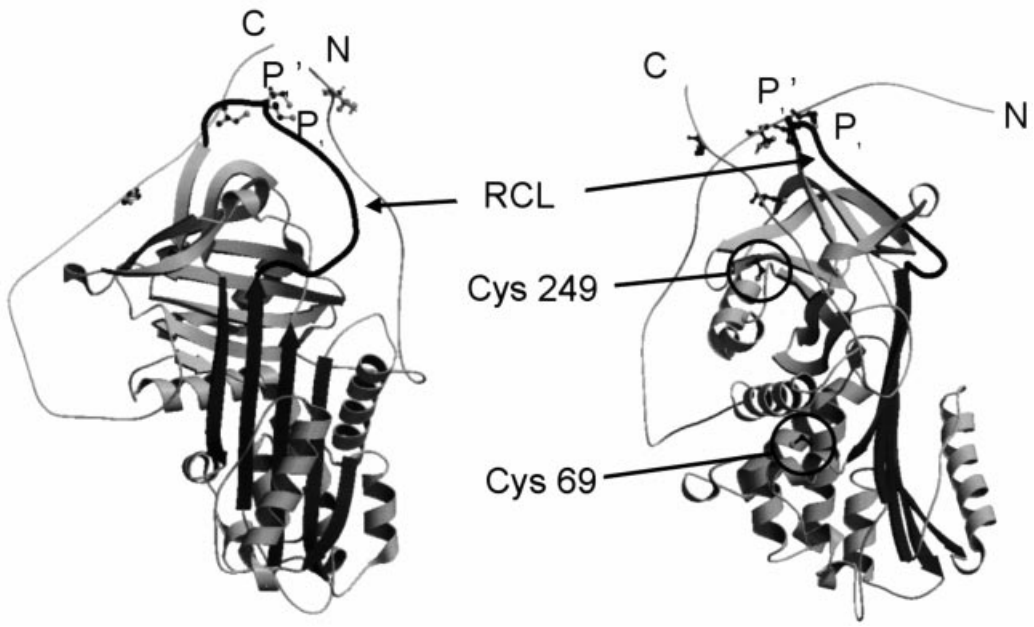

Figure 8 Molecular model of serpin 2A

Front and side view of an homology model of serpin 2A showing the reactive centre loop (RCL) in black and central A sheet in dark grey. Cysteines within the sequence are depicted in ball and stick representation. N- and C-termini have been modelled to show that the cysteines within these regions have enough steric freedom to form disulphide links with each other and/or those in the RCL. Cysteine residues at positions 69 and 249 are buried and are not able to form disulphides.

that either the C- or N-terminal cysteines in the protein could interact with the reactive-site cysteines (Figure 8).

In view of this predicted redox sensitivity of serpin 2A, we examined the structural stability of the protein by CD under reducing and oxidizing conditions. We were careful to avoid oxidation of the serpin during the extraction and purification process by using degassed buffers containing a low molarity of reducing agent and by storing the protein in the presence of 1 mM DTT and 0.1 mM EDTA. Therefore the purified recombinant serpin probably represents the condition of the protein in the cell before extraction. Thermal stability studies were performed in the presence of the oxidizing agent diamide or increasing concentrations of DTT (Figure 7C). The T_m of serpin 2A in 1 mM diamide and 1 mM DTT were identical. However, with increasing concentrations of DTT there was a progressive decrease in T_m from 69.3 to 65.4 °C (3.9 °C) (Figure 7C). Most of the change in T_m occurs between 1 and 10 mM DTT with a relatively small difference between 10 and 25 mM DTT (Figure 7D). To exclude a non-specific effect of DTT, we performed similar studies on antichymotrypsin, which has a single, buried cysteine side chain, and showed a change in T_m of only 0.06 (63.76–63.70 °C).

DISCUSSION

There are many features of the serpin 2A gene and its protein product which are surprising. Its tightly regulated expression in haemopoietic precursors and activated T-cells is striking. Its amino acid sequence indicates that it is a relatively distant relation of the ovalbumin–serpins and yet its lack of secretion signal peptide suggests an intracellular localization. It has a unique reactive-site sequence with the predicted scissile bond of Cys-Cys. Furthermore, it possesses a C-terminal extension containing two additional cysteine residues. The presence of redox-sensitive residues in the reactive site is another typical feature of intracellular serpins and oxidation of these residues has been shown to destroy protease inhibitory activity [21,31]. Our studies have confirmed the predicted intracellular location of serpin 2A and also demonstrated that a significant proportion of the serpin is present within the nucleus. A similar subcellular distribution is seen in endogenously expressing FDCP-Mix A4 cells and transfected COS-7 cells. Furthermore, there is minimal background staining of COS-7 cells not expressing serpin 2A, indicating that the antibody does not react non-specifically with nuclear proteins. In some FDCP-Mix A4 cells, serpin 2A appears to be predominantly intranuclear which argues against passive diffusion into the nucleus. On the other hand, the serpin does not possess a demonstrable nuclear localization sequence (NLS) and would therefore need to bind to an unidentified carrier protein to allow active transport into the nucleus. Similar nucleocytoplasmic localization has been demonstrated for other intracellular serpins which also lack nuclear import signals and there is evidence that this occurs by an active, but not ATP-dependent, mechanism [32]. We have attempted to corroborate the nuclear localization of the serpin using subcellular fractionation, but invariably find most of the protein in the cytosolic component. At this stage, there is no evidence to suggest that the serpin is attached to the nuclear matrix, chromatin or any other fixed nuclear structure and therefore fractionation methods which damage the nuclear membrane may allow rapid egress of the protein.

An increasing number of nuclear serpins have been reported in the literature. Previously, the chicken serpin myeloid and erythroid nuclear termination stage-specific protein (MENT) has been described within the nucleus and appears to have a role in DNA binding and chromatin condensation [33,34]. MENT is unusually basic compared with other serpins with a pI of 9.4 and, unlike serpin 2A, contains a putative NLS. Although MENT and serpin 2A share nuclear localization in haemopoietic cells, we have no

evidence to suggest that they have similar functions. Furthermore, MENT is predominantly expressed in terminally differentiated haemopoietic cells, especially of the granulocytic lineage, whereas serpin 2A is expressed in precursor cells. Another serpin which has been reported to be localized to the nucleus is PI-10 (bomapin) which is also expressed in haemopoietic cells [35,36]. Like MENT, PI-10 has a basic motif which serves as an NLS, but as yet has no defined function or target protease, although it is known to inhibit trypsin-like proteases *in vitro* [37]. Nucleocytoplasmic distribution has also been noted for PI-9, PI-6, PI-8, MNEI and plasminogen activator inhibitor-2, but as yet no functional significance has been attached to these observations [32]. Biologically important nuclear proteases such as signal transduction and activators of transcription 5 protease and separin are candidate targets for intracellular serpins and some more can be expected as interest in this field grows [38–40].

So far we have found no evidence of a human homologue for serpin 2A in expressed sequence tag databases or in searches of the human genome. The serpin 2A gene is located within a cluster of at least nine *Spi-2* serpins on murine chromosome 12 [19], which is syntenic with the human antitrypsin/antichymotrypsin locus on chromosome 14q22. The similarity between serpin 2A and human antichymotrypsin is interesting as there have been reports that antichymotrypsin, which is normally extracellular, can gain access to the interior of the cell and the nucleus [41,42].

One of the most puzzling features of serpin 2A is its apparent lack of protease inhibitory activity. The serpin 2A predicted reactive-site sequence of Cys-Cys is unusual and certainly does not correspond to the preferred cleavage site of any known protease. However, a scissile bond of Cys-Met has been reported for the MNEI [9] and it has also been suggested that PI-9, which inhibits granzyme B with a reactive site of Glu-Cys, is also capable of inhibiting elastase using the scissile bond of Cys-Met [43]. In spite of these results, indicating that a P_1 Cys is compatible with elastase inhibition, we were not able to demonstrate complex formation with this protease. Furthermore, there was no evidence of complex formation with endogenous proteases in Western blots of FDCP-Mix A4 cells (Figure 6, lanes 1 and 2). A trivial explanation of our findings is simply that we have not tested it against an appropriate target protease or that the complex formation assay is insensitive. Although this remains a possibility, it is unusual for serpins to demonstrate absolute specificity for a particular target and we have tested trypsin-, chymotrypsin- and elastase-like serine proteases together with the intracellular proteins granzyme B and caspase 3. More often, serpins will demonstrate some inhibitory or complex-forming activity with non-physiological targets, albeit with reduced efficiency or increased stoichiometry of inhibition, and we would therefore expect to detect some complex formation using a sensitive, Western-blot-based assay. In summary, although serpin 2A clearly undergoes the S–R transition typical of inhibitory serpins, we have not been able to identify positively a target protease.

The presence of vicinal cysteines at the reactive site, together with additional cysteines within the N- and C-terminal extensions, allowed us to believe that the function of the serpin would be redox-sensitive. In the absence of demonstrable protease inhibitor activity, we used thermal stability to inform us of the state of the protein under mildly oxidizing and stronger reducing conditions. The significance of the observed change in melting temperature (3.9 °C) can be judged by comparison of the difference between wild-type and Z-antitrypsin (8.0 °C) which leads to a 19-fold increase in the rate of polymerization [44]. The change in thermal stability observed on reduction of serpin 2A is consistent with rearrangement of contacts between secondary structural elements of the protein and we predict that this will involve interactions between the N- or C-terminus and the main body of the molecule. There are several well-described precedents for nuclear proteins with activities regulated by redox conditions [45,46]. The transcription factor, nuclear factor κB, possesses a cysteine residue, which is conserved in the c-Rel family of transcription factors, within its DNA-binding domain [45]. This cysteine residue must be in a reduced form for DNA binding to occur and its redox state is regulated by a thioredoxin-dependent mechanism [47]. In a striking convergence, it has recently been shown that nuclear factor κB is the major factor driving transcription of serpin 2A during T-cell activation [48]. It is therefore possible that redox conditions are important both for the regulation of serpin 2A production and for modulating its functional activity.

In the absence of a known target protease, our working hypothesis is that serpin 2A has a ligand-binding function and that this interaction(s) may be modulated by proteolytic cleavage of the reactive-site loop or changes in the prevailing redox conditions within the cell. Similar changes in the affinity of cortisol-binding globulin for its ligand, cortisol, have been reported upon reactive-site-loop cleavage [49]. We have used yeast 2-hybrid analysis to identify several non-protease-binding partners for serpin 2A and we are investigating these to shed further light on its biological activity and the reasons for its pattern of expression. Crystallographic studies in progress will further clarify the relationship between the structure and function of this unusual serpin.

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