The C-D interhelical domain of the serpin plasminogen activator inhibitor-type 2 is required for protection from TNF- α induced apoptosis

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Abstract

The serine proteinase inhibitor (serpin), plasminogen activator inhibitor type 2 (PAI-2), has been reported to inhibit tumor necrosis factor- α (TNF) induced apoptosis. In order to begin to understand the molecular basis for this protection, we have investigated the importance of a structural domain within the PAI-2 molecule, the C-D interhelical region, in mediating the protective effect. The C-D interhelical region is a 33 amino acid insertion which is unique among serpins and has been implicated in transglutaminase catalyzed cross-linking of PAI-2 to cell membranes. We have constructed a mutant of PAI-2 wherein 23 amino acids are deleted from the C-D interhelical region generating a structure predicted to be homologous to the closely related, but non-inhibitory serpin, chicken ovalbumin. The PAI-2 Δ 65/87 deletion mutant retained inhibitory activity against its known serine proteinase target, urokinase-type plasminogen activator (uPA); however expression of this mutant in HeLa cells failed to protect from TNF-induced apoptosis. Analyses of the cellular distribution of PAI-2 showed that intracellular PAI-2, and not secreted or cell-surface PAI-2, was likely responsible for the observed protection from TNF-induced apoptosis. No evidence was found for specific cross-linking of PAI-2 to the plasma membrane in either control or TNF/cycloheximide treated cells. The data demonstrate that the PAI-2 C-D interhelical domain is functionally important in PAI-2 protection from TNF induced apoptosis and suggest a novel function for the C-D interhelical domain in the protective mechanism.

Keywords: plasminogen activator inhibitor; tumor necrosis factor; apoptosis; cell death; serpin

Abbreviations: bp, base pairs; kb, kilobase(s); PAI-2, plasminogen activator inhibitor type-2; RSL, reactive site loop;

serpin, serine proteinase inhibitor; tumor necrosis factor- α , (TNF); urokinase-type plasminogen activator, uPA

Introduction

Plasminogen activator inhibitor type 2 (PAI-2) is a member of the serine proteinase inhibitor, or serpin, superfamily. Members of the serpin superfamily share a highly ordered tertiary structure defined by the crystal structure of the prototype serpin α 1-antitrypsin (Loebermann *et al*, 1984). PAI-2 is recognised as belonging to a subgroup of serpins designated the ov-serpin (ovalbumin-like) family, defined by several common features such as amino acid sequence homology and gene organization (Remold-O'Donnell, 1993). The specific functions of this diverse sub-family of molecules are only beginning to be elucidated. The best characterised of the ov-serpins, the viral serpin, *crmA*, is an inhibitor of interleukin-1 β converting enzyme (ICE)-like caspases involved in both TNF α and FAS induced apoptosis (Zhou *et al*, 1997).

PAI-2 has been well characterised as an inhibitor of the extracellular serine proteinase, urokinase-type plasminogen activator (uPA) (Kruithof *et al*, 1995). uPA catalyses the activation of plasminogen to plasmin, a broad spectrum serine proteinase which plays a central role in fibrinolysis, extracellular matrix degradation and in the activation of cytokines and growth factors in the extracellular milieu (Testa and Quigley, 1990). While PAI-2 can be found as a secreted extracellular form, the majority of PAI-2 synthesized by many cells remains intracellular (Kruithof *et al*, 1995). Recent studies (Kumar and Baglioni, 1991; Dickinson *et al*, 1995) demonstrate a novel function for PAI-2 in the regulation of apoptosis induced by the inflammatory cytokine, tumour necrosis factor- α (TNF) and in inhibition of apoptosis mediated by *Mycobacterium avian* (Gan *et al*, 1995). PAI-2 mediated resistance to TNF α is not due to the loss of TNF α -receptor or to the loss of TNF α -receptor binding activity (Dickinson *et al*, 1995). While the mechanism(s) by which PAI-2 protection occurs is as yet unknown, uPA does not appear to be the target of PAI-2 action (Dickinson *et al*, 1995) and PAI-2 has not been found to inhibit PAI-2 has not been found to inhibit ICE-like caspases associated with apoptosis (Dickinson *et al*, 1995; unpublished data).

The amino acid sequence of PAI-2, deduced from cDNA and genomic clones (Ye *et al*, 1987, 1989; Antalis *et al*, 1988; Schleuning *et al*, 1987; Samia *et al*, 1990) reveals several structural domains which may contribute to PAI-2 function. PAI-2 lacks a cleavable hydrophobic signal sequence, which is believed to account for the existence of both intracellular Mr 46 000 and secreted Mr 60–70 000

forms of PAI-2. By analogy with other members of the serpin superfamily, an exposed reactive site loop (RSL) contains the P₁-P₁' amino acid residues and acts as a bait for the target proteinase (Kiso *et al*, 1988). Mutagenesis studies show that the presence of the P₁-Arg³⁸⁰ amino acid residue of PAI-2 is required for inhibition of TNF-mediated apoptosis as cells expressing PAI-2 with an Ala in this position are sensitive to cytolysis by TNF (Dickinson *et al*, 1995).

A third feature unique to the ov-serpins is an extended amino acid domain located in the region between α -helices C and D based on the predicted structural model of PAI-2 (Antalis *et al*, 1988). This domain is non-homologous among the serpin family, is variable in length, and is unusually long in PAI-2 (33 residues) in comparison with other ovalbumin serpin members. *In vitro* studies show that glutamines contained within the C-D interhelical region can act as substrates for transglutaminase (Jensen *et al*, 1994). Recently several cellular proteins have been identified which reversibly bind to this PAI-2 domain, including annexins I and II, as well as several as yet unidentified proteins which associate with PAI-2 following crosslinking and immunoprecipitation (Jensen *et al*, 1996). However, an *in vivo* physiological role for this domain has not been demonstrated. In the present study, we have generated cells expressing a PAI-2 mutant lacking the C-D interhelical region and report for the first time an intracellular functional role for this domain in the protection of cells from TNF-induced apoptosis.

Results

The C-D interhelical region is highly conserved between species

Members of the serpin superfamily, including PAI-2, have a highly conserved tertiary structure. Alignment of the amino acid residues of PAI-2 with other members of the serpin superfamily using the crystal structure of α 1-antitrypsin as the prototype shows that PAI-2 contains an unusually long extended sequence located between predicted helices C and D and spanning amino acids 65–87 (Antalis *et al*, 1988). Comparison of the amino acid sequences of human, mouse, and rat PAI-2s shows that the amino acids contained within this region are highly conserved between species (Figure 1a).

Generation of mutant PAI-2 lacking the C-D interhelical region

In order to investigate the importance of the C-D interhelical region for the biological function of PAI-2, a mutant PAI-2 cDNA was engineered in which a 22 amino acid sequence within the C-D interhelical region was deleted (PAI-2 Δ 65/87) (diagrammed in Figure 1A). The rationale for this mutation was to remove amino acids not present in chicken ovalbumin, a structurally related serpin which has no known protease inhibitor activity. Thus possible secondary structural effects should be minimized. PAI-2 Δ 65/87 was inserted into the

Figure 1 (A) The PAI-2 C-D interhelical region. Alignment of the C-D interhelical amino acid sequences derived from the cDNA sequences of PAI-2 from human, mouse and rat PAI-2 relative to the other serpins, chicken ovalbumin and α 1-antitrypsin and showing the 22 amino acid deletion within the C-D interhelical region to generate PAI-2 Δ 65/87. The amino acid sequences were obtained from entries in the NCBI Genebank Database (Accession Numbers: human, J03603; mouse, X16490; rat, X64563). Conserved amino acids are indicated by asterisks. (B) PAI-2 Δ 65/87 expression in representative CDM transfectants. PAI-2 protein was detected in lysates from representative CDM transfectants following immunoblot analysis using anti-PAI-2 monoclonal antibody. CDM2, CDM7, CDM10, and CDM1 express PAI-2 Δ 65/87. S1n, S1/5 and S1a express different levels of wild-type 46 kD PAI-2 and ASM1 expresses Ala³⁸⁰-PAI-2. (C) Binding of PAI-2 Δ 65/87 to ¹²⁵I-uPA. Lane 1, S1/5 cells expressing wild-type PAI-2; Lane 2, CDM2 cells expressing PAI-2 Δ 65/87; Lane 3, A2/7 cells expressing anti-sense PAI-2; Lane 4, parental HeLa cells. The band at 75 kD represents ¹²⁵I-uPA complexed to the PAI-2 species. A 34 kD ¹²⁵I-uPA heavy chain is present in all lanes. The 60 kD band is an unidentified band present in all lanes but not in the tracer (data not shown). The difference in migration due to deletion of the C-D interhelical region is not visible.

Figure 2 Relative survival of representative PAI-2 clones expressing wild-type and PAI-2Δ65/87. (A) Cell survival following treatment with TNF and cycloheximide measured by crystal violet assay. The wild-type PAI-2 expressing HeLa transfectants S1a, S1/5 and S1n; PAI-2Δ65/87 expressing CDM7, CDM2, and CDM1; antisense PAI-2 expressing A2/7; the vector alone transfectant CMV; and parental HeLa cells were assayed untreated (□) or following treatment with 10 ng/ml TNF (□), 10 μg/ml cycloheximide (▨), or both of these agents (■) for 8 h. The data is expressed as the % survival relative to untreated cells. The bars represent the standard error. Absence of error bars indicates that the error was too small to allow display of the error in those columns. Identical results were obtained by MTT assay. Cycloheximide was not cytotoxic at this concentration during the time frame of these experiments. (B) Cell survival following treatment with TNF and interferon-γ measured by MTT assay. The HeLa transfectants described in (A) were assayed untreated (□), or following treatment with 10 ng/ml TNF (□), 1000 units/ml interferon-γ (▨), or both of these agents (■) for 8 h. (C) Relative survival of HeLa transfectants with increasing TNF concentration. Each transfectant was treated with increasing concentrations of TNF in the presence and absence of 10 μg cycloheximide, and with cycloheximide alone for 8 h and assayed for cell survival by crystal violet staining. The data is expressed as % survival relative to cells treated with

expression vector pRcCMV and a number of stable transfectants generated in HeLa cells. PAI-2Δ65/87 protein expression in the CD mutant clones (CDM) was monitored by immunoblot analysis as represented in Figure 1B. PAI-2Δ65/87 protein migrated with a slightly faster mobility than wild-type PAI-2 reflecting the absence of 23 amino acids or approximately 2.5 kD.

Biological activity of PAI-2Δ65/87

PAI-2Δ65/87 was evaluated for the ability to inhibit uPA, the known serine proteinase target of PAI-2. Cell lysates derived from CDM clones containing PAI-2Δ65/87 inhibited the uPA catalyzed cleavage of the synthetic substrate, Cbo-Glu-Gly-Arg-pNA, similar to lysates from wild-type PAI-2 as determined using the Spectrozyme UK assay (American Diagnostica, Greenwich, CT, USA) (see Materials and Methods). The ability of uPA and PAI-2Δ65/87 to form a stable inhibitory complex was monitored by ¹²⁵I-uPA binding assay. The assay is based on the formation of SDS-stable complexes between PAI-2 and ¹²⁵I-labelled low molecular weight uPA tracer containing the catalytic, but not the receptor binding domain of uPA. The uPA heavy chain-PAI-2 complex appears as a 75 kD band in cell lysates derived from both wild-type PAI-2 (S1/5) (Dickinson *et al*, 1995) and PAI-2Δ65/87 (CDM2) cells (Figure 1C, lanes 1 and 2), but not from antisense PAI-2 (A2/7) or parental HeLa cells (Figure 1C, lanes 3 and 4). These data demonstrate that PAI-2Δ65/87 is functional as an inhibitor of uPA, and show that the C-D interhelical domain is not required for the serpin inhibitory function of PAI-2.

PAI-2Δ65/87 does not confer protection against TNF-induced apoptosis

We have previously shown that expression of PAI-2 protects HeLa cells from apoptosis induced by TNF in the presence of the protein synthesis inhibitor cycloheximide (Dickinson *et al*, 1995). To investigate the importance of the C-D interhelical region in cell death, PAI-2Δ65/87 transfectants were assayed for sensitivity to TNF induced apoptosis. Cell death was quantitated by two independent methods, (i) crystal violet staining, which monitors cell survival as a function of cell adherence, and (ii) MTT assay, which provides a measure of viable cells. As represented in Figure 2A, cells expressing PAI-2Δ65/87 (CDM7, CDM2, CDM1) were as sensitive to TNF induced apoptosis as cells which do not express PAI-2 (A2/7, CMV, parental HeLa cells), while transfectants expressing different amounts of wild-type PAI-2 showed markedly increased cell survival, ranging from 80% in the high PAI-2 expressing line (S1a) to 40% in the low expressing cell line (S1n).

Expression of wild-type PAI-2 in HeLa cells abrogates the cytostatic effect of interferon-γ on HeLa cells as well as

cycloheximide alone. Each point represents the average of two experiments performed in triplicate. S1a (□), S1n (○), CDM1 (◊), CDM7 (◈), A2/7 (■), ASM1 (▨), and HeLa (◆)

confers resistance to apoptosis induced by the synergistic effects of TNF and interferon- γ (Dickinson *et al.*, 1995). As represented in Figure 2B, cells expressing PAI-2 Δ 65/87

(CDM7, CDM2, CDM1) are not resistant to the cytostatic effect of interferon- γ alone or the cytotoxic effects of interferon- γ and TNF, further confirming the importance of

Figure 3 Immunohistochemical analysis of PAI-2 expression in untreated cells and in cells undergoing TNF-induced apoptosis. (a) PAI-2 antibody staining of untreated S1a, 250 $\times$ magnification; (b) PAI-2 antibody staining of untreated S1a, 400 $\times$ magnification; (c) PAI-2 antibody staining of S1a cells treated for 1 h with TNF and cycloheximide, 250 $\times$ magnification; (d) PAI-2 antibody staining of S1a cells treated for 3 h with TNF and cycloheximide, 400 $\times$ magnification; (e) PAI-2 antibody staining of untreated A2/7, 250 $\times$ magnification; (f) cytochrome staining of untreated S1a, 400 $\times$ magnification

the C-D interhelical domain to PAI-2 mediated cell survival. We have previously shown that the level of protection by PAI-2 is dependent on the PAI-2 expression levels (Dickinson *et al.*, 1995). The observed loss of protection by cells expressing PAI-2 Δ 65/87 is unlikely due to the moderate expression levels of PAI-2 Δ 65/87, since S1n, a wild-type PAI-2 expressing clone, which expresses less PAI-2 than each of the CDM mutants, shows protection against both TNF and interferon- γ induced cytotoxic effects.

The sensitivity of cells expressing PAI-2 Δ 65/87 to the dose of TNF compared with wild-type PAI-2 expressing cells is shown in Figure 2C. Wild-type PAI-2 expressing HeLa cells (S1a and S1n) demonstrate enhanced survival at concentrations of TNF ranging from 0–10 ng/ml, relative to PAI-2 non-expressing cells, which show a drop in survival at 2 ng/ml that levels off at 10 ng/ml. CDM1 and CDM7 are as sensitive to TNF as the PAI-2 non-expressing cells and the ASM1 cell line, which expresses an active site mutant of PAI-2 (P₁-Ala³⁸⁰-PAI-2) that is sensitive to TNF-mediated apoptosis (Dickinson *et al.*, 1995). Increased cell death is obtained using higher concentrations of TNF, but survival below 20% is not observed even at 100 ng/ml TNF, as has been reported by others (Wong *et al.*, 1989). The reason for this is unknown but has been attributed to additional survival factors present in cells. These results demonstrate that the presence of the C-D interhelical domain in the PAI-2 molecule is required for PAI-2 protection against TNF-induced apoptosis.

Cellular distribution of PAI-2

In trophoblasts and during keratinocyte differentiation, PAI-2 exhibits a pericellular distribution, which has been attributed to transglutaminase cross-linking to the plasma membrane (Lyons-Giordano *et al.*, 1994). Given the suggested role of transglutaminase activity in the morphological changes associated with apoptosis (Jensen *et al.*, 1995; Knight *et al.*, 1991), we investigated whether PAI-2 may be associated with cellular structures in PAI-2 expressing cells and/or whether the apoptotic stimuli may induce changes in PAI-2 cellular distribution. Immunohistochemical analyses were performed to investigate the distribution of PAI-2 in S1a cells and PAI-2 localization during induction of apoptosis. As shown in Figure 3a and b, intense diffuse cytoplasmic staining of PAI-2 is observed in untreated S1a cells with no apparent accentuation of the cell membrane. This is in contrast to the distinct pattern of cytokeratin staining (Figure 3f) and the periplasmic staining of PAI-2 in human keratinocytes (Jensen *et al.*, 1995). No staining was detected in the absence of antibody (data not shown) or in the non-PAI-2 expressing A2/7 cells (Figure 3e). Immuno-detection of PAI-2 was dependent on permeabilization of the S1a cells (data not shown), indicating the absence of PAI-2 on the cell surface. Treatment with TNF and cycloheximide to induce apoptosis for 1 h (Figure 3c) had no discernible effect on the PAI-2 cytoplasmic staining pattern. Following treatment with TNF and cycloheximide for 3 h, when A2/7 cells show distinct morphological changes associated with apoptosis (Dickinson *et al.*, 1995; data not shown), the diffuse cytoplasmic distribution of PAI-2 is not changed (Figure 3d). These results show that PAI-2 does not

distribute pericellularly, in response to apoptotic stimuli in these cells.

Immunofluorescent staining of PAI-2 Δ 65/87 in CDM1 cells showed a diffuse, cytoplasmic staining pattern (Figure

Figure 4 Localisation of PAI-2 Δ 65/87 by immunofluorescence. PAI-2 polyclonal antibody staining of PAI-2 Δ 65/87 in untreated CDM1 cells (a), wild-type PAI-2 expressing untreated S1a cells (b), and anti-sense PAI-2 expressing untreated A2/7 cells (c) (100 $\times$ magnification). The stronger staining of S1a cells reflects the higher amount of PAI-2 species expressed in these cells relative to CDM1 cells. Staining of PAI-2 non-expressing A2/7 cells shows the level of nonspecific background staining associated with the primary polyclonal antibody, as no staining was detected in the absence of antibody

4a) similar to that observed for wild-type PAI-2 (Figure 4b), although the intensity of staining was less, likely due to the lower level of PAI-2 species expressed by these cells. Treatment with TNF and cycloheximide for 2 h had no apparent effect on the localisation of PAI-2 Δ 65/87 (data not shown).

Subcellular fractionation studies were performed with both untreated S1a cells and S1a cells treated with the TNF and cycloheximide death signal to further establish the subcellular location of PAI-2. Expression of PAI-2 in each subcellular fraction was monitored by immunoblot analysis. PAI-2 was associated with the soluble cytosolic fraction, in both untreated and TNF and cycloheximide treated cells (Figure 5, Lane 4), while no PAI-2 could be detected in the membranous fractions. A minimal amount of PAI-2 was associated with both the soluble and insoluble nuclear fractions (Figure 5, Lanes 6 and 7) both in the presence and absence of TNF and cycloheximide. The amount of PAI-2 associated with the nuclear fractions was very low to negligible and highly variable, suggesting that it is nonspecific and/or a result of minor whole cell contamination of the isolated nuclei. Thus, the apparent staining of some nuclei in Figure 3 is probably nonspecific, possibly resulting from procedures used in cell fixation. No evidence for secretion of PAI-2 could be detected in culture supernatants of *in vivo* labelled S1a cells either in the presence or absence of TNF and cycloheximide (data not shown).

Discussion

In this study we demonstrate that a second domain found between the C and D α -helices of PAI-2 is functionally important for protection of PAI-2 from TNF α induced apoptosis. Mutants of PAI-2 lacking the C-D interhelical domain, while retaining functional proteinase inhibitory activity against uPA, failed to protect from apoptosis induced by TNF and were not resistant to interferon- γ induced cytostasis. Further, PAI-2 protection against TNF induced apoptosis was demonstrated to be due to intracellular PAI-2, and was not associated with cross-linking of PAI-2 to the plasma membrane.

Figure 5 Subcellular distribution of PAI-2 in untreated cells and in cells undergoing TNF-induced apoptosis. S1a cells, either untreated (–) or treated with TNF and cycloheximide (+) for 4 h were fractionated as described in the Materials and Methods. Lane 1, heavy mitochondrial fraction (P1); Lane 2, light mitochondrial fraction (P2); Lane 3, microsomal fraction (P3); Lane 4, soluble cytosolic fraction; Lane 5, untreated whole cell S1a lysate; Lane 6, insoluble nuclear fraction; Lane 7, soluble nuclear fraction

Transglutaminase-catalyzed crosslinking to Gln⁸³, Gln⁸⁴, and Gln⁸⁶ within the C-D interhelical region of PAI-2 *in vitro* has been reported (Jensen *et al*, 1994; Jensen *et al*, 1993) and this has been postulated to contribute to the observed pericellular distribution of PAI-2 in terminally differentiated keratinocytes (Lyons-Giordano *et al*, 1991; Jensen *et al*, 1995). Transglutaminases are a family of calcium dependent enzymes that catalyse the cross-linking of cellular constituents resulting in stabilization of structures such as keratinocyte cornified envelopes and fibrin clots (Knight *et al*, 1991). Increased transglutaminase activity is associated with apoptosis where cross-linking of many cellular components occurs, forming the apoptotic envelope (Knight *et al*, 1991; Fesus *et al*, 1996; Gentile *et al*, 1992). Given the role of transglutaminases in apoptosis and the *in vitro* evidence linking PAI-2 and transglutaminase-catalyzed crosslinking through the C-D interhelical domain, it was hypothesized that crosslinking of PAI-2 to cellular membranes may be important for PAI-2 protection from apoptosis. The immunostaining and subcellular fractionation data show that treatment of PAI-2 expressing cells with TNF and cycloheximide under conditions that induce apoptosis in control cells, does not demonstrably change the diffuse cytoplasmic distribution of PAI-2, and no evidence from fractionation studies could be found to implicate attachment of PAI-2 to cell membranes during the apoptotic process. Thus it appears that pericellular distribution of PAI-2 is not a requirement for, or an indicator of, the protective function of PAI-2 from apoptosis in these cells.

While the C-D interhelical region does not appear to be involved in the crosslinking of PAI-2 during the apoptotic process, its presence was clearly important for PAI-2 protection. This may suggest that the C-D interhelical region is important for the maintenance of other functional domains within the PAI-2 molecule or more likely, that specific amino acids within the C-D interhelical region itself are involved in the protective mechanism. Comparison of the primary sequence of PAI-2 with the sequences and crystal structures of other serpins indicates that the C-D interhelical region is positioned at the opposite end of the molecule to the RSL. The high structural conservation amongst serpins and the maintenance of uPA binding activity in the C-D deletion mutants argue strongly for the deletion having a minimal effect on the integrity of the RSL domain.

Comparison of the species conservation among the amino acids contained within the C-D interhelical region of PAI-2 reveals several candidates that may be critical for its biological function (highlighted by asterisks in Figure 1A). The amino acids Cys⁷⁹, Gln⁸³, Ile⁸⁵ and Gln⁸⁶ are conserved among all three species. Cys⁷⁹ may play a role in intermolecular or intramolecular interactions involving disulfide cross-linking of PAI-2 (Wun *et al*, 1987; Radke *et al*, 1990). The region spanning amino acids 73–76 and containing the motif PENFT is also conserved and may have functional significance. Mutagenesis studies targeting specific amino acid motifs will be required to localize those amino acids critical for the PAI-2 protective mechanism.

Interaction of the C-D interhelical domain and other proteins is likely to be important for resistance to apoptosis.

Recent studies demonstrate that the C-D interhelical region may participate in reversible interactions with cytosolic proteins (Jensen *et al*, 1996). Annexins I, II, IV and V have been demonstrated to bind the C-D interhelical region of PAI-2 *in vitro* in a calcium independent manner (Jensen *et al*, 1996). The annexins are a family of Ca^{2+} and membrane phospholipid binding proteins that participate in membrane-related translocation, exocytosis and signalling events. The process of apoptosis leads to characteristic morphological changes in the plasma membrane and cytoplasm which may well involve annexins. While the role of annexins in apoptosis is only beginning to be elucidated, members of this family bind to phosphatidylserine, whose externalization is a marker of apoptosis (Vermes *et al*, 1995), and are involved in the regulation of phospholipase A_2 activity during apoptosis in thymocytes (Sakamoto *et al*, 1996). It is possible that binding of annexins to the C-D interhelical domain of PAI-2 could play a role in conferring resistance to apoptosis. As well as the annexins, as yet unidentified proteins of 100, 70 and 50 kDa co-precipitate with PAI-2 following immunoprecipitation of dithio-bis(succinimidylpropionate)-crosslinked proteins. It is interesting to speculate that one or more of these proteins may be involved in the protective action of PAI-2. If this were the case, PAI-2 would not be unique among serpins in possessing a secondary functional domain involved in intermolecular interactions, in addition to the RSL domain recognised by its cognate serine proteinase(s). PAI-1, an inhibitor of plasminogen activators, is synthesized in a conformationally latent form which becomes activated following binding to vitronectin through a domain between C and E helices which is distinct from its RSL domain (Lawrence *et al*, 1994). The inactivation of target enzymes by several serpins that regulate blood clotting in plasma, such as antithrombin III and heparin cofactor II, is greatly accelerated by binding to glycosaminoglycans through heparin binding domains located in the respective D-helices (Church *et al*, 1992). The C-D interhelical domain in PAI-2 may play a similar role in regulating PAI-2 mediated inhibition of proteolytic activity within the cell during apoptosis. Interactions with this domain may regulate, or potentially activate, PAI-2 inhibitory activity towards an intracellular proteinase target and/or may participate in intra-molecular protein interactions. The function of this PAI-2 domain clearly requires further study to elucidate these possibilities.

Materials and Methods

Cells and cell culture

HeLa S3 cells were obtained from the American Type Culture Collection (ATCC CLL 2). HeLa cell lines stably transfected with wild-type PAI-2 cDNA (S1a, S1/5, S1n), P₁-Ala³⁸⁰-PAI-2 (ASM1), antisense PAI-2 cDNA (A2/7), and vector alone (CMV) under the control of the cytomegalovirus promoter (pRcCMV) were generated as described (Dickinson *et al*, 1995). Cell lines were incubated in 5% CO_2 and 95% air atmosphere at 37°C and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamate, 25 mM HEPES buffer, 60 mg/ml penicillin G (CSL, Melbourne, Australia) and 100 mg/ml streptomycin sulfate (CSL). Transfected lines were

maintained in 300 $\mu\text{g}/\text{ml}$ G418 (Gibco-BRL, Melbourne, Australia). Cell viability was determined by trypan blue dye exclusion. All cultures were checked routinely and determined to be mycoplasma free.

cDNA constructs

The C-D interhelical mutant of PAI-2 was engineered using a PCR-based loop-out mutagenesis procedure allowing deletion of 69 bp spanning nt 267–336 of PAI-2 cDNA. A 317 bp *Pst*I/*Pvu*II fragment, extending from the *Pst*I restriction enzyme site in the pUC8 polylinker of pJ7/PAI-2 (Schleuning *et al*, 1987) to the *Pvu*II restriction enzyme site within PAI-2 was excised from J7/PAI-2. Using the 317 bp fragment as the template, a 326 bp DNA fragment extending from *Hind*III in the pUC8 polylinker of J7/PAI-2 to the *Pst*I site within the PAI-2 cDNA was generated by PCR amplification using primers E and F, listed below. Primer E spans the pUC8 polylinker in J7/PAI-2 from the *Hind*III restriction enzyme site and includes an inserted *Not*I site. Primer F spans bases 249–266 fused to 337–376 of PAI-2 cDNA, thus incorporating the deletion of 69 bp.

Primer E: 5'-ACGCCAAGCTTGGCGGCCGCTCGACGGATCCCCGG-GAATTC-3'

Primer F: 5'-TTTATCTGCAGCTTGTGCCTGCAAAATCGCATCAG-GATAACTACCTCCCACTTCATTAAGTG-3'

The generated 326 bp DNA fragment was digested with *Hind*III and *Pst*I and cloned into J7/PAI-2 following excision of the wild-type PAI-2 *Hind*III/*Pst*I fragment to generate J7/PAI-2 Δ CD. The 1811 bp full length PAI-2 Δ CD cDNA was excised from J7/PAI-2 Δ CD by *Eco*RI digestion, end-filled and cloned into *Hind*III digested, blunt-ended pRc/CMV generating PAI-2 Δ 65/87 (Figure 1A). The correct orientation and sequence was verified by DNA sequencing.

Transfection and selection of stable PAI-2 CD mutant clones in HeLa cells

Approximately 1×10^7 cells in log phase were electroporated with 20 μg plasmid DNA at 0.25 volts, 960 μFD in RPMI 1640 containing 10% fetal calf serum. Following culture for 16 h, transfectants were selected with 0.8 mg/ml G418 (Gibco-BRL, Melbourne, Australia) essentially as described (Dickinson *et al*, 1995). Expression of the transgene in isolated clones was monitored by Northern and immunoblot analyses as described (Dickinson *et al*, 1995). Antibodies used were an anti-PAI-2 monoclonal antibody (#3750, American Diagnostica, Greenwich, CT, USA) or goat anti-PAI-2 polyclonal antibody (Biotech Australia, Pty. Ltd., Sydney, Australia). Protein concentration was determined by Bio-Rad Protein assay.

uPA inhibitory activity

uPA inhibitory activity was determined by standard chromogenic assay (Spectrozyme UK, American Diagnostica). Crude protein extracts from cell lines expressing wild-type PAI-2 (S1a) and PAI-2 Δ 65/87 (CDM1) had a similar range of inhibitory activity against uPA (80–100 inhibitory units/mg) when the data was corrected for the amount of PAI-2 present in the extracts estimated by Western blot analysis.

uPA binding activity

Low molecular weight (LMW)-uPA (33 kD) was labelled with ^{125}I to a specific activity of 40 mCi/mg as previously described (Jensen *et al*,

1990). Cells were rinsed in ice cold PBS prior to lysis on ice in (120 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM EDTA, 3 mM EGTA, 0.05 mg/ml aprotinin, 1 mg/ml SBTI, 2 mM PMSF, 2 mM iodoacetamide, 0.25 M sucrose, 1% Triton X100) at a concentration of 4×10^6 /ml (Jensen *et al*, 1994). The extract was cleared by centrifugation for 15 min at 4°C at 15 000 g. 15 μ l of the supernatant was incubated with approximately 50 000 c.p.m. 125 I-LMW-uPA for 60 min. The incubation was terminated by adding 15 μ l 2 $\times$ SDS-loading buffer, heating to 95°C for 5 min followed by analysis on 8–16% gradient SDS–PAGE. The gels were stained, dried and subjected to autoradiography overnight at –80°C using Hyperfilm MP (Amersham International, Buckinghamshire, UK). Prestained molecular weight markers were from Novex, USA.

TNF cytotoxicity assays

Cells were seeded at 10^4 cells per well in triplicate in 96-well plates for 16 h prior to the treatments described in the Figure Legends. For assay by crystal violet staining, the cells were washed in PBS and stained with 0.2% crystal violet in 10% ethanol or 10% formaldehyde. The dye was eluted with 33% acetic acid and the absorbance was measured at 480 nm. For measurement by MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) was added to a final concentration of 0.4 mg/ml per well and incubated for 4 h. Plates were centrifuged at 800 $\times$ g for 5 min and supernatant removed. MTT crystals were dissolved in DMSO and the absorbance was measured at 570 nm.

Immunohistochemistry

S1a and A2/7 cells were seeded in sterile 8-well chamber slides (Nunc, Australia) and allowed to adhere overnight to replacing the supernatant with control media or media containing 10 ng/ml TNF and 10 μ g/ml CHX. After incubation for 1 h or 3 h, the cells were washed and fixed in 100% cold methanol for 2 min. Slides were then rinsed in PBS, and endogenous peroxidase activity was inhibited with 0.3% H₂O₂ and 0.1% sodium azide, and non-specific antibody binding was inhibited by immersion in 4% non-fat skim milk powder. Endogenous biotin activity was blocked with 0.1% avidin (Dakopatts, Carpinteria, CA) followed by 0.01% D-biotin. Non-specific antibody binding was further inhibited by pre-incubating the slides with 10% non-immune goat serum. Cells were incubated with primary antibody (anti PAI-2 monoclonal antibody, American Diagnostica), or no antibody, for 30 min at room temperature, then biotinylated goat anti-mouse immunoglobulins (Zymed, San Francisco, CA) followed by streptavidin-horseradish peroxidase complex (Zymed, San Francisco, CA). Antigenic sites were demonstrated using 3,3'-diaminobenzidine as chromogen with H₂O₂ as substrate. Cells were counterstained with Harris' haematoxylin and mounted in Permount.

Immunofluorescence

S1a, A2/7 and CDM1 cells were seeded in sterile 8-well chamber slides (Nunc, Australia) and allowed to adhere overnight prior to replacing the supernatant with control media or media containing 10 ng/ml TNF and 10 μ g/ml CHX. After incubation for 2 h, the cells were washed and fixed in 100% cold methanol for 2 min and allowed to dry. Slides were then rinsed in PBS, blocked with PBS containing 4% nonfat milk powder, and then with PBS containing 0.3% Triton X100. Non-specific antibody binding was further inhibited by pre-incubating the slides with 10% non-immune fetal calf serum. Cells were incubated with primary antibody (polyclonal anti-PAI-2 antibody, Biotech

Australia Pty. Ltd., Sydney, Australia), or no antibody, overnight at 4°C. After several washing and blocking steps, as above, antigenic sites were visualized with FITC-labelled sheep anti-goat antibody (Silenus, Melbourne, Australia).

Subcellular distribution of PAI-2

Subcellular fractionation was adapted from Yuan *et al*. (1996). Subconfluent S1a cells (2×10^7) were incubated with control media or media containing 10 ng/ml TNF and 10 μ g/ml CHX for 4 h. The cells were harvested by gentle scraping and after 3 $\times$ PBS washes, were resuspended in 100 μ l chilled hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.25 M sucrose, and 1 mM of the serine proteinase inhibitor, AEBSF). Suspensions were incubated for 30 min on ice and subjected to 20–25 strokes of Dounce homogenisation. The nuclei were then pelleted by centrifugation at 1000 g for 6 min at 4°C. The cytoplasmic fraction was subjected to sequential centrifugation at 4°C as follows: 300 g for 10 min to pellet the heavy mitochondrial fraction (P1), 17 000 g for 10 min to pellet the light mitochondrial fraction (P2), and 100 000 g for 45 min to pellet the microsomal fraction (P3). The P2 fraction contains mitochondria, lysosomes, peroxisomes, Golgi membranes and some endoplasmic reticulum. The P1 fraction contains predominantly mitochondria, but may also contain minor components from the P2 fraction entrapped during pelleting. The P3 fraction contains membrane vesicles, e.g. endosomes, trans-Golgi vesicles and disrupted plasma membrane. The final supernatant was the soluble cytosolic fraction. The nuclei pelleted in the first centrifugation were resuspended in 0.5 ml of hypotonic buffer without sucrose and washed through a 0.25 M sucrose cushion (0.5 ml) at 1600 g for 5 min. Purification through the sucrose cushion removes cell debris from the nuclear preparations, but does not remove possible minor contamination with whole, unbroken cells. The nuclei were lysed in hypotonic buffer containing 0.5% Triton X100 at 4°C for 30 min and centrifuged at 14 000 g for 10 min to pellet the insoluble nuclear fraction. The supernatant portion presents the Triton-soluble nuclear fraction. Aliquots of each fraction representing the same cellular volume were analyzed on 10% SDS–PAGE reducing gels and immunoblotted as above.

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References

- Antalis TM, Clark MA, Barnes T, Lehrbach PR, Devine PL, Schevzov G, Goss NH, Stephens RW and Tolstoshev P (1988) Cloning and expression of a cDNA coding for a human monocyte derived plasminogen activator inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* 85: 985–989

- Antalis TM and Dickinson JL (1992) Control of plasminogen-activator inhibitor type 2 gene expression in the differentiation of monocytic cells. *Eur. J. Biochem.* 205: 203–209
- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159
- Church FC, Pratt CW and Whinna HC (1992) A comparison of three heparin-binding serine proteinase inhibitors. *J. Biol. Chem.* 267: 8795–8801
- Dickinson JL, Bates EJ, Ferrante A and Antalis TM (1995) Plasminogen activator inhibitor-type 2 inhibits TNF α -induced apoptosis: Evidence for an alternate biological function. *J. Biol. Chem.* 270: 27894–27904
- Feinberg AP and Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6–13
- Fesus L, Madi A, Balaihy Z, Nemes Z, Szondy Z (1996) Transglutaminase induction by various cell death and apoptosis pathways. *Experientia* 52(10-11): 942–949
- Gan H, Newman GW and Remold HG (1995) Plasminogen activator inhibitor type 2 prevents programmed cell death of human macrophages infected with mycobacterium avium Serovar 4. *J. of Immunol.* 155: 1304–1315
- Gentile V, Thomazy V, Piacentini M, Fesus L and Davies PJA (1992) Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion. *J. Cell. Biol.* 119: 463–474
- Jensen PH, Christensen EI, Ebbesen P, Gliemann J and Andreasen PA (1990) Lysosomal degradation of receptor-bound urokinase-type plasminogen activator is enhanced by its inhibitors in human trophoblastic choriocarcinoma cells. *Cell Reg.* 1: 1043–1056
- Jensen PH, Cressey LI, Gjertsen BT, Madsen P, Melgren G, Hokland P, Gliemann J, Døskeland SO, Lanotte M and Vintermyr OK (1994) Cleaved intracellular plasminogen activator inhibitor 2 in human myeloleukaemia cells is a marker of apoptosis. *Brit. J. Cancer.* 70: 834–840
- Jensen PH, Jensen TG, Laug WE, Hager H, Gliemann J and Pepinsky B (1996) The exon 3 encoded sequence of the intracellular serine proteinase inhibitor Plasminogen activator inhibitor-2 (PAI-2) is a protein binding domain. *J. Biol. Chem.* 271: 26892–26899
- Jensen PH, Lorand L, Ebbesen P and Gliemann J (1993) Type-2 plasminogen-activator inhibitor is a substrate for trophoblast transglutaminase and Factor XIIIa. *Eur. J. Biochem.* 214: 141–146
- Jensen PH, Schuler E, Woodrow G, Richardson M, Goss N, Hojrup P, Peterson TE and Rasmussen LK (1994) A unique interhelical insertion in plasminogen activator inhibitor-2 contains three glutamines Gln⁸³ Gln⁸⁴ Gln⁸⁶ essential for transglutaminase-mediated cross-linking. *J. Biol. Chem.* 269: 15394–15398
- Jensen PJ, Wu Q, Janowitz P, Ando Y and Schechter NM (1995) Plasminogen activator inhibitor type 2: an intracellular keratinocyte differentiation product that is incorporated into the cornified envelope. *Exp. Cell Res.* 217: 65–71
- Kiso U, Kaudewitz H, Henschen A, Astedt B, Kruihof EKO and Bachmann F (1988) Determination of intermediates products and cleavage site in the reaction between plasminogen activator inhibitor type-2 and urokinases. *FEBS Letts.* 230: 51–56
- Knight CR, Rees RC and Griffin M (1991) Apoptosis: a potential role for cytosolic transglutaminase and its importance in tumour progression. *Biochem. Biophys. Acta.* 1096: 312–318
- Kruihof EKO, Baker M and Bunn CL (1995) Biological and clinical aspects of plasminogen activator inhibitor type 2. *Blood* 86: 4007–4024
- Kumar S and Baglioni C (1991) Protection from tumor necrosis factor-mediated cytotoxicity by overexpression of plasminogen activator inhibitor type 2. *J. Biol. Chem.* 266: 20960–20964
- Lawrence DA, Berkenpas MB, Palaniappan S and Ginsburg D (1994) Localization of vitronectin binding domain in plasminogen activator inhibitor-1. *J. Biol. Chem.* 269: 15223–15228
- Loebermann H, Tokuoka R, Deisenhofer J and Huber R (1984) Human α 1-proteinase inhibitor: Crystal structure analysis of two crystal modifications molecular model and preliminary analysis of the implications for function. *J. Mol. Biol.* 177: 531–556
- Lyons-Giordano B, Loskutoff D, Chen CS, Lazarus G, Keeton M and Jensen PJ (1994) Expression of plasminogen activator inhibitor type 2 in normal and psoriatic epidermis. *Histochem. J.* 101: 105–112
- Radtko KP, Wenz KH and Heimbürger N (1990) Isolation of plasminogen activator inhibitor-2 (PAI-2) from human placenta. Evidence for vitronectin/PAI-2 complexes in human placenta extract. *Biol. Chem. Hoppe-Seyler* 371: 1119–1127
- Remold-O'Donnell E (1993) The ovalbumin family of serpin proteins. *FEBS Letters* 315 (2): 105–108
- Sakamoto T, Repasky WT, Uchida K, Hirata A and Hirata F (1996) Modulation of cell death pathways to apoptosis and necrosis of H₂O₂-treated rat thymocytes by lipocortin I. *Biochem. Biophys. Res. Comm.* 220: 643–647
- Samia JA, Alexander SJ, Horton KW, Auron PE, Byers MG, Shows TB and Webb AC (1990) Chromosomal organization and localization of the human urokinase inhibitor gene: Perfect structural conservation with ovalbumin. *Genomics* 6: 159–167
- Schleuning W-D, Medcalf RL, Hession C, Rothenbühler R, Shaw A and Kruihof EKO (1987) Plasminogen activator inhibitor 2: regulation of gene transcription during phorbol ester-mediated differentiation of U-937 human histiocytic lymphoma cells. *Mol. Cell. Biol.* 7: 4564–4567
- Testa JE and Quigley JP (1990) The role of urokinase-type plasminogen activator in aggressive tumor cell behavior. *Canc. Metast. Rev.* 9: 353–367
- Vermes I, Haanen C, Steffensnacken H and Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods.* 184: 39–51
- Wong GHW, Elwell JH, Oberley L and Goeddel DV (1989) Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity to tumor necrosis factor. *Cell* 58: 923–931
- Wun T-C and Reich E (1987) An inhibitor of plasminogen activation from human placenta. Purification and characterization. *J. Biol. Chem.* 262: 3636–3653
- Ye RD, Ahern SM, Le Beau MM, Lebo RV and Sadler JE (1989) Structure of the gene for human plasminogen activator inhibitor-2. The nearest mammalian homologue of chicken ovalbumin. *J. Biol. Chem.* 264: 5495–5502
- Ye RD, Wun TC and Sadler JE (1987) cDNA cloning and expression in *Escherichia coli* of a plasminogen activator inhibitor from human placenta. *J. Biol. Chem.* 262: 3718–3725
- Yuan Z-M, Huang Y, Kraeft S-K, Chen LB, Kharbada S and Kufe D (1996) Interaction of cyclin-dependent kinase 2 and the Lyn tyrosine kinase in cells treated with 1-beta-D-arabinofuranosylcytosine. *Oncogene* 13: 939–946
- Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM and Salvensen GS (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. *J. Biol. Chem.* 272: 7797–7800