Acta Biochimica Polonica

Vol. 43 No. 3/1996

419–430

QUARTERLY

Minireview

The intracellular serpin family*

Renata Korpula-Mastalerz and Adam Dubin[a]

Institute of Molecular Biology, Jagiellonian University, Al. A. Mickiewicza 3, 31-120 Cracow, Poland

Received: 5 June, 1996

Key words: elastase inhibitor, ovalbumin family, cytosol inhibitor, inhibitor function

The serpins are widely distributed, structurally related family of proteins with diverse functions. Most of the known serpins are proteinase inhibitors, the majority being found as secreted species, however, there are a few that occur intracellularly and their physiological role remains unknown. Most of the intracellularly occuring serpins have been classified into the ovalbumin subfamily. The possible phytogenetic tree of 14 intracellular serpins is presented.

The serpins are a constantly expanding family of structurally related proteins, similar in amino-acid sequence, overall tertiary structure and mechanism of inhibition. Most of the known serpins are inhibitors of proteinase, the majority being found as secreted species. However, there are a few serpins which occur intracellularly and their role in the organism, while still being elucidated, nonetheless in most cases remains unknown. The ovalbumin family of intracellular serpins consists of members which are largely non-secreted due to the fact that they lack a cleavable signal sequence. Ovalbumin serpins share similarities in gene structure indicating their close evolutionary links and a possibility of divergent evolution from a common ancestor. A few other characteristic features distinguish this subfamily from a large superfamily of serpins, the criteria being: lack of a cleavable translocation signal, lack of the N-terminal extension regions common to other serpins, as well as lack of the C-terminal extension. Moreover, ovalbumin serpins have a serine rather than asparagine, as have most serpins, at the penultimate position 390 and a variable residue rather than valine at position 388. A peculiarity of this group of proteins is that some of its members, such as ovalbumin, plasminogen activator inhibitor-2, or maspin can be fully or partially secreted due to the possession of an internal signal sequence. However, most other members, such as leucocyte elastase inhibitors from human (HEI), horse (HLEI) or pig (PLEI), human proteinase

*This work was supported in part by a grant No. 0639/P2/94/07 to A.D. from the State Committee for Scientific Research (KBN).

[a]To whom correspondence should be addressed.

Abbreviations: α_1-PI, α_1-proteinase inhibitor; ATIII, antithrombin III; B-43, inhibitor from bovine brain; BLEI, bovine leucocyte elastase inhibitor; CrmA, cytokine response modifier A (cowpox virus serpin); HEI, human elastase inhibitor; HLEI, horse leucocyte elastase inhibitor; ICE, interleukine-1β converting enzyme; LICI-1 and -2, *Limulus* intracellular coagulation inhibitor; LPS, lipopolysaccharide; PAPI-2, crayfish *Pacifastacus* proteinase inhibitor; t-PA and u-PA, tissue- and urokinase-plasminogen activator; PAI-1 and -2, plasminogen activator inhibitor type-1 and -2; PCR, polymerase chain reaction; PI-6, PI-8 and PI-9, human proteinase inhibitors; PI-10, bone marrow-associated serpin (bomapin); PLEI, pig leucocyte elastase inhibiotr; PMN, polymorphonuclear leucocyte; PTI, placental thrombin inhibitor; SCCA, squamous cell carcinoma antigen; TNF-α, tumour necrosis factor α.

inhibitor (PI-6), its relatives: PI-8 and PI-9, squamous carcinoma cell antigen (SCCA), leupin, bomapin (PI-10), *Limulus* intracellular coagulation inhibitors (LICI-1, LICI-2) are bound to remain in the cytosol. However, some new, recently defined serpins were also found to be non-secreted, such as: B-43 from bovine brain, viral serpin CrmA, crayfish PAPI-2 or plant serpins: barley protein Z and wheat like-Z serpin. Since the pool of intracellularly occurring serpins is still enlarging it is becoming hard to classify the new emerging species; possibly, after further examination of their structure, some of them will meet the criteria classifying them to the ovalbumin family of intracellular serpins. Nonetheless, they share two important features in common, which are the intracellular localization and a more or less miscellaneous role in the organism.

GENERAL CHARACTERISTICS OF SERPINS

Serpins are a large family of structurally related proteins most of which act as inhibitors of serine proteinases and many of which are involved in regulation of important physiological processes such as blood coagulation, fibrinolysis and inflammation. In some cases, proteins classified as serpins have lost their inhibitory activity and developed specialized functions such as hormone transport (cortisol-binding globulin and thyroxin-binding globulin), blood pressure regulation (angiotensinogen) or, like ovalbumin, have no recognized function [1, 2]. Serpins share a few very distinctive features which account for their classification into a single, structurally related superfamily. The family members have a remarkably highly conserved three dimensional structure despite the lack of significant homology in the amino-acid sequence between the members [3]. Inhibitory serpins are characteristic in having a mobile reactive centre which is exposed on a peptide loop. They are also known to form stable complexes with their respective proteinases because the reactive centre residue functions as an ideal substrate [4]. The inhibition is due to a tight 1:1 molar association between a proteinase and serpin, which is best modeled as formation of a Michaelis-like complex [5]. This relationship is similar to a proteinase's interaction with its substrate, progressing to the point of cleavage of the P_1-P'_1 peptide bond and formation of the first covalent acyl-intermediate. Cleavage is coupled to a rapid shift in the relative position of the enzyme and its inhibitor, sufficiently distorting an active site geometry to prevent deacylation and eventually effectively trapping the enzyme. Upon cleavage, a reactive centre loop is incorporated into the body of the inhibitor bringing the inhibitor closer to the lowest free energy state resulting in trapping of the proteinase in the acyl-enzyme intermediate form [5]. The association of proteinase and inhibitor is very rapid, with second order rate constants of 10^5–10^7 $M^{-1}s^{-1}$, however, the complex dissociates very slowly yielding an active proteinase and a cleaved inhibitor.

A typical structure of serpin resembles that of an α_1-proteinase inhibitor, a molecule where helices constitute 30% of the structure and three β-sheets 40%. One of the sheets (sheet A), made of six anti-parallel strands, is a predominant feature of serpins with the reactive-centre peptide extending from the middle strand, designated S4 [6]. The similarity in tertiary structure of serpins allows for classification of its members, however, there are regions where some members have a different amino-acid sequence and these differences give clues as to their individual functions. The exposed portion of the loop P_8-P'_5 is one of the few regions of the molecule that is highly variable, being specific for the target proteinase. Immediately proximal to the exposed loop (towards the amino end) is the hinge area of the molecule, P_9-P_{15}, where strand 5 of the β-sheet turns to re-enter as strand 4 and then leaves the sheet to form the reactive-centre loop. The hinge region is strongly conserved in all the inhibitory serpins, with P_9-P_{12} being primarily alanines [7]. Mutations in this region result in a loss of inhibitory activity when the reactive-centre loop is locked in the extended position, as it can be seen in molecules of angiotensinogen or egg-white ovalbumin. Serpins can adopt alternative conformations depending on the degree to which a peptide loop has been incorporated into the sheet to form the middle, fourth strand. Crystallographic structures show that a cleaved inhibitory serpin completely incorporates its cleaved loop back into the A sheet with a consequent separation of the P_1 residue from the

P'_1 residue by 70 Å. The shift is accompanied by an increase in overall stability of the molecule, as it gains thermal stability and becomes less susceptible to SDS treatment, presumably due to reorganization of the five-stranded β-sheet A from a mixed parallel-antiparallel arrangement to a six-stranded, predominantly anti-parallel β-sheet [2]. The molecule is said to undergo from a stressed (S) to relaxed (R) conformation. The serpins ability to vary conformations of the reactive-centre allows modulation of their inhibitory activity and also protects the circulating inhibitor against a proteolytic attack. The external position of the reactive-centre loop makes it vulnerable to proteolytic cleavage and the serpins are readily inactivated in this way by numerous proteinases. In fact, the inhibitory activity of a serpin depends on its ability to fold its reactive-centre loop. Serpins which are not proteinase inhibitors are susceptible to proteolytic cleavage in the exposed loop but do not undergo a stressed to relaxed conformation switch [8].

Two structural extremes of the potential loop configuration have been determined based on crystal structures of non-functional serpins: ovalbumin and latent plasminogen activator inhibitor-1 (PAI-1) [9, 10]. In ovalbumin, strand S4 is not inserted at all into sheet A and a pseudo-reactive site peptide loop is exposed on the final turn of a mobile three-turn helix extending from the underlying molecule [9]. Latent PAI-1, on the other hand, has a reactive site loop fully incorporated into sheet A as observed with cleaved serpins [10]. Other serpins when exposed to mild denaturing agents at low temperatures can also be induced to form an inactive-locked conformation. Also, incubation with small synthesized reactive-site loop peptides can induce formation of a binary complex in which a synthesized peptide will insert into the space otherwise occupied by strand S4 [3]. Only recently the spatial structure of active, functional antithrombin has been described by Carrell *et al.* [11] and for the first time a reconstruction of the intact reactive-centre loop was possible. The study was based on crystal structure of a dimer formed by linkage of an inactive molecule of antithrombin with an incorporated reactive loop and an active molecule of antithrombin which resembles that of ovalbumin, with an exposed reactive loop and a five-stranded β-sheet A. As opposed to ovalbumin, there is a complete loss of helicity in the loop and a slight opening of the β-sheet A which would allow potential insertion of the loop. In fact, the active molecule has a conformation which keeps the molecule ready to react with its proteinase, that is, its reactive loop is neither fully exposed nor totally incorporated into the A-sheet. In keeping with earlier predictions, Carrell *et al.* [11] confirmed that the active molecule of antithrombin has a flexible reactive loop that can be considered to adopt a series of conformations, and this appears to be a major characteristic of inhibitory serpins. The most recent communication of Song *et al.* [12] reveals a crystal structure of uncleaved α_1-PI where a reactive loop adopts a distorted helical conformation similar to that of antithrombin. However, in contrast to antithrombin, no wider opening between the strands s3A and s5A in the β-sheet A is observed, implying that partial insertion of N-terminal residues of the reactive loop does not takes place in α_1-PI [12].

The vast majority of serpins is being secreted as their protease targets function in the extracellular environment. These serpins, however, have been closely investigated and described in much detail. In contrast to the amount of information available on the regulatory activities of the extracellular serpins that function either in plasma, the pericellular space of tissue or at the cell surface, very little is known regarding the intracellular members of this superfamily. Since, in most cases, the intracellular inhibitor is spatially separated from the proteinases it can be postulated that its physiological function is protection of cytoplasmic proteins in the intact cytosol of cells, or inactivation *in vivo* of released proteinases only in the case of total cell disruption. Most of the intracellularly occurring serpins have been classified into a subfamily of ovalbumin serpins which is continuously expanding. Nonetheless, there are still emerging new intracellular serpins which are hard to classify. But still, one feature distinguishing all of them is their more or less miscellaneous role in the organism.

SUBFAMILY OF OVALBUMIN SERPINS (OV-SERPINS)

The ovalbumin family consists of serpins which are largely non-secreted, with its repre-

sentative — ovalbumin as a major exception. The species so far classified as ovalbumin serpins are: a chicken oviduct protein — ovalbumin, a product of chicken gene Y (not studied at the protein level), human plasminogen activator inhibitor-2 (PAI-2), squamous cell carcinoma antigen (SCCA), leucocyte elastase inhibitor derived from humans (HEI), horse (HLEI), and pig (PLEI), human proteinase inhibitor (PI-6), its relatives: PI-8 and PI-9, leupin, bomapin (PI-10) and maspin. Some recently defined serpins such as: B-43 isolated from bovine brain, *Limulus* (horseshoe crab) intracellular coagulation inhibitors (LICI-1 and LICI-2), PAPI-2 a crayfish serpin or a viral serpin CrmA seem to be close in structure to ovalbumin serpins, however, they either do not fulfill all the requirements to be classified into this family or still remain to be examined more closely.

A classification of serpins to ovalbumin family is based on a few commonly shared characteristics. Ov-serpins share similarities in gene structure pointing to close evolutionary links and a possibility of divergent evolution from a common ancestor [13]. In most cases, the genes of the ovalbumin family members which have been characterized contain seven exons with nearly identical placement of the same junctional type introns within the coding sequence [13, 14]. Very characteristic of ovalbumin serpins is the lack of a typical, cleavable signal peptide that would promote their translocation across a membrane. Despite that, ovalbumin, PAI-2, SCCA and maspin are still able to pass through the endoplasmic reticulum and be efficiently secreted [13]. Most serpins possess an amino-terminal hydrophobic signal sequence which is removed by signal peptidase during translocation. Therefore it has been suggested, that efficient secretion requires a hydrophobic sequence to initiate translocation and a site for signal peptidase cleavage to separate the hydrophobic sequence from the secreted protein [15]. Ovalbumin and PAI-2 apparently possess an internal sequence, made up of hydrophobic regions which act as a translocation sequence, the difference being that it is not cleaved when the protein passes through the endoplasmic reticulum membrane [15]. A signal probably resides in the first 70 amino acids of ovalbumin and PAI-2 and it remains fused to the protein upon its translocation [15]. Most probably, in the case of other non-secreted ov-serpins a charge distribution around a potential hydrophobic signal sequence accounts for their cytosolic accumulation; for example HLEI contains six charged amino acids [16] in positions which in PAI-2 are occupied by a hydrophobic or a polar amino-acid residues [14]. Nevertheless, all ov-serpins can be said to share a common feature, which is the lack of a cleavable translocation signal. Ov-serpins also lack the N-terminal extension regions common to other serpins. Relative to α_1-PI they begin at amino acid number 23. Another feature distinguishing them from a larger family of serpins is the lack of C-terminal extension (they terminate at Pro376, the equivalent of Pro391 in α_1-PI), even though a structural importance has been suggested for these last residues because mutants of α_1-PI shorter by even one residue are degraded in the cell [14]. Ov-serpins have serine rather than asparagine, as in most serpins, at the position 390 of α_1-PI and a variable residue rather than valine at position 388 [14]. Nearly the same gene organization pattern for all members of ov-serpins suggests very close evolutionary links between them even though they have different functions and come from different species. Albeit a structural resemblance allows ovalbumin serpins to belong to the same class of related proteins, their more or less speculative functions are strikingly varied. Some, like ovalbumin, have lost their original inhibitory function and the character of inhibitory specificity of others is largely determined by the nature of the reactive site P_1-P'_1 peptide bond.

PAI-2, originally derived from human placenta, is a serine proteinase inhibitor which regulates plasmin generation by inhibiting urokinase and tissue plaminogen activator. It is a unique example of protein that by design has an inefficient translocation signal allowing, as already mentioned, the synthesis of both extra and intracellular forms. PAI-2 is supposed to secure homeostasis during pregnancy and delivery which implies its functional relationship to ovalbumin [17]. It was noted that the synthesis of PAI-2 in monocytes or endothelium can be drastically affected by inflammatory mediators and, considering its insensitivity to oxidants, it is possible that it has a function in the inflammatory reaction [14, 15, 18]. The latter argument is strengthened by the observation that TNF-α induces PAI-2 gene transcription in mononuclear phagocytes and fibroblasts [14].

Since TNF-α induces most inflammatory and cell mediated immune reactions, it is possible that the induced release of PAI-2 may allow deposition of fibrin by inhibiting plasminogen activation [14, 19]. According to Belin [14], PAI-2 can also have a cytoprotective role resembling the anti-apoptotic action of the oncogene *bcl-2* gene which is in close proximity of the PAI-2 gene on the chromosome 18.

Another member of the ovalbumin family, homologous in 50.1% to PAI-2, is a human leucocyte elastase inhibitor (HEI) which was first found in the cytosol of human monocyte-derived macrophages [20] and then in the granules of neutrophils — PMNs [21]. It has been long investigated with respect to its possible extracellular localization but can be quite surely said to occur intracellularly. HEI is an efficient inhibitor of neutrophil elastase, proteinase-3 and, to a lesser degree, cathepsin G [22]. Its close relatives were found in horse, ovine and porcine PMNs [23–26]. They all function to inhibit elastase showing more or less broad specificity. For example HLEI, unlike human elastase inhibitor, shows a broad specificity and inhibits not only elastases found in human and horse leucocytes but also elastase from pig pancreas and dog leucocyte [23, 27]. It also shows a remarkably high antichymotrypsin activity while antitrypsin activity is very low [23]. A peculiar aspect of the intracellular elastase inhibitor is that the content of the inhibitor with respect to its substrate found in the granules varies quite significantly from species to species and is the main cause of the debate about the inhibitor's hypothetical function. For example, in human PMNs the content of elastase is much higher than that of its inhibitor (3 pg and 0.03 pg per cell) [21], while in horse PMNs both proteins occur in similar amounts (0.5 pg per cell) [27]. This difference is substantial but its role remains to be explained. When it comes to primary structure, elastase inhibitors purified from bovine (BLEI), porcine (PLEI), or horse (HLEI) leucocytes are very highly homologous (78% identity for P_1-P'_{22} position) [28]. They all show an outstanding similarity of primary structures and this similarity extends to active site sequences, reflecting functional relationship of elastase inhibition. Similarly, no carbohydrate attachment to either species of elastase inhibitors from mammalian PMNs was found suggesting their intracellular localization. The only communication we have of a secreted leucocyte elastase inhibitor is that which Remold-O'Donnell & Lewandowski found in guinea pig macrophages [29]. However, our reluctance to assume the extracellular presence of the elastase inhibitors from earlier described species is due to the uncertain mechanism of their release. Nevertheless, many workers have postulated that compartmentation of the elastase inhibitor and its confinement to cytosol of leucocytes of most investigated species suggests its main function to be the control intracellular protein turnover rather than proteolysis outside the cell [21, 23].

More recently, a novel intracellular thrombin inhibitor was isolated from human placenta and later identified in monkey kidney epithelial cell line BSC-1. It was designated CAP (cytoplasmic antiproteinase) or PTI (placental thrombin inhibitor) [30, 31] but to avoid confusion the Genome Data Base organization has recommended that it be known as proteinase inhibitor 6 (PI-6). The purified 38-kDa protein was found to inhibit thrombin, trypsin, urokinase and factor Xa, but not elastase [30]. Its amino-acid sequencing showed the highest homology to HLEI (51%) and PAI-2 (49%), and some other common features like lack of classical signal sequence, blockage of N-terminus, and sensitivity to oxidation [32]. All these features and a comparatively lower molecular mass suggesting the absence of covalently attached carbohydrate, are in agreement with intracellular localization of PI-6 [30–32]. However, the physiological function of this novel cytoplasmic inhibitor is still unknown. Coughlin *et al.* [32] suggest that due to the inhibitors intracellular confinement, thrombin is not a natural target for PI-6, unless it can be secreted under certain circumstances like PAI-2. In this case, however, the oxidation sensitivity of PI-6 should limit its action to the immediate vicinity of the site of its release. Even though all evidences based on molecular structure and amino-acid similarity suggest that PI-6 belongs to the ov-serpin subfamily. Sun *et al.* [33] propose that PI-6 has recently diverged from ov-serpins but might not belong to the same group. The main reason lays in one intron (intron C), which is present in PAI-2 and ovalbumin genes, and absent in the PI-6 gene; other than that it is identical as to the number, position, and phas-

ing of the intron/exon boundaries. Also localization of the genes for PI-6 and for other ov-serpin family members on different chromosomes implies that they can not be part of the same gene cluster [33]. Nevertheless, PI-6 is very closely related to ov-serpins. In fact, two novel gene sequences encoding regions very similar to those of PI-6 have been identified by Sprecher *et al.* [34] on screening human placental cDNA library. These two genes code for proteins designated PI-8 and PI-9 which are both very similar in structure to ov-serpins, demonstrating all the unique features mentioned above. The PI-8 was shown to form a complex with human thrombin, and its P_1-P'_1 centre was identified as Arg-Cys [34]. PI-9 has Glu-Cys as its P_1-P'_1 residues, but still its possible target protein has not been found. PI-9 is, however, expected to be an inhibitory serpin, since its hinge region located close to that reactive centre is highly homologous with that of all inhibitory serpins. It is a peculiar feature of PI-9 that it seems to be the first human serpin identified which has an acidic residue in the reactive-centre P_1 position [34]. In addition, the reactive centre loop of PI-9 exhibits 54% identity with the residues found in the reactive-centre loop of the cowpox virus CrmA serpin (described below) also having an acidic P_1 residue. Thus, PI-9 appears to be the closest mammalian serpin relative of the viral serpin reported to date [34].

Another member of ovalbumin family is a tumour associated protein first isolated from squamous cell carcinoma tissue of the uterine cervix and referred to as squamous cell carcinoma antigen (SCCA). It is closely homologous to chicken gene Y protein (47%), PAI-2 (45%) and ovalbumin (42%) [35]. Because of the highly conserved hinge region consisting mainly of alanines it possesses an inhibitory function [35]. In fact, it was found to inhibit cystein proteinases such as papain and cathepsin L but showed no inhibitory activity against a wide range of serine proteinases [36]. Its reactive site P_1-P'_1 is Ser-Ser, which has not been found in other members of the serpin family. Considering that cancer tissue is more apt to release SCCA than normal squamous epithelium, Suminami *et al.* [35] speculate that SCCA may act as a modulator of a host immune response against tumour cells. Only recently, a very peculiar novel serpin gene has been identified using PCR for sequence alignment of conserved regions of several proteins — members of the intracellular ov-serpin subfamily. The gene product, designated leupin, is expressed in human placenta and in HeLa cell lines [36]. The predicted protein shows high sequence similarity of 91.8% to one of the ov-serpins — SCCA. There are only six amino-acid differences in the reactive loop sequence including the predicted P_1 position which is a leucine residue in leupin, as opposed to a serine residue in SCCA [36]. These serpins are likely to have different target proteases; it may be expected that leupin will inhibit chymotrypsin or cathepsin G because it has the same cleavage site as α_1-antichymotrypsin, or perhaps it will also show inhibitory activity against a cysteine proteinase in the same manner as does SCCA. Barnes & Worrall [36] suggest that leupin and SCCA represent a relatively recent gene duplication event, since the two serpins show a closer evolutionary divergence than previously seen in mammalian serpins.

The cross-class inhibitory activity demonstrated by SCCA is not an only exception. Such activity has previously been seen in the viral serpin CrmA. CrmA is a 38-kDa protein encoded by a viral gene *crmA* (cytokine response modifier gene) the amino-acid sequence of which is similar to those of members of the serpin superfamily [37]. It can not be classified as an ovalbumin serpin, however, it probably belongs to a group of intracellular species since it was not found to be secreted. This serpin's predicted reactive site is not similar to any of the known serine proteinase inhibitors, as it has aspartic acid and cysteine as residues at the P_1-P'_1 position, and its target was found to be specifically an interleukin-1β converting enzyme (ICE). ICE is responsible for processing of interleukin-1β, therefore the serpin encoded by *crmA* gene can prevent proteolytic activation of interleukin-1β, and thereby suppress the host response to infection. However, preliminary characterizations of ICE suggest that it is not a serine proteinase but a cysteine proteinase [37]. These findings are significant since they confirm that the proteinase specificity of other serpins may extend beyond serine proteinases, as in the case of SCCA and CrmA.

Another novel serpin which is closest in its structure to ov-serpins has been described by Riewald & Schleef [38]. It is bomapin (bone

marrow-associated serpin), expressed specifically in the human bone marrow. It has a low molecular mass of 45 kDa and is closest in structure to PAI-2 (they share 48% amino-acid homology). It was found to form SDS-stable complexes with thrombin and trypsin. All of the structural features that distinguish ov-serpins from the family of serpin proteins are also met by bomapin, that is lack of a C-terminal extension region (ending with the equivalent of Pro391 in α_1-PI), serine rather than asparagine at the penultimate position, no valine at Val388 relative to α_1-PI, and lack of an N-terminal extension. Bomapin has Arg-Ile as its P_1-P'_1 residues and a highly conserved hinge region responsible for mobility of the reactive-site loop. The expression of bomapin restricted to the bone marrow raises the possibility that this serpin may play a role in regulation of proteinases activity during hematopoiesis [38].

A quite interesting member of ovalbumin serpins is a tumour suppressor — maspin which can be produced by a number of cells of epithelial origin and which in fact is reported to occur extracellularly [39]. It has an arginine residue at the P_1 position, but most likely it is not an inhibitor due to the lack of homology in the hinge region, a region very conserved in all inhibitory serpins. It does not undergo a stressed to relaxed transition in its conformation and does not inhibit any known proteinase. Hypothetically, it should be able to inhibit u-PA and t-PA similarly as does PAI-2 because they both have arginine at the P_1 position [39]. Maspin, however, has been shown to block the growth, invasion and metastatic properties of mammary tumour cell lines [39, 40]. According to Hopkins [41] maspin might function as a ligand binding serpin — possibly of thymosin β_4 and in this respect might have a binding domain similar to that hypothesized for HLEI. HLEI was found to possess a short loop between strand 3B and helix G which is exposed on the protein surface and consists of a group of negatively charged residues (Asp-Ile-Glu-Asp-Glu) [16]. The charge distribution in this region is complementary to that of β_4-thymosin, suggesting that HLEI can bind thymosin β_4. In fact, during sequencing, Dubin *et al.* [16] found that about 70% of the inhibitor in the preparation is associated with thymosin β_4. This is in contrast to other mammalian elastase inhibitors which, when purified, are not found to be associated with substantial amounts of thymosin β_4. Maspin has a structure that is identical in 43% to HLEI and a similar insertion between strand 3B and helix G [41]. Maspin has a sequence Asp-Val-Glu-Asp-Glu inserted at the equivalent position as in HLEI (Asp-Ile-Glu-Asp-Glu), complementary to thymosin [41]. All this might suggest that HLEI is an evolutionary ancestor of the recently identified maspin. Most probably, maspin has evolved a new function, since its P_8 to P_{12} residues show no homology with any other known inhibitory serpin. This speculative association with thymosin has no defined meaning, however, it has been suggested that thymosin β_4 binds actin monomers and controls their polymerization [42]. On the other hand, Hopkins & Whisstock [41] suggest that maspin suppresses cell motility and tumour metastasis, which is a logical continuation of thymosin properties. Maspin, therefore, might bind elements involved in regulating cell shape change and cell mobility.

The pool of newly defined serpins is continuously increasing and in accordance with this trend, a novel serpin designated B-43 has been described [43]. It is of a particular interest since again it is most homologous to PAI-2, HEI and PI-6. B-43 has been isolated from bovine brain and is present in both neurons and astrocytes. It was reported not to form SDS-resistant complexes with thrombin, urokinase, elastase or plasmin, suggesting other unknown targets. Nishibori *et al.* [43] suggest that B-43 may be involved in the barrier function of blood vessels in the brain through the inhibition of the activity of proteinases extravasated from blood; it might also be involved in the regulation of neuronal plasticity in cooperation with proteinase nexin-1 and α_1-antichymotrypsin.

Another recently described possible relatives of the ovalbumin family of serpins are *Limulus* intracellular coagulation inhibitor type-1 and type-2 (LICI-1, LICI-2) isolated from Japanese horseshoe crab [44, 45]. They occur specifically in the large granules of the hematocytes and show closest homology to such ovalbumin serpins as PAI-2 (40%) and HEI (39%). They are both inhibitory serpins, LICI-1 inhibits lipopolysaccharide-sensitive serine proteinase, factor C, while LICI-2 in addition to factor C, also inhibits the limulus clotting enzyme and mammalian serine proteinases: α-thrombin, kallikrein, plasmin, and tissue plasminogen

activator [44, 45]. The primary function of these inhibitors might be to regulate the clotting enzyme cascade; this function can be even more effective as the proenzymes of the clotting cascade are co-localized with the inhibitors in the granules of hematocytes. The only restriction which would account for placing these inhibitors outside the ovalbumin family of serpins and not within it is the fact that even though being intracellular, they possess a typical N-terminal signal destining them for endoplasmic reticulum [45]. Miura *et al.* [45] suggest they might be co-released with several coagulation factors and bactericidal peptides in response to external LPS stimulation.

Also recently, a serpin isolated from freshwater crayfish *Pacifastacus leniusculus* was described as an intracellular protein found to be expressed in the crayfish hematocytes [46, 47]. The serpin — *Pacifastacus* proteinase inhibitor 2 (PAPI-2) has the highest sequence similarity to PAI-2 (38%) and PLEI (38%), however, on the genetic tree it is clustered with serpins of insects, therefore can not be regarded as a potential ovalbumin serpin [46, 47]. In contrast, the horseshoe crab serpin described above is classified with mammalian ovalbumin serpins.

PLANT SERPINS

Serpins present in plants are allowed a separate section because they are on a far end of the genetic tree when compared with ovalbumin serpins. So far, however, they have been found specifically in the grain and therefore can be classified as intracellularly occurring serpins. Barley seeds seem to be a source of a unique serpin with unidentified target proteinase. A serpin, described by Brandt *et al.* [48] as barley protein Z_4, is accumulated and stored in the endosperm but neither the site of its synthesis and deposition within the cell or its function are known. Protein Z_4, a protein with molecular mass of about 40 kDa, displays extreme heat stability after cleavage of its P_1-P'_1 bond, which, as for most inhibitory serpins, is Met-Ser [48]. Protein Z_4 is synthesized both by the free and membrane bound polysomes, moreover, its sequence contains a hydrophobic section in the amino-terminal region in a position similar to that of the hydrophobic region of PAI-2 [48] which, as earlier emphasized, is synthesized both as a secreted and cytosolic form. This

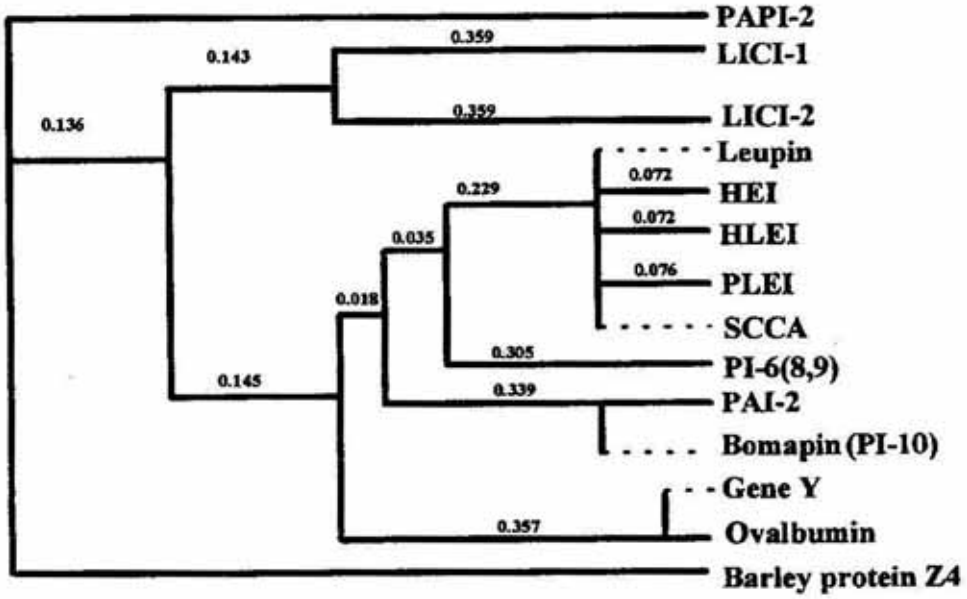

Fig. 1. Phylogenetic tree of some intracellular serpin family members (prepared after modification [45, 46, 50]).

Dash lines represent only hypothetical position without phylogenetic analysis. PAPI-2, crayfish hematocytes serpin [46]; LICI-1 and LICI-2, inhibitor from horseshoe crab [45]; HEI, human elastase inhibitor [51]; HLEI, horse leucocyte elastase inhibitor [16]; PLEI, porcine leucocyte elastase inhibitor [26]; SCCA, squamous cell carcinoma antigen [35]; leupin [36]; PI-6, PI-8 and PI-9, placental thrombin inhibitor [33, 34]; PAI-2, plasminogen activator inhibitor [17]; bomapin, bone marrow-associated serpin [38]; ovalbumin [52]; barley protein Z_4 [48, 49]. The number along the line is the mutation distance between each branch point.

could imply that protein Z_4 has a dual mode of synthesis and possesses no cleavable signal peptide as it is the case with members of the ov-serpin family.

Other serpins, collectively called Z proteins, have been isolated from barley seeds which for some time continued to be the only source of plant serpins. Only lately a Z-like protein, with molecular mass of also about 40 kDa, was isolated from wheat grain. Wheat Z proteins have Gln as a P_1 residue instead of Ser or Thr, as in most serpins, nonetheless they form with chymotrypsin stable complexes capable of withstanding treatment with SDS or boiling [49] which is a unique property of serpins. Sequencing of amino acids of Z proteins isolated from barley or wheat confirmed a substantial homology with mammalian members of serpin family, therefore it would be of future interest to find possible target proteinases and investigate the role of serpins in plants.

CONCLUSION

It seems reasonable to classify the intracellular serpins as a separate family because they appear to share many substantial features in common and, most importantly, many seem to be quite close evolutionary relatives. The possible phylogenetic tree of 14 intracellularly occurring serpins is shown in Fig. 1. In fact, almost all of them can be classified into the already existing subfamily of ovalbumin serpins, grouping together serpins of different origin and, in most instances, with unknown function. It is hoped and in fact most expected to find the explanation for their specific role which remains to be speculative.

REFERENCES

1. Carrell, R.W. & Boswell, D.R. (1986) Serpins: The superfamily of plasma serine proteinase inhibitors; in *Proteinase Inhibitors* (Barrett, A.J. & Salveson, G., eds.) pp. 403–420, Elsevier, New York.
2. Carrell, R. & Travis, J. (1985) Alpha-1-antitrypsin and the serpins: Variation and countervariation. *Trends Biochem. Sci.* **10**, 20–24.
3. Potempa, J., Korzus, E. & Travis, J. (1994) The serpin superfamily of proteinase inhibitors: Structure, function and regulation. *J. Biol. Chem.* **269**, 15957–15960.
4. Carrell, R.W. & Evans, D.L. (1992) Serpins: Mobile conformations / in a family of proteinase inhibitors. *Curr. Opin. Struct. Biol.* **2**, 438–446.
5. Lawrence, D.A., Ginsburg, D., Day, D.E., Berkenpas, M.B., Verhamme, I.M., Kvassman, J.O. & Shore, J.D. (1995) Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. *J. Biol. Chem.* **270**, 25309–25312.
6. Huber, R. & Carrell, R.W. (1989) Implication of three-dimensional structure of α_1-antitrypsin for structure and function of serpins. *Biochemistry* **28**, 8951–8966.
7. Hopkins, P.C.R., Carrell, R.W. & Stone, S.R. (1993) Effects of mutations in the hinge region of serpins. *Biochemistry* **32**, 7650–7657.
8. Carrell, R.W., Evans, D.L. & Stein, P.E. (1991) Mobile reactive centre of serpins and the control of thrombosis. *Nature (London)* **353**, 576–578.
9. Stein, P.E., Leslie, A.G.W., Finch, J.T., Turnell, W.G., McLaughlin, P.J. & Carrell, R.W. (1990) Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature (London)* **347**, 99–102.
10. Mottonen, J., Strand, A., Symersky, J., Sweet, R.M., Danley, D.E., Geoghegan, K.F., Gerard, R.D. & Goldsmith, E.J. (1992) Structural basis of latency in plasminogen activator inhibitor-1. *Nature (London)* **355**, 270–273.
11. Carrell, R.W., Stein, P.E., Fermi, G., Wardell, M.R. (1994) Biological implications of a 3A structure of dimeric antithrombin. *Structure* **2**, 257–270.
12. Song, H.K., Lee, K.N., Kwon, K., Yu, M. & Suh, S.W. (1995) Crystal structure of an uncleaved α_1-antitrypsin reveals the conformation of its inhibitory reactive loop. *FEBS Lett.* **377**, 150–154.
13. Remold O'Donnell, E. (1993) The ovalbumin family of serpin proteins. *FEBS Lett.* **315**, 105–108.
14. Belin, D. (1993) Biology and facultative secretion of plasminogen activator inhibitor-2. *Thromb. Haemostasis* **70**, 144–147.
15. Ye, D.R., Wun, T.C. & Sadler, J.E. (1988) Mammalian protein secretion without signal peptide removal. *J. Biol. Chem.* **263**, 4869–4875.
16. Dubin, A., Travis, J., Enghild, J.J. & Potempa, J. (1992) Equine leukocyte elastase inhibitor. Primary structure and identification as a thymosin-binding protein. *J. Biol. Chem.* **267**, 6576–6583.
17. Ye, D.R., Ahern, M.S., LeBeau, M.M., Lebo, V.R. & Sadler, J.E. (1989) Structure of the gene for

human plasminogen activator inhibitor-2. *J. Biol. Chem.* **264,** 5495–5502.

18. Arndt, A.D., Gohill, J., Rankin, K., Houwen, B. & Hart, D.A. (1989) Differentiation-linked expression of the plasminogen activator inhibitor type-2 gene in the human HL-60 promyelocytic cell line. *Exp. Cell Res.* **185,** 473–481.
19. Schwartz, B.S. & Bradshaw, J.D. (1989) Differential regulation of tissue factor plasminogen activator inhibitor by human mononuclear cells. *Blood* **74,** 1644–1650.
20. Remold-O'Donnell, E. (1985) A fast-acting elastase inhibitor in human monocytes. *J. Exp. Med.* **162,** 2142–2154.
21. Thomas, R.M., Nauseef, W.M., Iyer, S.S., Peterson, M.W., Stone, P.J. & Clark, R.A. (1991) A cytosolic inhibitor of human neutrophil elastase and cathepsin G. *J. Leukocyte Biol.* **50,** 568–579.
22. Evans, E., Cooley, J. & Remold-O'Donnell, E. (1995) Characterization and chromosomal localization of ELANH2, the gene encoding human monocyte/neutrophil elastase inhibitor. *Genomics* **28,** 235–240.
23. Dubin, A. (1977) A polyvalent proteinase inhibitor from horse-blood-leucocyte cytosol. *Eur. J. Biochem.* **73,** 429–435.
24. Junger, W., Hallström, S., Redl, H. & Schlag, G. (1988) Preliminary data on isolation of an elastase-like proteinase and its inhibitor from ovine neutrophil granulocytes. *Biol. Chem. Hoppe-Seyler* **369,** 63–65.
25. Kopitar, M. & Lebez, D. (1975) Intracellular distribution of neutral proteinases and inhibitors in pig leucocytes: isolation of two inhibitors of neutral proteinases. *Eur. J. Biochem.* **56,** 571–577.
26. Teschauener, W.F., Mentele, R. & Sammerhoff, C.P. (1993) Primary structure of porcine leukocyte serpin. *Eur. J. Biochem.* **217,** 519–526.
27. Dubin, A. & Koj, A. (1986) Proteinase inhibitors from horse plasma and leucocytes: properties and biological functions. *Biomed. Biochim. Acta* **45,** 1391–1396.
28. Korzus, E., Dubin, A., Potempa, J. & Travis, J. (1991) Elastase inhibitor from mammalian neutrophil cytosol. *Biomed. Biochim. Acta* **50,** 687–690.
29. Remold-O'Donnell, E. & Lewandowski, K. (1983) Two proteinase inhibitors associated with peritoneal macrophages. *J. Biol. Chem.* **258,** 3251–3257.
30. Morgenstern, K.A., Henzel, W.J., Baker, J.B., Wong, S., Pastuszyn, A. & Kisiel, W. (1993) Isolation and characterization of an intracellular serine proteinase inhibitor from a monkey kidney epithelial cell line. *J. Biol. Chem.* **268,** 21560–21568.
31. Coughlin, P.B., Tetaz, T. & Salem, H.H. (1993) Identification and purification of a novel serine proteinase inhibitor. *J. Biol. Chem.* **268,** 9541–9547.
32. Coughlin, P., Sun, J., Cerruti, L., Salem, H.H. & Bird, P. (1993) Cloning and molecular characterization of a human intracellular serine proteinase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9417–9421.
33. Sun, J., Rose, J.B. & Bird, P. (1995) Gene structure, chromosomal localization, and expression of the murine homologue of human proteinase inhibitor 6 (PI-6) suggests divergence of PI-6 from the ovalbumin serpins. *J. Biol. Chem.* **270,** 16089–16096.
34. Sprecher, C.A., Morgenstern, K.A., Mathews, S., Dahlen, J.R., Schrader, S.K., Foster, D.C. & Kisiel, W. (1995) Molecular cloning, expression, and partial characterization of two novel members of the ovalbumin family of serine proteinase inhibitors. *J. Biol. Chem.* **270,** 29854–29861.
35. Suminami, Y., Kishi, F., Sekiguchi, K. & Kato, H. (1991) Squamous cell carcinoma antigen is a new member of the serine proteinase inhibitors. *Biochem. Biophys. Res. Commun.* **181,** 51–58.
36. Barnes, R.C. & Worrall, D.M. (1995) Identification of a novel human serpin gene; Cloning sequencing and expression of leupin. *FEBS Lett.* **373,** 61–65.
37. Ray, C.A., Black, R.A., Kronheim, S.R., Greenstreet, T.A., Sleath, P.R., Salvesen, G.S. & Pickup, D.J. (1992) Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell* **69,** 597–604.
38. Riewald, M. & Schleef, R.R. (1995) Molecular cloning of bomapin (proteinase inhibitor 10), a novel human serpin that is expressed specifically in the bone marrow. *J. Biol. Chem.* **270,** 26754–26757.
39. Pemberton, P.A., Wong, D.T., Gibson, H.L., Kiefer, M.C., Fitzpatrick, P.A., Sager, R. & Barr, P.J. (1995) The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. *J. Biol. Chem.* **270,** 15832–15837.
40. Zou, Z., Anisowicz, A., Hendrix, M.J.C., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E. & Sager, R. (1994) Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* **263,** 526–529.

41. Hopkins, P. & Whisstock, J. (1994) Function of maspin. *Science* **265,** 1893–1894.

42. Shafer, D., Elzinga, M. & Nachmias, V.T. (1991) Thymosin β_4 and Fx, an actin-sequestering peptide, are indistinguishable. *J. Biol. Chem.* **266,** 4029–4032.

43. Nishibori, M., Ohtsuka, A., Kawabata, M., Nakaya, N., Murakami, T. & Saeki, K. (1995) A novel serpin-like protein, B-43, exists in both neurons and astrocytes: an immunohistochemical study in the parietal region of the bovine brain. *Neurosci. Lett.* **200,** 125–128.

44. Miura, Y., Kawabata, S. & Iwanaga, S. (1994) A *Limulus* intracellular coagulation inhibitor with characteristics of the serpin family. *J. Biol. Chem.* **269,** 542–547.

45. Miura, Y., Kawabata, S., Wakamiya, Y., Nakamura, T. & Iwanaga, S. (1995) A Limulus intracellular coagulation inhibitor type-2. *J. Biol. Chem.* **270,** 558–565.

46. Liang, Z. & Soderhall, K. (1995) Isolation of cDNA encoding a novel serpin of crayfish hematocytes. *Comp. Biochem. Physiol.* **112B,** 385–391.

47. Aspan, A., Huang, T.S., Cerenius, L. & Soderhall, K. (1995) cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 939–943.

48. Brandt, A., Svendsen, I. & Hejgaard, J. (1990) A plant serpin gene; Structure, organization and expression of the gene encoding barley protein Z_4. *Eur. J. Biochem.* **194,** 499–505.

49. Rosenkrands, I., Hejgaard, J., Rasmusen, S.K. & Bjorn, S.E. (1994) Serpins from the wheat grain. *FEBS Lett.* **343,** 75–80.

50. Carrell, R.W., Pemberton, P.A. & Boswell, D.R. (1987) The serpins: Evolution and adaptation in a family of protease inhibitors. *Cold Spring Harbor Symp. Quant. Biol.* **52,** 527–535.

51. Remold-O'Donnell, E., Chin, J. & Alberts, M. (1992) Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5635–5639.

52. Woo, S.L.C., Beattie, W.G., Catterall, J.F., Dugaiczyk, A., Staden, R., Brownlee, G.G. & O'Malley, B.W. (1981) Complete nucleotide sequence of the chicken chromosomal ovalbumin gene and its biological significance. *Biochemistry* **20,** 6437–6446.