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Biochemical properties of plasminogen activator inhibitor-1

Daniel Miotto Dupont[1,2], Jeppe Buur Madsen[1], Thomas Kristensen[1], Julie Stove Bodker[1], Grant Ellsworth Blouse[1], Troels Wind[1], Peter Andre Andreasen[1]

[1]Department of Molecular Biology and [2]Interdisciplinary Nanoscience Center (iNANO), University of Aarhus, Gustav Wieds Vej 10C, 8000 Aarhus C, Denmark

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1. ABSTRACT

PAI-1 is a M_r ~50,000 glycoprotein, which is the primary physiological inhibitor of the two plasminogen activators uPA and tPA. PAI-1 belongs to the serpin protein family. Studies of PAI-1 have contributed significantly to the elucidation of the protease inhibitory mechanism of serpins, which is based on a metastable native state becoming stabilised by insertion of the RCL into the central beta-sheet A and formation of covalent complexes with target proteases. In PAI-1, this insertion can occur in the absence of the protease, resulting in generation of a so-called latent, inactive form of the protein. PAI-1, in its active state, also binds to the extracellular protein vitronectin. When in complex with its target proteases, it binds with high affinity to endocytosis receptors of the low density receptor family.

2. INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is a fast and specific inhibitor of the serine proteases urokinase-type (uPA) and tissue-type (tPA) plasminogen activator, the second order rate constants for the inhibition reaction being between 10^6 and 10^7 $M^{-1}s^{-1}$. It is therefore the primary inhibitor of generation of the active, broad-spectrum serine protease plasmin from the zymogen plasminogen, and thus a main regulator of fibrin degradation and turnover of the extracellular matrix. PAI-1 belongs to the serpin family. X-ray crystal structure analyses have shown that serpins, including PAI-1, are globular proteins containing 3 beta-sheets and 9 alpha-helices. They have an exposed peptide loop, the reactive center loop (RCL), which is of decisive importance for the inhibitory mechanism (for reviews, see 1-4). The general inhibitory mechanism of serpins has been

Table 1. Second order rate constants for various plasminogen activator inhibiting serpins

Serpin	Second order rate constants ($M^{-1}s^{-1}$)			Ref
	uPA	sc-tPA[1]	tc-tPA[2]	
PAI-1	2.0×10^7	5.0×10^6	2.0×10^7	224
PAI-2	$2.4 - 2.8 \times 10^6$	$2.5 - 2.7 \times 10^5$	$0.8 - 1.2 \times 10^4$	225, 226
PAI-3	2.2×10^3		8.0×10^2	227
PN-1	1.5×10^5	1.5×10^3	3×10^4	228, 229
Neuroserpin	2.5×10^4	8.0×10^4	6.2×10^5	230

Abbreviations: [1]single-chain tPA, [2]two-chain tPA

elucidated in work published since the mid 80´s (for reviews, see 5-7), beginning with the first X-ray crystal structure of a serpin (8). Important contributions to the now generally accepted ideas about serpin inhibitory mechanisms were made from studies of PAI-1. In addition, PAI-1 has a number of unusual and unique properties of special interest for the understanding of the biochemical properties of serpins, including its ability to assume the latent conformation, to bind to the extracellular protein vitronectin, and to be endocytosed upon complex formation with uPA or tPA. We will here review some of the biochemical properties of PAI-1, seen in relation to general serpinology. This review is not meant to be a comprehensive one. Rather, we will emphasise aspects which have not been well covered in other reviews. Also, the reader is referred to other recent reviews for coverage of the physiological and pathophysiological functions of PAI-1 (9-12).

3. GENERAL BIOCHEMISTRY OF PAI-1

Functional evidence for the existence of the fibrinolytic inhibitor later to be known as PAI-1 was published in the late 70´s (13, 14). Early on, PAI-1 was also called the fast acting inhibitor, the endothelial cell inhibitor and the beta-migrating endothelial inhibitor. Human PAI-1 protein was first purified to apparent homogeneity in 1984 (15). Monoclonal antibodies against human PAI-1 became available in 1986 (16, 17). cDNA for human PAI-1 was cloned by 4 groups in 1986 (Figure 1) (18-21). The amino acid sequence of PAI-1 is now known in numerous mammalian species but in contrast, very little is known about the protein in non-mammalian species. In fact, an experimentally verified full-length mRNA or protein sequence has yet to be reported in any non-mammalian species although predicted protein sequences in the databases indicate that PAI-1 is also present in non-mammalian vertebrate species, including the phylogenetically relatively distantly related teleosts (e.g. zebrafish (*Danio rerio*) GenBank XP_684545). In pufferfish (*Fugu rubripes*), 26 proteins involved in clotting and fibrinolysis were searched against predicted protein sequences based on the full genome and although 21 orthologs were found, PAI-1 was one of the proteins that did not survive the backsearch as a true homologue in this study (22). Interestingly, no convincing homologue was found for any of the 26 genes in the urochordate, the sea squirt (*Ciona intestinalis*) genome, thereby strongly suggesting that the main lines of the vertebrate clotting pathway evolved in the interval between the last common ancestor of the two (22).

In higher primates, including humans and orangutans and old-world monkeys such as African green monkey, the PAI-1 gene gives rise to two different mature mRNA transcripts of 2.6 and 3.6 kb, respectively, due to the presence of two alternative polyadenylation sites in these species (23). The two transcripts result in translation into the same protein sequence. However, new-world monkeys, such as owl monkey, bushbaby and lemur only contain the larger transcript (3.6 kb) as do more distantly related mammalian species including mouse, rat and cow, thus providing evidence that the mutation(s) giving rise to the additional shorter 2.6 kb transcript appeared 20-65 million years ago in the time between the divergence of the new-world monkeys and the old-world monkeys from the evolutionary line that leads to higher primates and humans (23).

Phylogenetically, PAI-1 (SERPINE1) is most closely related to protease nexin-1 (PN-1; SERPINE2) and myxoma virus SERP-1 (SERPIN SPI-1) (4). Several serpins, such as PAI-2 (SERPINB2; also known as the placental inhibitor), PAI-3 (SERPINA5; also known as activated protein C inhibitor), PN-1 and neuroserpin (SERPINI1) can inhibit uPA and tPA in vitro but generally not as fast as PAI-1 (see Table 1).

Human PAI-1 has either 379 or 381 amino acids, which is caused by the presence of two possible cleavage sites for the signal peptidase in the N-terminus of the nascent protein (Figure 1). The signal peptide has either 23 or 21 amino acids, respectively. In purified preparations of PAI-1 from natural sources, each isoform constitutes about 50% (18, 24). After cleavage, the amino acid sequence from the N-terminus is either VHHPP- or SAVHHPP-. The correct numbering of the amino acids is then S1-A2-etc, but most workers in the field have chosen the numbering V1-H2-etc. In this review the S1-A2-etc.-numbering will be used (18) with the serpin consensus alpha1-proteinase inhibitor (alpha1-PI) numbering in parenthesis (1). A peculiar, but technically useful property of human PAI-1 is the lack of cysteines, which enables experimental insertion of cysteines into the PAI-1 structure for labelling with fluorescent probes. Mammalian PAI-1 has 3 potential sites for N-linked glycosylation, N211(228a), N267(278), and N331(341), and the glycosylation sites are conserved in all mammalian species. Although the predicted non-mammalian PAI-1 mRNA and protein sequences in the databases remain to be experimentally confirmed, an alignment between mammalian PAI-1 and the corresponding best hit in zebrafish (GenBank XP_684545), and thereby a putative teleost fish PAI-1 homologue, shows conservation of N211(228a) but substitutions of N267(278) and N331(341) by G and T, respectively, thus providing preliminary evidence that the mammalian PAI-1 glycosylation sites may not be conserved across vertebrate species. The actual glycosylation pattern has been analysed in PAI-1 naturally expressed by the human cell line HT-1080 or recombinantly expressed by the human cell line HEK293. Only the sites at N211(228a) and N267(278) are utilised, carrying glycans of a high mannose type and a bi-antennary complex type, respectively (25). A reported crystal structure of glycosylated PAI-1 confirms the

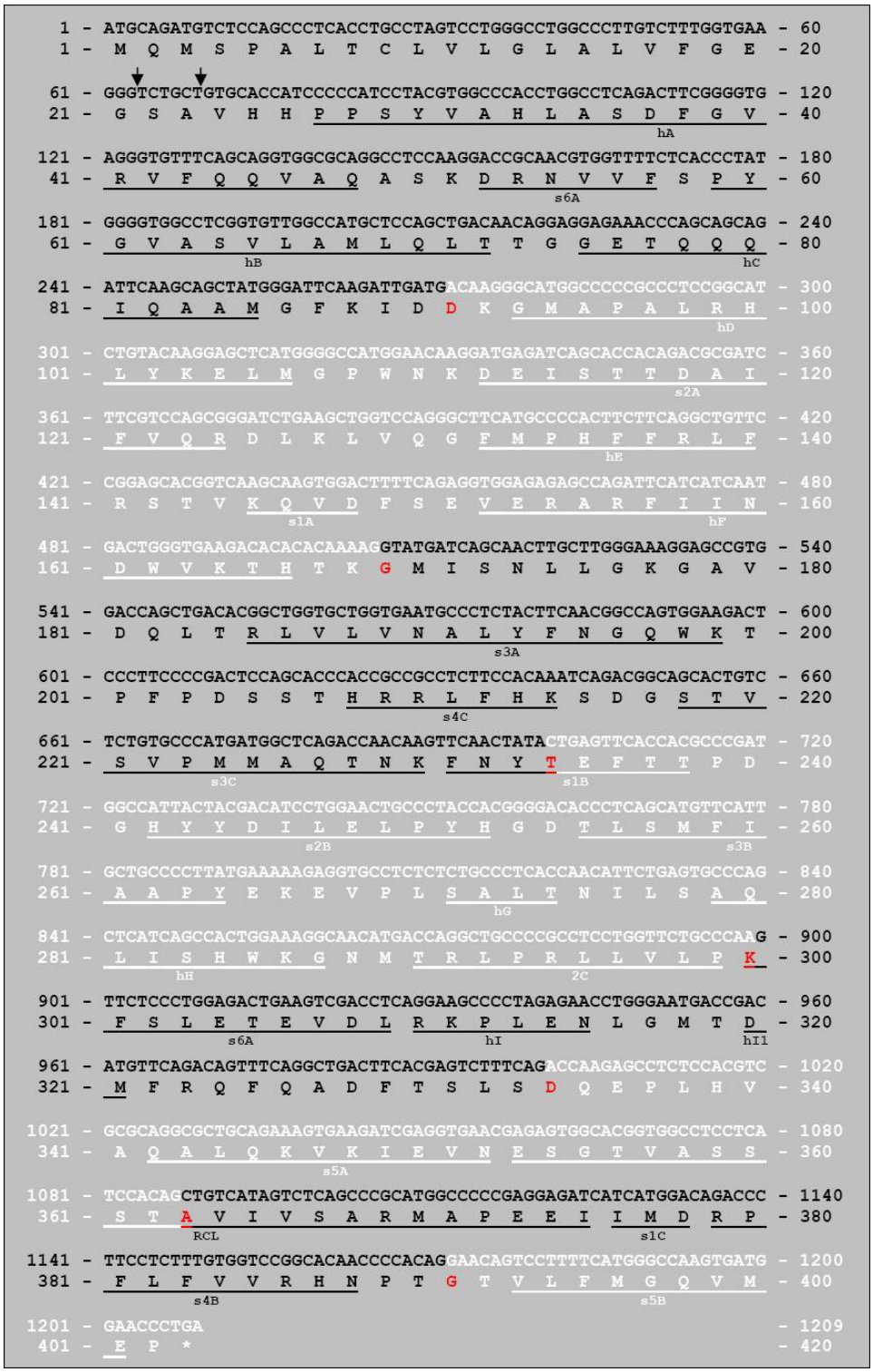

Figure 1. Intron-exon structure of human plasminogen activator inhibitor 1 (PAI-1) gene with the protein sequence indicated below the nucleotide sequence. Protein secondary structure elements are based on pdb file 1LJ5 and are indicated by underlinings. Black/white highlighting indicates alternate exons. Red highlighting indicates amino acids encoded across a splice junction. The two alternative signal peptidase cleavage sites are indicated by black arrows above the sequence.

existence of glycans at position 211(228a) and 267(278) but only a small part of the glycan moieties is visible suggesting pronounced flexibility (26). Recent data suggest, however, that the bi-antennary glycan complex at position 267(278) covers a hydrophobic cleft in the PAI-1 structure, exposed upon detachment of beta-strand 1C from beta-sheet during the process of PAI-1 latency transition (27) (see also below).

The human PAI-1 gene and its 5'-flanking region were cloned in 1987 and 1988 (28-30). The human PAI-1 gene is localised on chromosome 7, in the region q21.3-q22 (31). It contains 9 exons and 8 introns (Figure 1). The transcription of the PAI-1 gene is regulated by a large number of hormones, cytokines, and growth factors. A number of transcription factor binding sites in the 5'-flanking region of the gene has been identified (for reviews, see 11, 32, 33). The PAI-1 mRNA level is also regulated post-transcriptionally by regulation of mRNA stability (for a review, see 34). Historically, the induction of PAI-1 expression by glucocorticoid hormones is of special interest, as PAI-1 was discovered as a dexamethasone-induced fibrinolytic inhibitor (14).

4. INHIBITORY PROPERTIES OF PAI-1 AND OTHER SERPINS

By a combination of biochemical and biophysical experimental strategies, including X-ray crystal structure analysis, the major steps of the serpin inhibitory mechanism have been elucidated since the mid 80´s (Figure 2). The major steps in the reaction are the following: 1, formation of a reversible Michaelis complex, in which the P_1-P_1' bond in the RCL docks into the active site of the protease; 2, formation of an acyl-enzyme intermediate, in which the active site serine of the protease is attached to the hydroxyl group of the P_1 residue by an ester bond; 3, insertion of the N-terminal side of the RCL into beta-sheet A, thereby dragging the protease to the other pole of the serpin; 4, distortion of the active site of protease, preventing completion of the catalytic cycle (for reviews, see 1, 5-7).

Step 1 - The P_1-P_1' bond of PAI-1 is R348(358)-M349(359). In general, the P_1 residue is the major determinant of serpin specificity (for a review, see 1), and the P_1 residue in plasminogen, the major substrate for uPA, is also an arginine. Swapping residues P_{17} to $P_{2'}$ in PAI-1 with the corresponding sequence in other serpins have shown that this region plays a significant role for PAI-1 specificity (35). The importance of the P_1 and P_1´ residues in PAI-1 have been investigated by saturation mutagenesis revealing a number of mutants that were relative specific for either uPA or tPA (36). Additional plasminogen activator specificity has been obtained in PAI-1 variants carrying substitutions from P_3 to P_1´ (37, 38). A series of site-directed mutagenesis studies showed that charge-charge interactions between the E-residues in the P_4' and P_5' positions of PAI-1 and basic residues in the so-called 37-loop of uPA and tPA are important for formation of the initial, reversible docking complex (39-49). The importance of the P' residues of the serpin and the 37-loop of uPA and tPA for formation of the docking complex is in good agreement with the three-dimensional structures of reversible, non-covalent Michaelis complexes formed between a serpin and protease, in which the active site serine had been replaced by an alanine: *Manduca sexta* serpin 1B A353K with rat trypsin (50); $alpha_1$-proteinase inhibitor Pittsburgh with bovine trypsin (51); heparin cofactor II with thrombin (52); antithrombin III with thrombin (53); and antithrombin III and factor Xa (54), and also the Michaelis complex formed between antithrombin III and inactive anhydrothrombin (55).

Step 2 - The docking step is followed by a locking step in which the P_1-P_1' bond is being cleaved, the P_1 residue coupled to the active site serine of the protease by an ester bond. The existence of the ester bond was expected on the basis of the generally accepted theory for serine protease hydrolysis. However, its existence was directly established by the isolation of a fragment of the uPA - PAI-1 complex in which the presence of the ester bond could be unambiguously demonstrated by direct, protein chemical methods (56).

On the basis of more detailed analyses by the use of stopped flow reaction analysis, rapid acid quench, and a fluorescent PAI-1 derivative, in which RCL insertion was prevented by pre-insertion of a peptide corresponding to the RCL into beta-sheet A, a theory for the reactions immediately before and immediately after acyl-enzyme complex formation was formulated. It is believed that the exosite bonds between the distal hinge loop of the RCL and the 37-loop of the protease retain specifically the distal part of the PAI-1 RCL in the substrate pocket, favouring reversibility of the acylation step. Acylation of the protease becomes effective only by physical separation of the products of the acylation step. This step occurs only when the initial few residues of the proximal hinge loop of the RCL insert into the top of beta-sheet A in the breach region in the position of beta-strand 4A. The process is dependent on the strictly conserved W177(194). Breaking of the exosite interactions are needed for the ensuing full insertion of the RCL into beta-sheet A (39, 57-59).

Step 3 - Three-dimensional structures of the stable complex between $alpha_1$-PI and trypsin (60) and between $alpha_1$-PI and porcine pancreatic elastase (PPE) (61) show full insertion of the N-terminal part of the RCL as strand 4 in beta-sheet A and translocation of the protease completely to the opposite pole of the molecule. The same conclusion was reached from distance measurements by various fluorescence methods (62-65). However, recent evidence obtained by FRET-measurements on a single molecule level suggested the existence of complexes with partial insertion of the RCL (66, 67). Whether these represent intermediates or alternative forms of stable complexes remain unclear.

Step 4 - From the fact that the protease moiety of the three-dimensional structure of the trypsin - $alpha_1$-PI complex (60) was partially disordered in the crystal, it was inferred that the catalytic mechanism becomes stopped at the acyl-enzyme intermediate stage due to distortion of the

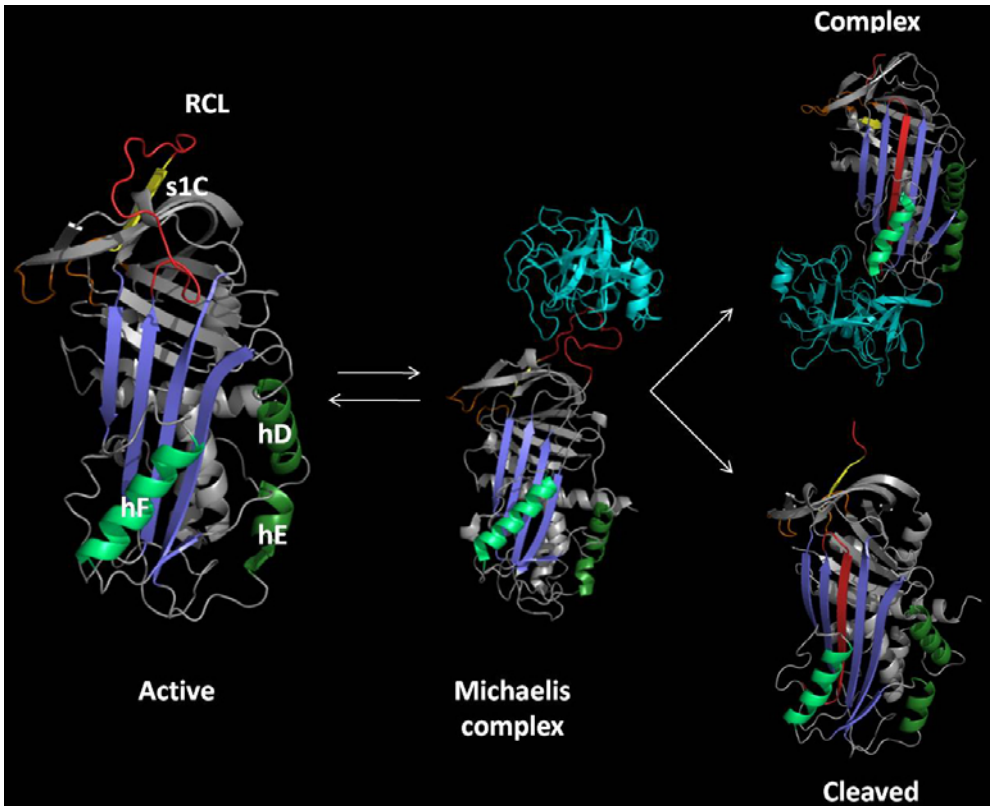

Figure 2. The inhibitory mechanism of PAI-1. On the left, the three-dimensional structure of an active PAI-1 variant with 4 mutations, introduced to delay latency transition and maintain the active state during crystallisation (120). The following structural elements are highlighted: the surface exposed or inserted reactive center loop (red); hF (limegreen); hD and hE (dark green); gate region (orange); s1C (yellow); beta-sheet A (blue). In the middle, the Michaelis complex of a serpin and its target protease is shown, represented by the *Manduca sexta* serpin 1B A353K - trypsin S195A complex (50). The protease is shown in cyan. At the right, the two possible outcomes of the reaction between a serpin and a protease is shown. The upper structure illustrates the covalent complex formed, in which the protease has been moved to the opposite pole of the serpin and inactivated. The complex shown is the one between alpha$_1$-PI and porcine pancreatic elastase (PPE) (61). In some cases, the protease can complete the hydrolysis of the RCL without being inactivated, which results in the reactive center cleaved variant (lower structure at the right). The structure shown is that of cleaved PAI-1 (124). The structures are displayed with PyMOL version 0.99rc6, DeLano Scientific LLC.

active site of the protease (6). The more recent X-ray structure of the stable complex between alpha$_1$-PI and PPE also contains a distorted active site but an overall much more defined protease moiety. The protease moiety was rotated approximately 40 degrees relative to the protease moiety in the previous trypsin - alpha$_1$-PI complex (61). Thus, the extent of the serpin-induced protease-distortion remains unclear and may indeed vary between different serpin-protease pairs. Jointly, the two X-ray crystal structure analyses show that the P_1 residue of the serpin is pulled out of the protease´s S_1 pocket; that the S and the H residue of the protease active site are pulled too far apart to be efficient in catalysis; and that the protease oxyanion hole is distorted. Similar conclusions were drawn from observations of differential proteolytic susceptibility of free and serpin-complexed proteases (61, 68-70), differential rates of reduction of disulfide linkages in free and alpha$_1$-PI-complexed trypsin (71), fluorescence studies (72), and NMR studies (73). The energy needed for the distortion is believed to stem from stabilisation of the serpin in the 'relaxed' conformation by insertion of the RCL into beta-sheet A, as opposed to the 'stressed', relatively unstable active conformation where the RCL is exposed on the surface of the serpin (1). It is possible, however, that the serpin-trapped inactive protease exists in equilibrium with an active conformation that can complete the catalytic cycle and thereby slowly be released from a cleaved serpin (74).

However, the protease can also cleave the serpin as if it was a substrate, which renders the serpin inactive (Figure 2), while the protease regains full activity. Two different hypotheses have been forwarded about the two alternative pathways of the protease-serpin reaction. According to the "branched pathway" hypothesis, the covalent acyl-enzyme intermediate can go either of two paths, leading to the stable serpin-protease complex formation or to reactive centre-cleaved serpin and active protease. In this model, the rate constants for the two branches of the bifurcation can be affected by outer stimuli, such as temperature, pH, ionic strength, detergents, etc.,

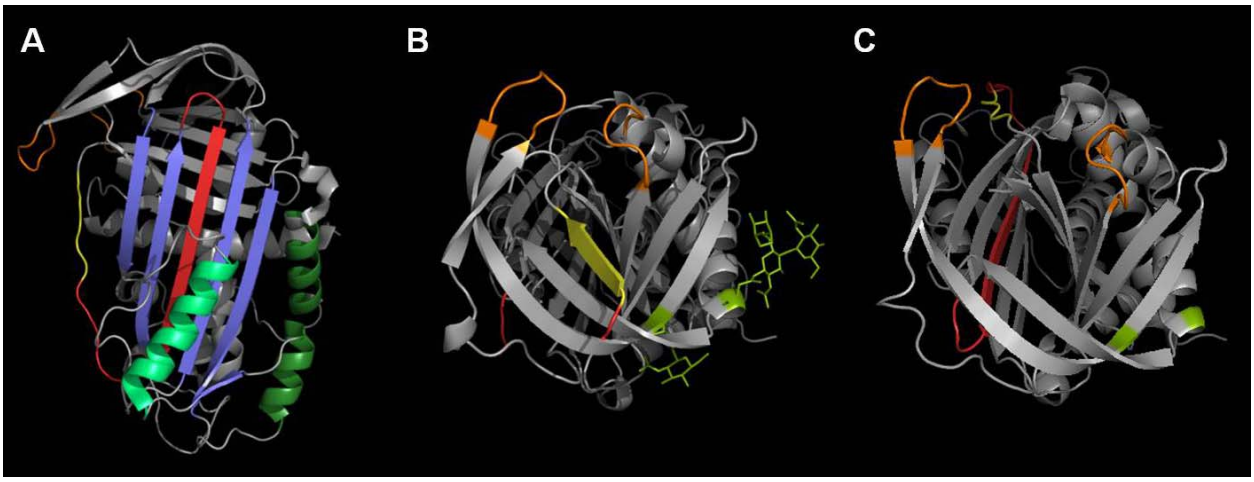

Figure 3. The latent form of PAI-1, and structural features of the area of PAI-1 surrounding beta-sheet C and the glycosylation area. A) The latent form of PAI-1 (pdb-file 1LJ5). The various secondary structural elements of PAI-1 have the same colours as in figure 2. B) RCL-cleaved glycosylated PAI-1 (pdb 1A7C). C) Latent, non-glycosylated PAI-1 (pdb 1LJ5). In B) and C), the RCL is coloured red; s1C is coloured yellow; the glycans at position N211 (228a) and Asn267 (278) are coloured green; and the gate-region is coloured orange.

thereby affecting the ratio of substrate-cleaved versus inhibitory serpin. In the "initial pathway" hypothesis, the serpin is believed to be in a conformation, that is destined to be either inhibitory or act as a substrate upon the encounter with the protease. Following this hypothesis, the fate of the serpin is pre-decided and not sensitive to changes in the environment of the encounter although external stimuli effecting the initial conformation of the serpin will change the outcome of the mechanism. The proposed models are not mutually exclusive (for reviews, see 3, 75).

5. PAI-1 LATENCY TRANSITION

Premature RCL insertion into beta-sheet A is the basis for the unique spontaneous conversion of active PAI-1 into the so-called latent state (76), in which the N-terminal part of the intact RCL is inserted as s4A and its C-terminal extension, forming s1C in the active conformer, stretched out along the surface of the molecule (77) (Figure 3). PAI-1 latency transition is one of the largest structural rearrangements known for a folded protein without a concomitant change in covalent structure, yet the sequence of conformational changes during latency transition remains largely unknown. Reported half-lives for latency transition of PAI-1 *in vitro* vary between 1 and 2 hours at physiological conditions, depending on protein source and experimental setup.

Several mutations in PAI-1 have been found to affect PAI-1 latency transition and are scattered throughout the molecule. Some PAI-1 variants with single mutations and modest decreases in the rate of latency transition have been obtained through heuristic protein engineering (78, 79) while others have been identified by chance (80-83). The variants with the slowest latency transition carry multiple mutations and have been obtained through *in vitro* molecular evolution, *i.e.* the creation of libraries of PAI-1 variants followed by screening (84) or selection (85) to isolate members with a slow latency transition. An interesting trait observed in the two examples of molecular evolution of PAI-1 is that the identified stabilising mutations in many cases replace the wild type PAI-1 residue with a serpin consensus residue, indicating that the instability of PAI-1 has resulted from positive evolutionary selection (84, 85).

As the P_1-P_1' bond is not cleaved during latency transition, the intact RCL must be stretched out and completely extracted from beta-sheet A to pass the so-called gate region between the loop between s3C and s4C (s3C/s4C-loop) and the loop between s3B and hG, before insertion of the RCL as s4A can be completed (77, 78, 86) (Figure 3). In contrast, RCL insertion during complex formation as well as during substrate behaviour implicates P_1-P_1' bond cleavage (see above) so that the insertion can proceed directly. This implies that the RCL movements during the transition from stressed to relaxed PAI-1 follows different pathways. Considering that RCL insertion into beta-sheet A is several orders of magnitude faster during complex formation than during latency transition, a reasonable suggestion is that the passage of the RCL through the gate region is rate limiting for latency transition. This presumption is supported by the observation that substitutions of basic residues in the s3C/s4C-loop with acidic ones accelerate latency transition (87). Several antibodies have been found to accelerate PAI-1 latency transition and, since the transition is an irreversible event, the effect of such antibodies cannot be explained by a stabilisation of the latent conformation. It has therefore been hypothesised that the antibodies accelerate latency transition by binding to and stabilising an intermediate, pre-latent conformation existing in an equilibrium with active PAI-1 (27, 88, 89). By mapping the epitopes of antibodies accelerating latency transition, it has been suggested that the pre-latent form of PAI-1 is

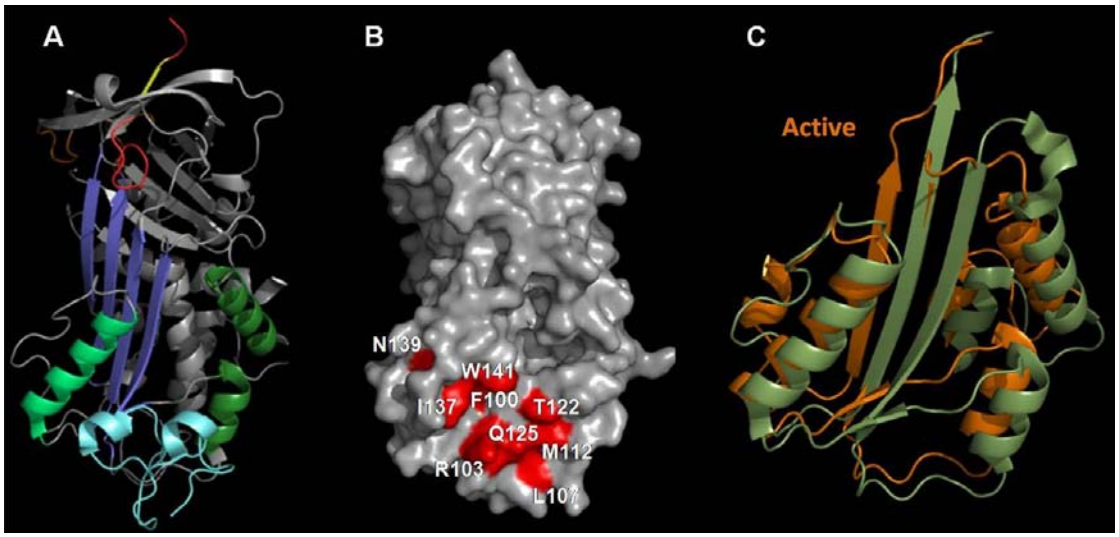

Figure 4. Structural aspects of PAI-1 - vitronectin binding. A) The three-dimensional structure of the somatomedin B domain of vitronectin complex with the PAI-1 variant stabilised in the active conformation, as determined by X-ray crystal structure analysis (pdb 1OC0). The somatomedin B domain is shown in cyan. The various secondary structural elements of PAI-1 have the same colours as in Figure 2. B) PAI-1´s vitronectin-binding residues, as determined using the ability of vitronectin to protect PAI-1 against bis-ANS inactivation (112), displayed on a surface presentation of PAI-1 (pdb 1OC0) having the same orientation as in A). C) The flexible joint region of active PAI-1 (pdb 1B3K) and latent PAI-1 (pdb 1LJ5). The two structures were superimposed by aligning s5A.

characterised by insertion of N-terminal residues of the RCL at the top of beta-sheet A (88-90) and partial detachment of beta-strand 1C from beta-sheet C (27). The structure of the predicted pre-latent form could resemble that of the delta-conformation of alpha$_1$-antichymotrypsin (91). The reversible partial insertion of the N-terminal end of the RCL and extraction of s1C would enable an irreversible passage of the C-terminal end of the RCL through the gate region. The importance of the release of s1C is supported by the observation that mutations expected to weaken the interations at the s1C region facilitated the conformational transition to the latent state and affected the overall structural stability of PAI-1 (92).

6. PAI-1 - VITRONECTIN BINDING

Vitronectin is an M_r ~78,000 extracellular glycoprotein, which is found in blood plasma in a concentration of 2-4 micromolar (93, 94) mainly in a monomeric closed conformation, and in the ECM in an open multimeric conformation (for a review, see 95). It contains, as mentioned from the N-terminus: a 44-residues long somatomedin B domain (SMB), followed by a connecting region, containing an integrin binding RGD sequence, and two hemopexin-like domains (Hp1 and Hp2), one with a heparin binding sequence. The three-dimensional structure of the SMB domain has been revealed both using NMR and X-ray crystallography (96-98). A disulphide bridge-pattern in full agreement with the reported three-dimensional structures were determined by rigorous protein chemical methods (99, 100).

PAI-1 is present in blood and extracellular matrix in a complex with vitronectin and can bind to vitronectin in purified systems (93, 101-103), as well as in cell culture (104). The K_d value for binding of vitronectin to active PAI-1 is in the subnanomolar range, while latent PAI-1 and other relaxed forms bind with an at least 200-fold lower affinity (105). The high affinity binding site resides in vitronectin's N-terminal somatomedin B domain (96, 106). Recently, it has been suggested that vitronectin may contain a second vitronectin binding site with a lower affinity (107). The binding site for somatomedin B in PAI-1 was first suggested to reside in the flexible joint region on the basis of random mutagenesis, implicating Q125(144) and L118(137) (108). A later report implicated residues 116(135)-120(139) (109). Investigations by the less accurate methods of antibody competition (110) and PAI-1 - PAI-2 chimeras (111) also gave results compatible with vitronectin binding to the flexible joint region. Analysis of the vitronectin binding surface in PAI-1 by site-directed mutagenesis is complicated by the fact that any mutation in PAI-1 observed to reduce vitronectin-binding may actually do so by increasing the fraction of relaxed PAI-1, *e.g.* by increasing the rate of latency transition, rather than by directly disrupting the vitronectin-binding surface. Using an alternative approach, in which the ability of vitronectin to protect active PAI-1 against an inactivating organochemical ligand was used as a basis for finding PAI-1 residues involved in PAI-1-vitronectin binding, an extensive interaction surface in PAI-1's flexible joint region was defined (Figure 4) (112). The information obtained by that approach was later used to construct a PAI-1 variant without measurable vitronectin affinity, but normal with respect to a variety of other tested parameters, including the rate of latency transition (113). The vitronectin interaction surface defined by these methods were in complete agreement with the X-ray crystal structure analysis of a

complex between stable PAI-1 and the somatomedin B domain of vitronectin (Figure 4) (96).

The PAI-1 binding site in the somatomedin B domain of vitronectin overlaps with binding site for the uPA receptor (uPAR) (114-116). The binding of PAI-1 to vitronectin also competes with the binding of integrins to the adjacent RGD sequence (117, 118).

The forms of vitronectin found in plasma and in the ECM have been shown to be very different in terms of conformation and ligand/cell binding properties. Binding of PAI-1 to vitronectin is known to cause monomeric vitronectin to multimerise into both disulphide-bridged and non-covalently multimers (119). Multimeric vitronectin, opposed to plasma vitronectin, is able to interact with a number of molecules, which allow the protein to bind to cells and ECM (for a review, see 95). The effect of the PAI-1 – vitronectin interaction may therefore be two-fold, resulting in stabilisation but also localisation of PAI-1 activity at sites of action.

7. GLOBAL CONFORMATIONAL CHANGES OF PAI-1 IN ASSOCIATION WITH RCL INSERTION

When looking only at the initial and the final structures, the intramolecular rearrangements that occur during the transition from the stressed to the relaxed serpin conformation are well described by the X-ray crystal structure analyses, the only main reservation being that the structure of active PAI-1 is that of a variant with 4 mutations, introduced to delay latency transition and maintain the active state during crystallisation (120, 121). The most prominent change is the insertion of the RCL as s4A (Figure 2 and 3). It is clear, however, that several other structural elements are involved during the stressed-to-relaxed transitions of PAI-1, and transient conformational changes during RCL insertion cannot be understood by X-ray crystal structure analysis.

Insertion of the RCL as s4A involves the translocation of s1A, s2A, s3A, hF and the loop connecting hF and s3A (the hF/s3A-loop), collectively known as the small serpin fragment, relative to the rest of the molecule (the large serpin fragment) and subtle structural changes in the region around hE and hD, known as the flexible joint-region (122, 123) (Figure 2 and 4). In the case of PAI-1, evidence for conformational flexibility of the flexible joint-region and for its differential conformation in stressed and relaxed states of PAI-1 was obtained by X-ray crystal structure analysis (77, 120, 121, 124, 125), limited proteolysis analysis (126), and differential binding of organochemicals (127, 128). The structural changes following the stressed-to-relaxed transition of PAI-1 include a narrowing of a solvent-accessible hydrophobic cavity between s2A and hD. The differential vitronectin-affinity of the active, stressed form of PAI-1 on one side and of latent PAI-1 and other relaxed forms on the other and the localisation of the vitronectin binding area (see above; Figure 4) are in good agreement with the different conformations of the flexible joint-region in stressed and relaxed forms (77, 120, 121, 124, 125).

On the other hand, binding of vitronectin to PAI-1 delays its rate of latency transition by a factor of 1.5 - 3 fold (93, 113). Vitronectin also decreases the k_{lim} for loop insertion during reaction with proteases (129-131). Thus, there seems to be a bi-directional communication between the movements of the RCL and the vitronectin-binding area. How does vitronectin, when binding to the flexible joint region, affect the movements of the RCL? On the basis of biochemical studies, it has been suggested that vitronectin induces a conformational change of the RCL (132, 133), which could be the explanation for a delayed latency transition due to an impeded passage through the gate region, a stabilisation of beta-sheet C, or a slower rate of RCL insertion. On the basis of the three-dimensional structure of the PAI-1 - somatomedin B complex, Zhou *et al.* (2003) suggested that vitronectin simply sterically hinders the shift of beta-strands 2A and 3A towards hE and thus delays the necessary opening of beta-sheet A. However, certain observations concerning K325(335)A in beta-strands 5A argues against this mechanism. Residue K325(335), which is well conserved in the serpin family (4) appears to form an important link between the small and the large serpin fragment. It is situated above the shutter region with its side chain pointing towards the hF/s3A-loop. In PAI-1, the side chain of K325(335) was suggested to interact with the small serpin fragment *via* a hydrogen bond to the backbone of the hF/s3A-loop (134) or *via* a chloride ion coordinated between two lysine residues in s5A (K325(335) and K327(337)) and two backbone amides from the hF/s3A-loop (125). In $alpha_1$-PI, hydrophobic interactions between K325(335) and residues in the hF/s3A-loop were suggested (135). Substitutions of K325(335) with small hydrophobic amino acids, *e.g.* alanine, led to increased thermal stability and substrate behaviour of $alpha_1$-PI, suggesting that burial of the side chain of K325(335) between beta-sheet A and the hF/s3A-loop contributes to the metastability of the stressed conformation (135, 136). Substitution of K325(335) in PAI-1 with alanine delays latency transition, which could be interpreted as an increased stability of the stressed conformation (137). In addition, several observations suggested that K325(335) in PAI-1 is involved in the communications that take place within active PAI-1 as a response to vitronectin and certain monoclonal antibodies (for a review, see 7). For example, whereas the K325(335)A substitution delayed latency transition in the absence of vitronectin, it accelerated latency transition in the presence of vitronectin (137). It is the latter observation, which is difficult to reconcile with vitronectin simply forming a physical barrier to expansion of beta-sheet A. It rather argues that the effect of vitronectin is a conformational one.

During beta-sheet A opening and RCL insertion, s3A and s5A must slide apart in a shutter-like movement over the underlying hB (122). The central part of s3A and s5A and the N-terminal part of hB are therefore referred to as the shutter region (2). The shutter region contains a buried cluster of residues with a complicated hydrogen bonding network, involving the amino acids in positions 53(69) and 56(72) in hB, 186(203) in s3A, and 334(344) in s5A (1), but differently organised in the stressed and the

relaxed conformations (138). Also, the clustering of naturally occurring, disease-causing mutations in the shutter region of various serpins other than PAI-1 highlights the importance of the integrity of its hydrogen-bonding network for the normal orientation of the RCL, most of the mutations appearing to cause loop-sheet polymerisation (139-145). Although PAI-1 has an unusual amino acid composition in these positions, the less well resolved X-ray crystal structures of the various forms of PAI-1 are compatible with a similar network in this serpin (77, 120, 121, 124, 125). A number of substitutions in positions 53(69), 56(72) and 334(344) in PAI-1 strongly accelerated latency transition (134), while the substitutions P54(70)S and P54(70)T delayed latency transition (84). Thus, the amino acid composition of the shutter region of PAI-1 affects the rate of latency transition, either by affecting beta-sheet A opening, the release of s1C, the rate of passage of the RCL through the gate region, or by further destabilising the stressed conformation of PAI-1.

In its translocation away from s5A during RCL insertion, other secondary structural elements in the small serpin fragment change position relative to each other. Based on an analysis of the three-dimensional structures of native and P_1-P_1' cleaved $alpha_1$-PI, Whisstock *et al.* (2000) concluded that hF changes its packing relative to beta-sheet A during RCL insertion with part of the hF/s3A-loop as a flexible hinge for this rearrangement (123). With PAI-1, a variety of observations have implicated movements of the elements of the small serpin fragment during latency transition as well as complex formation. Significant effects on the rate of latency transition were observed by substitutions of residues involved in interactions connecting different secondary structural elements, including residues in hF, the hF/s3A-loop, and s1A (82, 84, 85, 120, 121, 125). Intriguingly, the significant acceleration of latency transition of PAI-1 conferred by certain point mutations in s1A or hF was counteracted by vitronectin (82), implying that the latency transition-delaying effect of vitronectin might also involve conformational changes of s1A and hF. Some point mutations in s1A and hF also increased the tendency to substrate behaviour (82, 146). Furthermore, antibodies with epitopes encompassing hF and the hF/s3A-loop can induce substrate behaviour (147, 148), presumably by inducing a rigidity in the small serpin fragment which is not compatible with a rate of strand insertion fast enough for normal complex formation (148). The observation that the substrate behaviour induced by one of these antibodies is strongly potentiated by vitronectin also points towards regulation of the flexibility of this region by vitronectin (129-131, 148, 149). On the basis of separate substitutions of all residues in hF with alanine, Wind *et al.* (2003) concluded that interactions of hF and its neighbouring structural elements regulate the rate of latency transition (146). Gettins (2002) proposed that serpins´ alpha-helix F must be temporally displaced towards alpha-helix E to make space for the protease as it translocates from its position in the Michaelis complex to its position in the stable complex (75).

8. PAI-1 POLYMERISATION

Serpin polymerisation can be induced by specific mutations and underlies several inherited diseases, "serpinopathies" (for a review, see 150). Serpins may polymerise by the RCL of one molecule forming an additional strand in beta-sheet A or beta-sheet C of another molecule (for a review, see 2). PAI-1 has been shown to be able to polymerise *in vitro*. *In vitro,* the plasminogen activator inhibitory activity of PAI-1 can be neutralised by a number of structurally diverse organochemical compounds with IC_{50} values in the micromolar range (for a review, see 9). Mutational studies and fluorimetric binding analysis indicated that some of these compounds bind to a hydrophobic cavity in the flexible joint-region (127, 128). Activity neutralisation proceeded through two consecutive steps, the first one consisting in conversion to a form displaying substrate behaviour, the second one consisting in conversion to forms inert to plasminogen activators. The second step was associated with polymerisation of PAI-1 (128, 151). As investigated by native gel electrophoresis, the size of the PAI-1 polymers ranged from dimers to multimers of more than 20 units (152). During incubations with urokinase-type plasminogen activator, the polymers were slowly converted to reactive centre-cleaved monomers, indicating substrate behaviour of the terminal PAI-1 molecules in the polymers. Interestingly, PAI-1 could form co-polymers with the other serpin heparin cofactor II. On the basis of these results, it was suggested that the binding of ligands in a specific region of PAI-1 leads to insertion of the RCL of one molecule into the upper part of the cleft between s3A and s5A of another molecule. Induction of polymerisation by small organochemical ligands have not been observed with other serpins (152). PAI-1 has also been reported to polymerise at pH 4, but since both latent and active PAI-1 polymerised under these acidic conditions (153), the underlying molecular mechanism must differ from that induced by PAI-1-neutralisers. It is not known whether PAI-1 polymerisation is of physiological relevance.

9. BIOCHEMICAL IMPORTANCE OF PAI-1 GLYCOSYLATION

With few exceptions, all biochemical studies on PAI-1 have been performed with PAI-1 expressed in *E. coli*. Because of the short culture time needed to achieve a high level of expression, PAI-1 expressed in *E. coli* remains in the active form, in contrast to PAI-1 expressed naturally or recombinantly by mammalian cells, which must be reactivated after purification from conditioned culture medium collected after culturing cells for several days, during which time the secreted PAI-1 is converted to the latent form. However, PAI-1 expressed in *E. coli* lacks glycosylation. While the basic serpin inhibitory mechanism from all available evidence does not depend on the glycosylation, the binding and effect of certain artificial ligands have been shown to differ between glycosylated and non-glycosylated PAI-1. This was true for the binding of PAI-1 by 4 activity-neutralising monoclonal antibodies. For example, MAI-12 had a more than 10 fold higher affinity to glycosylated PAI-1 than to non-glycosylated PAI-1. Expression in mammalian cells of PAI-1 variants, in which one or the other of the utilised glycosylation sites was mutationally inactivated, showed that the higher affinity of MAI-12 to glycosylated PAI-1 depended on

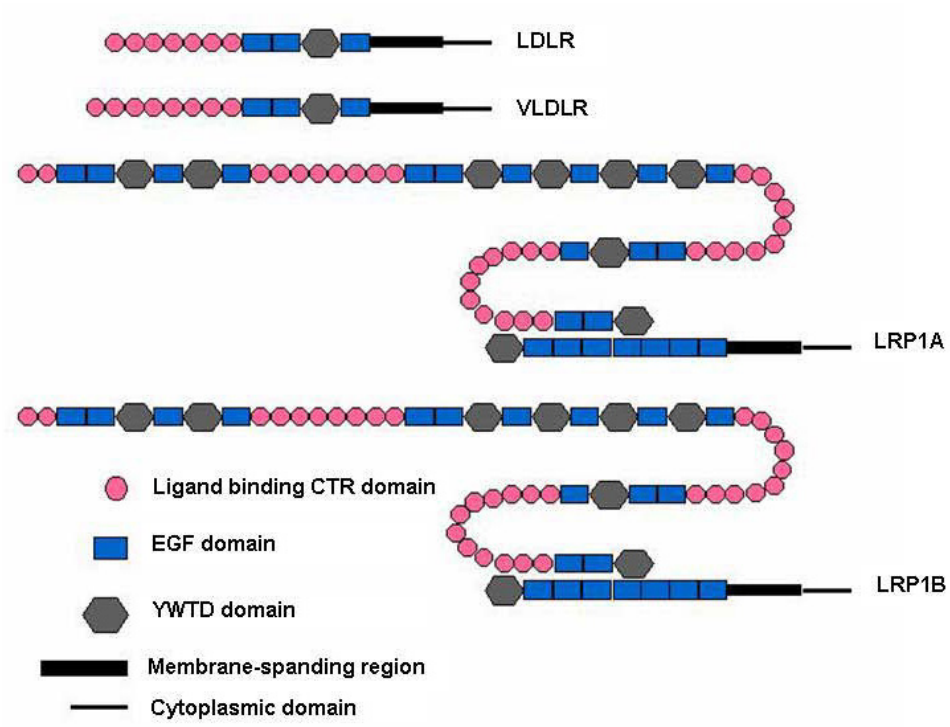

Figure 5. Cartoon presentation of the domain structure of endocytosis receptors of the LDLR family.

glycosylation at N267 (278) (25). Results like these stress the importance of the expression system used when developing and studying the mechanism of action of PAI-1 inactivating compounds of potential clinical importance. However, it remains unknown whether glycosylation variants of PAI-1 exist *in vivo*.

10. BINDING OF PLASMINOGEN ACTIVATOR - PAI-1 COMPLEXES TO ENDOCYTOSIS RECEPTORS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR FAMILY

The low density lipoprotein receptor (LDLR) family of endocytosis receptors includes a number of receptors composed from the same type of domains, but with variable sequence and variable number of each type of domain. The known mammalian members of the family are, besides LDLR itself, low density lipoprotein receptor-related protein-1A (LRP-1A), LRP-1B, megalin or LRP-2, very low density lipoprotein receptor (VLDLR), and apolipoprotein receptor 2 (apoER2) (Figure 5). The domains include complement-type repeats (CTRs), YWTD repeats, and epidermal growth factor precursor domains. All the receptors have a transmembrane alpha-helix and a cytoplasmic C-terminal domain with one or more sequences mediating endocytosis *via* clathrin-coated pits (for a review, see 154-157). The classical ligands for LDLR are apolipoproteins B100 and E (155), and these apolipoproteins are also ligands to the other receptors. In 1990, however, it was discovered that receptors of the family also have non-apolipoprotein ligands, as the receptor for protease-complexed alpha$_2$-macroglobulin was found to be identical to LRP-1A (158, 159), the amino acid sequence of which had been reported two years earlier (160).

Also in 1990, it was reported that uPAR-bound uPA - PAI-1 complex is endocytosed efficiently, at least by some cell types (161-163). However, the molecular mechanism behind this phenomenon remained unknown until 1992, when it was reported that tPA - PAI-1 complex (164) and uPA - PAI-1 complex (165) can bind to and be endocytosed by LRP-1A. Slightly later, it was realised that LRP-1A can function as a clearance receptor for tPA (164, 166, 167). However, the free proteases and free PAI-1 are endocytosed much slower than their complexes by these receptors (164, 165, 168). The differential endocytosis rate of free proteases and free PAI-1 on one side and their complexes on the other is reflected in a differential affinity of the complexes and the free components to the receptors *in vitro*: uPA - PAI-1 complex binds to LRP-1A with an affinity which is approximately 100-fold higher than that of free uPA, active PAI-1, latent PAI-1 and cleaved PAI-1 (K_d of 0.5 and 50 nM, respectively) (168). Later, it was shown that also megalin (169, 170), VLDLR (171, 172), and LRP-1B (173, 174) bind and mediate endocytosis of plasminogen activator - PAI-1 complexes. Besides, sorting protein-related receptor (sorLA; LR11), a receptor related to the LDLR family by containing a cluster of 11 CTRs, but also other types of domains, and having a ligand repertoire overlapping with that of LDLR family, also binds plasminogen activator - PAI-1 complexes (175).

The binding of the complexes to these receptors may function in the clearance of the complexes both locally in the tissues and systemically in the liver, which has an

abundant expression of LRP-1A. In spite of the low affinity of free uPA, tPA or PAI-1 to these receptors, the receptors may still be important for clearance of the free components, as a high receptor density may compensate for a low affinity. Certainly, LRP-1A is able to mediate endocytosis and degradation of tPA (164, 166, 167), also *in vivo* (176). But locally, on cell surfaces, the different endocytosis rate of uPA and uPA - PAI-1, respectively, is functionally important. Another important aspect is that uPAR-bound uPA - PAI-1 complex is endocytosed and degraded much faster than uPA - PAI-1 complex free in the fluid phase, presumably because endocytosis mediated by the relatively low affinity binding to the endocytosis receptors (K_d around 500 pM) is facilitated by accumulation of complex on the cell surface by the relatively high affinity binding to uPAR (K_d around 50 pM) (165, 168, 177). uPAR is, as a consequence of its binding to the uPA moiety of the uPA - PAI-1 - endocytosis receptor complex, co-endocytosed (178), and later recirculated to the cell surface (179).

Since the early 90es, evidence has been accumulating that each of these receptors mediate endocytosis of many structurally unrelated ligands. For instance, the receptors bind matrix metalloproteases, extracellular matrix proteins, and viruses. The binding specificities of individual receptors are overlapping, but still distinct. A ligand of special interest is the M_r ~40,000 receptor-associated protein (RAP), first identified because it co-purifies with LRP-1A (159) and later found to be an endoplasmic reticulum-localised chaperone, ensuring correct folding of receptors from the LDLR family (for reviews, see 180-182). RAP consists of 3 homologous domains (183-186).

Moreover, some receptors of the LDLR family may also have a signalling function (for reviews, see 154, 156, 187), and binding of uPA - PAI-1 complex to VLDLR has also been reported to initiate an intracellular signal transduction cascade (188). In this connection, it is of interest to note that among the endocytosis receptors binding the uPA - PAI-1 complex, endocytosis occurs at very diverse rates: LRP fast, VLDLR moderate, LRP-1B and sorLA slow (174, 175, 189). The slowly endocytosing receptors seem to be most relevant when considering a potential signaling function.

Most interesting in the present context, these receptors' ligands include many serine proteases, serpins, and their complexes (uPA - PN-1, uPA - PCI, uPA - C1 inhibitor, tPA - PAI-1, tPA, pro-uPA, PAI-1, neutrophil elastase - $alpha_1$-PI, trypsin - $alpha_1$-PI, C1 inhibitor - C1s, thrombin - antithrombin III, thrombin - heparin cofactor II, thrombin - PCI, thrombin - PN-1, factor IXa, cathepsin G - $alpha_1$-antichymotrypsin). Many, although not all, bind with a subnanomolar K_d (for reviews, see 154, 155, 157, 187). The uPA - PN-1 complex appears to represent a special case as far as the binding to LRP and VLDLR is concerned. The uPA - PN-1 complex binds as well to LRP as uPA - PAI-1, and appears to have a particularly high affinity to VLDLR, 5-15 fold higher than that of uPA - PAI-1 complex to VLDLR (190) and is the only protease-serpin complex known to be able to compete the binding of RAP (191).

Because of the high-affinity binding of so many structurally unrelated ligands by these receptors, understanding the biochemistry of this type of receptor-ligand interaction is a special challenge. A specific challenge in the case of plasminogen activator - PAI-1 complexes is understanding the biochemistry of the different binding affinity of each component separately and of the complex, being related to the inhibitory mechanism of serpins. What is the biochemical mechanism behind the increased affinity of the complex? *A priory*, several possibilities could be contemplated, including 1, an increased affinity associated with a conformational change of PAI-1 following complex formation; 2, an increased affinity associated with a conformational change of the protease; 3, an increased affinity associated with conformational changes of both components; 4, an avidity effect due to the combination of two binding sites on the same ligand, one from uPA and one from PAI-1. There is no X-ray crystal structure analysis of any complex between an endocytosis receptor and a serine protease, a serpin, or their complex. But some inferences can be made from the crystal structures of the third domain of RAP in complex with a CTR pair from LDLR and of human rhinovirus serotype 2 in complex with CTRs from VLDLR (192, 193). Also of relevance is an X-ray structure analysis of LDLR crystals obtained at pH 5, in which the beta-propeller bends back and makes contact with the CTR cluster in a way believed to mimic ligand binding (194). Moreover, a model of a complex between a pair of CTR repeats and RAP domain 1, based on their separate NMR structures, were published recently (195). Common to all of these structures is the fact that binding to the CTRs is mediated through basic and hydrophobic residues in the ligand. Also, the three-dimensional structures of several CTR repeats from LDLR and LRP have been determined (196-203).

But so far, the only available direct knowledge about contacts between plasminogen activator - PAI-1 complexes and the endocytosis receptors has been obtained by site-directed mutagenesis and the use of deletion variants of uPA. The initial search for receptor-binding residues in serpins focused on the flexible joint region, because the flexible joint region is known to change conformation upon RCL insertion and because it is not covered by the protease in the complex. Also, the initial search concentrated on basic residues, as heparin was known to inhibit the binding of several ligands to receptors of the LDLR family (for a review, see 204). Binding of uPA - PAI-1 complex to LRP and VLDLR requires basic residues in hD and hE in the flexible joint-region in the inhibitor. The implicated residues were R78(97), K82(101), R120(139), and K124(143) (205); K71(90) and K82(101) (206); R78(97) (207), K71(90), R78(97), K82(101), K90(109), R117(136), R120(139), R270(281) (208) (Figure 6).

The observation that complexes of the same serpin with different proteases have different affinities to LRP and VLDLR (190) suggests that contacts between protease and receptor as well as between PAI-1 and receptor contribute to the increased affinity, but the contribution may come from the A-chains as well as from

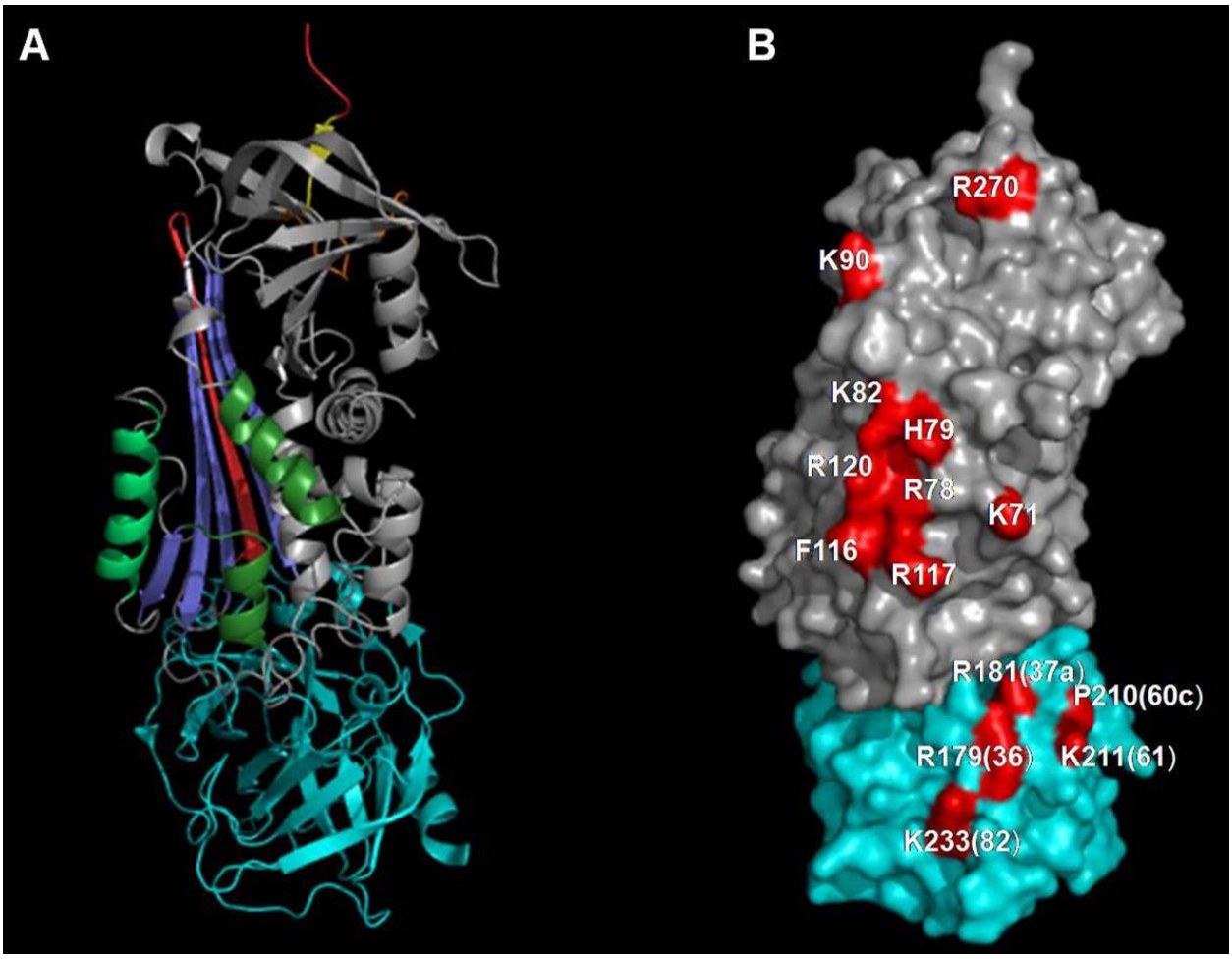

Figure 6. The receptor binding surface in the uPA - PAI-1 complex. The uPA - PAI-1 complex shown is a model constructed from the structure of the α_1-PI in complex with PPE 61 (pdb file 2D26), by aligning the alpha$_1$-PI part of that structure with the structure of the cleaved PAI-1 (pdb file 9PAI) and the PPE part of the structure with that of uPA (pdb file 1F92). Thus, the orientation of the uPA - PAI-1 complex is realistic but it does not allow any predictions of exact distances between amino-acid residues. A) The uPA - PAI-1 complex with the different structures coloured in the same way as in Figure 2. uPA is coloured cyan. B) Surface presentation of the uPA - PAI-1 complex shown in the same orientation as in A. PAI-1 is coloured grey and uPA cyan. Residues important for binding to VLDLR are coloured red in both structure. PAI-1 numbering is according to (18). uPA numbering is according to (231) with the chymotrypsin template numbering in parenthesis.

the serine protease domain. In fact, the 10-fold difference in affinity between the complexes of PAI-1 with full length uPA and LMW-uPA, lacking almost the entire A-chain (168, 207, 208) proves the involvement of the A-chain in the binding to the endocytosis receptors. Deletion of the N-terminal growth factor domain of uPA alone reduced the affinity 2-4-fold, indicating that both the growth factor domain and the kringle contributes to receptor binding (208). Stefansson *et al.* (207) supported a theory of lack of importance of the serine protease domain by their report of equal receptor-binding affinities of PAI-1 in complex with the serine protease domain of uPA and with trypsin, respectively. However, a more detailed analysis showed that substitution of a cluster of basic residues near the 37-loop and 60-loop of uPA reduced the receptor-binding affinity of the uPA - PAI-1 complex approximately twofold. The localization of the implicated residues in the three-dimensional structures of uPA and PAI-1 shows that they form a continuous receptor-binding area spanning the serpin as well as the A-chain and the serine protease domain of uPA. Thus, the 10-100 fold higher affinity of the uPA - PAI-1 complex compared with the free component seems to depend on the bonus effect of bringing the binding areas on uPA and PAI-1 together on the same binding entity. Whether or not conformational changes of the protease domain or the serpin upon complex formation also contribute to the increased affinity can probably best be decided on the basis of X-ray crystal structural analysis.

Ligand binding by receptors of the LDLR family is critically dependent on the CTR clusters (Figure 5). LDLR, VLDLR, apoER2, and sorLA have only one such cluster, with 7 (LDLR), 8 (VLDLR, apoER2), or 11 (sorLA) CTRs, respectively. LRP-1A has 4 clusters, of 2, 8,

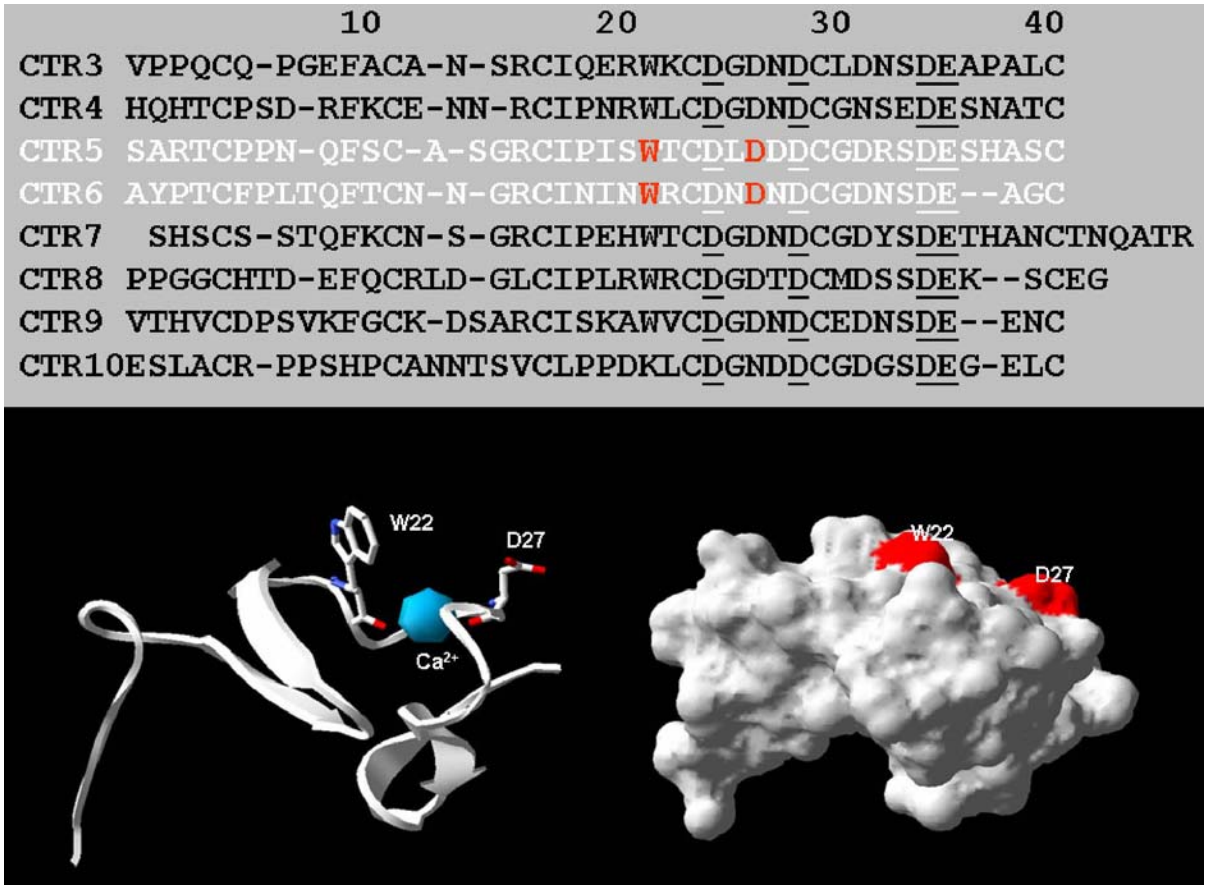

Figure 7. uPA - PAI-1 binding site of LRP. Top panel, the amino acid sequence of the 8 CTRs of the 2nd cluster in LRP. CTR5 and CTR6, the pair of which has the highest affinity to uPA - PAI-1, are shown in white. Residues implicated in the binding are highlighted. Bottom panel, the three-dimensional structure of CTR3 from LRP (200), in a ribbon presentation (left) and surface presentation (right). Indicated are W22 and D27, which were implicated in uPA - PAI-1 binding. In both panels, the numbering of the amino acids in the CTRs are according to (198).

10, and 11 CTRs, respectively (for a review, see 155). LRP CTR clusters II and IV have been shown to bind uPA - PAI-1 complex (209-212). LRP-1B has 4 clusters, of 2, 8, 10, and 12 CTRs, respectively, and CTR clusters II and IV have been shown to bind plasminogen activator - PAI-1 complexes (173). Megalin has 4 clusters, of 7, 8, 10, and 11 CTRs and the second cluster has been implicated in ligand binding (213). As originally concluded for LDLR itself (214, 215), the ligand-receptor binding specificity seems to depend on each individual ligand making contacts to a specific set of CTRs in each receptor, and this also appears to be true in the case of plasminogen activator - PAI-1 complexes. By expression of VLDLR variants lacking specific complement-type repeats, it was found that the second CTR in the cluster of 8 in this receptor was required for maximal affinity to uPA - PAI-1 complex (216). Reportedly, tPA - PAI-1 complex, PAI-1, and pro-uPA can bind to a fragment encompassing CTR3-CTR4-CTR5-CTR6-CTR7 (the CTRs of the N-terminal CTR-cluster 1 being numbered as number 1 and 2) of CTR cluster II of LRP (211, 217, 218). Andersen *et al.* (2001) showed that the minimal functional unit capable of mimicking the binding of uPA - PAI-1 complex to LRP CTR cluster II is a 2-CTR fragment, and that the CTR5-CTR6 pair had the highest affinity to the uPA - PAI-1 complex (219). The substitutions W22S and D27N (using the numbering of (198) in either CTR5 or CTR6 resulted in a reduced binding of uPA - PAI-1, whereas substitutions of two different R residues had no effect on the binding. The substitution D28N in CTR5 also resulted in a reduced binding. W22 and D27 are centrally placed in the three-dimensional structure of the CTRs, as indicated on the three-dimensional structure of LRP CTR3 (Figure 7). Whereas W22 and D27 are important for the binding of uPA - PAI-1 complex, the observations with these 2 residues do not directly or totally explain the specificity underlying the specific binding of uPA - PAI-1 complex to CTR5-CTR6, as the W22 and D27 are also found in CTR3, CTR4, CTR7, CTR8, and CTR9. The D28 residue may contribute to the specificity, as it is only found in CTR5. Among the CTRs of LRP CTR cluster II, this residue is only found in CTR5 and could for that reason be assumed to be implicated in conferring specificity to the binding, but then again, none of the CTRs in VLDLR has the combination W22, D27, D28.

From the dimensions of the CTRs emerging from the X-ray crystal structure analysis of LDLR (194), the uPA-serine protease domain and the PAI-1 moieties could each make contact to a CTR. The A-chain of uPA would bind to a third CTR. Thus, whereas the individual components uPA and PAI-1 would only be able to make contact to one or two CTRs, the complex would be able to make contact to three CTRs and thus bind with a high affinity.

11. CONCLUSIONS AND PERSPECTIVES

Studies of PAI-1 and other serpins in the latest two decades have shown that their distinguishing feature is the conversion from the metastable native, "stressed" conformation to the loop-inserted, "relaxed" conformation. This conformational change forms the basis for their inhibitory mechanism. RCL insertion is coupled to both transient and permanent global conformational changes. Thus, during the insertion of the RCL, the shift of the small serpin fragment, and the conformational change of the flexible joint region in connection with both complex formation and latency transition, there are concerted conformational changes of the shutter region and the surrounding part of s5A, and the small serpin fragment. On the other side, binding of vitronectin to the small serpin fragment of PAI-1 may affect the movements of the RCL. The elucidation of serpin inhibitory mechanism and serpin conformational changes has thus contributed to the general understanding of protein conformational changes and of enzymes catalytic and inhibitory mechanisms.

PAI-1 is a potential therapeutic target in cardiovascular diseases as well as in cancer (for a review, see 9, 12, 220-222). The detailed knowledge about the molecular interactions of PAI-1, as reviewed here, has laid a solid foundation for the development of PAI-1 inactivating compounds, be they organochemicals, monoclonal antibodies, or peptides (for a review, see 10, 12). Such inactivating agents may be used both for target validation in animal disease models and leads in drug development.

In spite of the substantial progress, many challenges of a basic protein chemical nature still lie ahead. Thus, the early steps of the protease - serpin interaction still contain considerable elements of "standard mechanism inhibition" characteristic for inhibitors of the Kunitz, Kazal and other families, in which an exposed loop also inserts into the active site, but in which there are no ensuing global conformational change of the inhibitor (for a review, see 223). The importance of exosite interactions for the progression from standard mechanism inhibition to the RCL insertion-based mechanism is of considerable interest. The latency transition is one of the largest structural rearrangements known for a folded protein without a concomitant change in covalent structure. Yet, the sequence of conformational changes during latency transition remains largely unknown. It is a challenge to understand the conformational changes induced into PAI-1 by its natural ligand vitronectin and the many organochemicals, monoclonal antibodies, and peptides, which have been developed in order to be able to inactivate PAI-1 *in vivo*. Finally, a structural characterisation of the binding of protease - PAI-1 complexes to the endocytosis receptors would be an important step.

12. ACKNOWLEDGEMENTS

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13. REFERENCES

1. R. Huber and R. W. Carrell: Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins. *Biochemistry*, 28, 8951-66 (1989)

2. R. W. Carrell and P. E. Stein: The biostructural pathology of the serpins: critical function of sheet opening mechanism. *Biol Chem Hoppe Seyler*, 377, 1-17 (1996)

3. A. Gils and P. J. Declerck: Structure-function relationships in serpins: current concepts and controversies. *Thromb Haemost*, 80, 531-41 (1998)

4. J. A. Irving, R. N. Pike, A. M. Lesk, and J. C. Whisstock: Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res*, 10, 1845-64 (2000)

5. S. Ye and E. J. Goldsmith: Serpins and other covalent protease inhibitors. *Curr Opin Struct Biol*, 11, 740-5 (2001)

6. J. A. Huntington: Shape-shifting serpins--advantages of a mobile mechanism. *Trends Biochem Sci*, 31, 427-35 (2006)

7. T. Wind, M. Hansen, J. K. Jensen, and P. A. Andreasen: The molecular basis for anti-proteolytic and non-proteolytic functions of plasminogen activator inhibitor type-1: roles of the reactive centre loop, the shutter region, the flexible joint region and the small serpin fragment. Biol Chem, 383, 21-36 (2002)

8. H. Loebermann, R. Tokuoka, J. Deisenhofer, and R. Huber: Human alpha 1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. J Mol Biol, 177, 531-57 (1984)

9. M. K. Durand, J. S. Bodker, A. Christensen, D. M. Dupont, M. Hansen, J. K. Jensen, S. Kjelgaard, L. Mathiasen, K. E. Pedersen, S. Skeldal, T. Wind, and P. A. Andreasen: Plasminogen activator inhibitor-I and tumour growth, invasion, and metastasis. *Thromb Haemost*, 91, 438-49 (2004)

10. A. Gils and P. J. Declerck: The structural basis for the pathophysiological relevance of PAI-I in cardiovascular diseases and the development of potential PAI-I inhibitors. *Thromb Haemost*, 91, 425-37 (2004)

11. C. Dellas and D. J. Loskutoff: Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thromb Haemost*, 93, 631-40 (2005)

12. P. A. Andreasen: PAI-1 - a potential therapeutic target in cancer. *Curr Drug Targets*, 8, 1030-41 (2007)

13. D. J. Loskutoff and T. E. Edgington: Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc Natl Acad Sci U S A*, 74, 3903-7 (1977)

14. S. C. Seifert and T. D. Gelehrter: Mechanism of dexamethasone inhibition of plasminogen activator in rat hepatoma cells. *Proc Natl Acad Sci U S A*, 75, 6130-3 (1978)

15. J. A. van Mourik, D. A. Lawrence, and D. J. Loskutoff: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J Biol Chem*, 259, 14914-21 (1984)

16. L. S. Nielsen, P. A. Andreasen, J. Grondahl-Hansen, J. Y. Huang, P. Kristensen, and K. Dano: Monoclonal antibodies to human 54,000 molecular weight plasminogen activator inhibitor from fibrosarcoma cells--inhibitor neutralization and one-step affinity purification. *Thromb Haemost*, 55, 206-12 (1986)

17. M. Philips, A. G. Juul, S. Thorsen, J. Selmer, and J. Zeuthen: Immunological relationship between the fast-acting plasminogen activator inhibitors from plasma, blood platelets and endothelial cells demonstrated with a monoclonal antibody against an inhibitor from placenta. *Thromb Haemost*, 55, 213-7 (1986)

18. P. A. Andreasen, A. Riccio, K. G. Welinder, R. Douglas, R. Sartorio, L. S. Nielsen, C. Oppenheimer, F. Blasi, and K. Dano: Plasminogen activator inhibitor type-1: reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing. *FEBS Lett*, 209, 213-8 (1986)

19. T. Ny, M. Sawdey, D. Lawrence, J. L. Millan, and D. J. Loskutoff: Cloning and sequence of a cDNA coding for the human beta-migrating endothelial-cell-type plasminogen activator inhibitor. *Proc Natl Acad Sci U S A*, 83, 6776-80 (1986)

20. H. Pannekoek, H. Veerman, H. Lambers, P. Diergaarde, C. L. Verweij, A. J. van Zonneveld, and J. A. van Mourik: Endothelial plasminogen activator inhibitor (PAI): a new member of the Serpin gene family. *Embo J*, 5, 2539-44 (1986)

21. D. Ginsburg, R. Zeheb, A. Y. Yang, U. M. Rafferty, P. A. Andreasen, L. Nielsen, K. Dano, R. V. Lebo, and T. D. Gelehrter: cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. *J Clin Invest*, 78, 1673-80 (1986)

22. Y. Jiang and R. F. Doolittle: The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proc Natl Acad Sci U S A*, 100, 7527-32 (2003)

23. G. T. Cicila, T. M. O'Connell, W. C. Hahn, and J. G. Rheinwald: Cloned cDNA sequence for the human mesothelial protein 'mesosecrin' discloses its identity as a plasminogen activator inhibitor (PAI-1) and a recent evolutionary change in transcript processing. *J Cell Sci*, 94 (Pt 1), 1-10 (1989)

24. J. G. Rheinwald, J. L. Jorgensen, W. C. Hahn, A. J. Terpstra, T. M. O'Connell, and K. K. Plummer: Mesosecrin: a secreted glycoprotein produced in abundance by human mesothelial, endothelial, and kidney epithelial cells in culture. *J Cell Biol*, 104, 263-75 (1987)

25. A. Gils, K. E. Pedersen, P. Skottrup, A. Christensen, D. Naessens, J. Deinum, J. J. Enghild, P. J. Declerck, and P. A. Andreasen: Biochemical importance of glycosylation of plasminogen activator inhibitor-1. *Thromb Haemost*, 90, 206-17 (2003)

26. Y. Xue, P. Bjorquist, T. Inghardt, M. Linschoten, D. Musil, L. Sjolin, and J. Deinum: Interfering with the inhibitory mechanism of serpins: crystal structure of a complex formed between cleaved plasminogen activator inhibitor type 1 and a reactive-centre loop peptide. *Structure*, 6, 627-36 (1998)

27. D. M. Dupont, G. E. Blouse, M. Hansen, L. Mathiasen, S. Kjelgaard, J. K. Jensen, A. Christensen, A. Gils, P. J. Declerck, P. A. Andreasen, and T. Wind: Evidence for a pre-latent form of the serpin plasminogen activator inhibitor-1 with a detached beta-strand 1C. *J Biol Chem*, 281, 36071-81 (2006)

28. D. J. Loskutoff, M. Linders, J. Keijer, H. Veerman, H. van Heerikhuizen, and H. Pannekoek: Structure of the human plasminogen activator inhibitor 1 gene: nonrandom distribution of introns. *Biochemistry*, 26, 3763-8 (1987)

29. A. Riccio, L. R. Lund, R. Sartorio, A. Lania, P. A. Andreasen, K. Dano, and F. Blasi: The regulatory region of the human plasminogen activator inhibitor type-1 (PAI-1) gene. *Nucleic Acids Res*, 16, 2805-24 (1988)

30. L. Strandberg, D. Lawrence, and T. Ny: The organization of the human-plasminogen-activator-inhibitor-1 gene. Implications on the evolution of the serine-protease inhibitor family. *Eur J Biochem*, 176, 609-16 (1988)

31. K. W. Klinger, R. Winqvist, A. Riccio, P. A. Andreasen, R. Sartorio, L. S. Nielsen, N. Stuart, P. Stanislovitis, P. Watkins, R. Douglas, and *et al.*: Plasminogen activator inhibitor type 1 gene is located at region q21.3-q22 of chromosome 7 and genetically linked

with cystic fibrosis. *Proc Natl Acad Sci U S A*, 84, 8548-52 (1987)

32. P. A. Andreasen, B. Georg, L. R. Lund, A. Riccio, and S. N. Stacey: Plasminogen activator inhibitors: hormonally regulated serpins. *Mol Cell Endocrinol*, 68, 1-19 (1990)

33. B. De Taeye, L. H. Smith, and D. E. Vaughan: Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease. *Curr Opin Pharmacol*, 5, 149-54 (2005)

34. Y. Nagamine, R. L. Medcalf, and P. Munoz-Canoves: Transcriptional and posttranscriptional regulation of the plasminogen activator system. *Thromb Haemost*, 93, 661-75 (2005)

35. D. A. Lawrence, L. Strandberg, J. Ericson, and T. Ny: Structure-function studies of the SERPIN plasminogen activator inhibitor type 1. Analysis of chimeric strained loop mutants. *J Biol Chem*, 265, 20293-301 (1990)

36. P. M. Sherman, D. A. Lawrence, A. Y. Yang, E. T. Vandenberg, D. Paielli, S. T. Olson, J. D. Shore, and D. Ginsburg: Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. *J Biol Chem*, 267, 7588-95 (1992)

37. P. M. Sherman, D. A. Lawrence, I. M. Verhamme, D. Paielli, J. D. Shore, and D. Ginsburg: Identification of tissue-type plasminogen activator-specific plasminogen activator inhibitor-1 mutants. Evidence that second sites of interaction contribute to target specificity. *J Biol Chem*, 270, 9301-6 (1995)

38. J. D. York, P. Li, and S. J. Gardell: Combinatorial mutagenesis of the reactive site region in plasminogen activator inhibitor I. *J Biol Chem*, 266, 8495-500 (1991)

39. C. A. Ibarra, G. E. Blouse, T. D. Christian, and J. D. Shore: The contribution of the exosite residues of plasminogen activator inhibitor-1 to proteinase inhibition. *J Biol Chem*, 279, 3643-50 (2004)

40. H. M. Tucker and R. D. Gerard: Sequence requirements in the reactive-center loop of plasminogen-activator inhibitor-1 for recognition of plasminogen activators. *Eur J Biochem*, 237, 180-7 (1996)

41. E. L. Madison, E. J. Goldsmith, R. D. Gerard, M. J. Gething, and J. F. Sambrook: Serpin-resistant mutants of human tissue-type plasminogen activator. *Nature*, 339, 721-4 (1989)

42. E. L. Madison, E. J. Goldsmith, R. D. Gerard, M. J. Gething, J. F. Sambrook, and R. S. Bassel-Duby: Amino acid residues that affect interaction of tissue-type plasminogen activator with plasminogen activator inhibitor 1. *Proc Natl Acad Sci U S A*, 87, 3530-3 (1990)

43. E. L. Madison, E. J. Goldsmith, M. J. Gething, J. F. Sambrook, and R. D. Gerard: Restoration of serine protease-inhibitor interaction by protein engineering. *J Biol Chem*, 265, 21423-6 (1990)

44. D. S. Adams, L. A. Griffin, W. R. Nachajko, V. B. Reddy, and C. M. Wei: A synthetic DNA encoding a modified human urokinase resistant to inhibition by serum plasminogen activator inhibitor. *J Biol Chem*, 266, 8476-82 (1991)

45. A. J. Horrevoets, G. Tans, A. E. Smilde, A. J. van Zonneveld, and H. Pannekoek: Thrombin-variable region 1 (VR1). Evidence for the dominant contribution of VR1 of serine proteases to their interaction with plasminogen activator inhibitor 1. *J Biol Chem*, 268, 779-82 (1993)

46. M. van Meijer, Y. Roelofs, J. Neels, A. J. Horrevoets, A. J. van Zonneveld, and H. Pannekoek: Selective screening of a large phage display library of plasminogen activator inhibitor 1 mutants to localize interaction sites with either thrombin or the variable region 1 of tissue-type plasminogen activator. *J Biol Chem*, 271, 7423-8 (1996)

47. J. D. Sipley, D. S. Alexander, J. E. Testa, and J. P. Quigley: Introduction of an RRHR motif into chicken urokinase-type plasminogen activator (ch-uPA) confers sensitivity to plasminogen activator inhibitor (PAI)-1 and PAI-2 and allows ch-uPA-mediated extracellular matrix degradation to be controlled by PAI-1. *Proc Natl Acad Sci U S A*, 94, 2933-8 (1997)

48. R. J. Dekker, A. Eichinger, A. A. Stoop, W. Bode, H. Pannekoek, and A. J. Horrevoets: The variable region-1 from tissue-type plasminogen activator confers specificity for plasminogen activator inhibitor-1 to thrombin by facilitating catalysis: release of a kinetic block by a heterologous protein surface loop. *J Mol Biol*, 293, 613-27 (1999)

49. H. H. Petersen, M. Hansen, S. L. Schousboe, and P. A. Andreasen: Localization of epitopes for monoclonal antibodies to urokinase-type plasminogen activator: relationship between epitope localization and effects of antibodies on molecular interactions of the enzyme. *Eur J Biochem*, 268, 4430-9 (2001)

50. S. Ye, A. L. Cech, R. Belmares, R. C. Bergstrom, Y. Tong, D. R. Corey, M. R. Kanost, and E. J. Goldsmith: The structure of a Michaelis serpin-protease complex. *Nat Struct Biol*, 8, 979-83 (2001)

51. A. Dementiev, M. Simonovic, K. Volz, and P. G. Gettins: Canonical inhibitor-like interactions explain reactivity of alpha1-proteinase inhibitor Pittsburgh and antithrombin with proteinases. *J Biol Chem*, 278, 37881-7 (2003)

52. T. P. Baglin, R. W. Carrell, F. C. Church, C. T. Esmon, and J. A. Huntington: Crystal structures of native and thrombin-complexed heparin cofactor II reveal a multistep

allosteric mechanism. *Proc Natl Acad Sci U S A*, 99, 11079-84 (2002)

53. W. Li, D. J. Johnson, C. T. Esmon, and J. A. Huntington: Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat Struct Mol Biol*, 11, 857-62 (2004)

54. D. J. Johnson, W. Li, T. E. Adams, and J. A. Huntington: Antithrombin-S195A factor Xa-heparin structure reveals the allosteric mechanism of antithrombin activation. *Embo J*, 25, 2029-37 (2006)

55. A. Dementiev, M. Petitou, J. M. Herbert, and P. G. Gettins: The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. *Nat Struct Mol Biol*, 11, 863-7 (2004)

56. R. Egelund, K. W. Rodenburg, P. A. Andreasen, M. S. Rasmussen, R. E. Guldberg, and T. E. Petersen: An ester bond linking a fragment of a serine proteinase to its serpin inhibitor. *Biochemistry*, 37, 6375-9 (1998)

57. J. O. Kvassman, I. Verhamme, and J. D. Shore: Inhibitory mechanism of serpins: loop insertion forces acylation of plasminogen activator by plasminogen activator inhibitor-1. *Biochemistry*, 37, 15491-502 (1998)

58. S. T. Olson, R. Swanson, D. Day, I. Verhamme, J. Kvassman, and J. D. Shore: Resolution of Michaelis complex, acylation, and conformational change steps in the reactions of the serpin, plasminogen activator inhibitor-1, with tissue plasminogen activator and trypsin. *Biochemistry*, 40, 11742-56 (2001)

59. G. E. Blouse, M. J. Perron, J. O. Kvassman, S. Yunus, J. H. Thompson, R. L. Betts, L. C. Lutter, and J. D. Shore: Mutation of the highly conserved tryptophan in the serpin breach region alters the inhibitory mechanism of plasminogen activator inhibitor-1. *Biochemistry*, 42, 12260-72 (2003)

60. J. A. Huntington, R. J. Read, and R. W. Carrell: Structure of a serpin-protease complex shows inhibition by deformation. *Nature*, 407, 923-6 (2000)

61. A. Dementiev, J. Dobo, and P. G. Gettins: Active site distortion is sufficient for proteinase inhibition by serpins: structure of the covalent complex of alpha1-proteinase inhibitor with porcine pancreatic elastase. *J Biol Chem*, 281, 3452-7 (2006)

62. E. Stratikos and P. G. Gettins: Major proteinase movement upon stable serpin-proteinase complex formation. *Proc Natl Acad Sci U S A*, 94, 453-8 (1997)

63. E. Stratikos and P. G. Gettins: Mapping the serpin-proteinase complex using single cysteine variants of alpha1-proteinase inhibitor Pittsburgh. *J Biol Chem*, 273, 15582-9 (1998)

64. M. Wilczynska, M. Fa, J. Karolin, P. I. Ohlsson, L. B. Johansson, and T. Ny: Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat Struct Biol*, 4, 354-7 (1997)

65. M. Fa, F. Bergstrom, P. Hagglof, M. Wilczynska, L. B. Johansson, and T. Ny: The structure of a serpin-protease complex revealed by intramolecular distance measurements using donor-donor energy migration and mapping of interaction sites. *Structure*, 8, 397-405 (2000)

66. L. Liu, N. Mushero, L. Hedstrom, and A. Gershenson: Conformational distributions of protease-serpin complexes: a partially translocated complex. *Biochemistry*, 45, 10865-72 (2006)

67. L. Liu, N. Mushero, L. Hedstrom, and A. Gershenson: Short-lived protease serpin complexes: partial disruption of the rat trypsin active site. *Protein Sci*, 16, 2403-11 (2007)

68. M. I. Plotnick, L. Mayne, N. M. Schechter, and H. Rubin: Distortion of the active site of chymotrypsin complexed with a serpin. *Biochemistry*, 35, 7586-90 (1996)

69. E. S. Stavridi, K. O'Malley, C. M. Lukacs, W. T. Moore, J. D. Lambris, D. W. Christianson, H. Rubin, and B. S. Cooperman: Structural change in alpha-chymotrypsin induced by complexation with alpha 1-antichymotrypsin as seen by enhanced sensitivity to proteolysis. *Biochemistry*, 35, 10608-15 (1996)

70. R. Egelund, T. E. Petersen, and P. A. Andreasen: A serpin-induced extensive proteolytic susceptibility of urokinase-type plasminogen activator implicates distortion of the proteinase substrate-binding pocket and oxyanion hole in the serpin inhibitory mechanism. *Eur J Biochem*, 268, 673-85 (2001)

71. G. Kaslik, J. Kardos, E. Szabo, L. Szilagyi, P. Zavodszky, W. M. Westler, J. L. Markley, and L. Graf: Effects of serpin binding on the target proteinase: global stabilization, localized increased structural flexibility, and conserved hydrogen bonding at the active site. *Biochemistry*, 36, 5455-64 (1997)

72. D. J. Tew and S. P. Bottomley: Intrinsic fluorescence changes and rapid kinetics of proteinase deformation during serpin inhibition. *FEBS Lett*, 494, 30-3 (2001)

73. F. C. Peterson and P. G. Gettins: Insight into the mechanism of serpin-proteinase inhibition from 2D (1H-15N) NMR studies of the 69 kDa alpha 1-proteinase inhibitor Pittsburgh-trypsin covalent complex. *Biochemistry*, 40, 6284-92 (2001)

74. S. V. Calugaru, R. Swanson, and S. T. Olson: The pH dependence of serpin-proteinase complex dissociation reveals a mechanism of complex stabilization involving inactive and active conformational states of the proteinase which are perturbable by calcium. *J Biol Chem*, 276, 32446-55 (2001)

75. P. G. Gettins: The F-helix of serpins plays an essential, active role in the proteinase inhibition mechanism. *FEBS Lett*, 523, 2-6 (2002)

76. C. M. Hekman and D. J. Loskutoff: Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem*, 260, 11581-7 (1985)

77. J. Mottonen, A. Strand, J. Symersky, R. M. Sweet, D. E. Danley, K. F. Geoghegan, R. D. Gerard, and E. J. Goldsmith: Structural basis of latency in plasminogen activator inhibitor-1. *Nature*, 355, 270-3 (1992)

78. H. M. Tucker, J. Mottonen, E. J. Goldsmith, and R. D. Gerard: Engineering of plasminogen activator inhibitor-1 to reduce the rate of latency transition. *Nat Struct Biol*, 2, 442-5 (1995)

79. D. A. Lawrence, S. T. Olson, S. Palaniappan, and D. Ginsburg: Engineering plasminogen activator inhibitor 1 mutants with increased functional stability. *Biochemistry*, 33, 3643-8 (1994)

80. G. C. Sui and B. Wiman: The Bbeta-sheet in the PAI-1 molecule plays an important role for its stability. *Thromb Haemost*, 83, 896-901 (2000)

81. H. Mangs, G. C. Sui, and B. Wiman: PAI-1 stability: the role of histidine residues. *FEBS Lett*, 475, 192-6 (2000)

82. G. C. Sui and B. Wiman: Stability of plasminogen activator inhibitor-1: role of tyrosine221. *FEBS Lett*, 423, 319-23 (1998)

83. T. H. Ngo, M. F. Hoylaerts, I. Knockaert, E. Brouwers, and P. J. Declerck: Identification of a target site in plasminogen activator inhibitor-1 that allows neutralization of its inhibitor properties concomitant with an allosteric up-regulation of its antiadhesive properties. *J Biol Chem*, 276, 26243-8 (2001)

84. M. B. Berkenpas, D. A. Lawrence, and D. Ginsburg: Molecular evolution of plasminogen activator inhibitor-1 functional stability. *Embo J*, 14, 2969-77 (1995)

85. A. A. Stoop, E. Eldering, T. R. Dafforn, R. J. Read, and H. Pannekoek: Different structural requirements for plasminogen activator inhibitor 1 (PAI-1) during latency transition and proteinase inhibition as evidenced by phage-displayed hypermutated PAI-1 libraries. *J Mol Biol*, 305, 773-83 (2001)

86. P. Kruger, S. Verheyden, P. J. Declerck, and Y. Engelborghs: Extending the capabilities of targeted molecular dynamics: simulation of a large conformational transition in plasminogen activator inhibitor 1. *Protein Sci*, 10, 798-808 (2001)

87. A. Gils, J. Lu, K. Aertgeerts, I. Knockaert, and P. J. Declerck: Identification of positively charged residues contributing to the stability of plasminogen activator inhibitor 1. *FEBS Lett*, 415, 192-5 (1997)

88. N. V. Gorlatova, H. Elokdah, K. Fan, D. L. Crandall, and D. A. Lawrence: Mapping of a conformational epitope on plasminogen activator inhibitor-1 by random mutagenesis. Implications for serpin function. *J Biol Chem*, 278, 16329-35 (2003)

89. I. Verhamme, J. O. Kvassman, D. Day, S. Debrock, N. Vleugels, P. J. Declerck, and J. D. Shore: Accelerated conversion of human plasminogen activator inhibitor-1 to its latent form by antibody binding. *J Biol Chem*, 274, 17511-7 (1999)

90. D. Naessens, A. Gils, G. Compernolle, and P. J. Declerck: Elucidation of the epitope of a latency-inducing antibody: identification of a new molecular target for PAI-1 inhibition. *Thromb Haemost*, 90, 52-8 (2003)

91. B. Gooptu, B. Hazes, W. S. Chang, T. R. Dafforn, R. W. Carrell, R. J. Read, and D. A. Lomas: Inactive conformation of the serpin alpha (1)-antichymotrypsin indicates two-stage insertion of the reactive loop: implications for inhibitory function and conformational disease. *Proc Natl Acad Sci U S A*, 97, 67-72 (2000)

92. Y. R. Na and H. Im: Specific interactions of serpins in their native forms attenuate their conformational transitions. *Protein Sci*, 16, 1659-66 (2007)

93. P. J. Declerck, M. De Mol, M. C. Alessi, S. Baudner, E. P. Paques, K. T. Preissner, G. Muller-Berghaus, and D. Collen: Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). *J Biol Chem*, 263, 15454-61 (1988)

94. M. G. Conlan, B. R. Tomasini, R. L. Schultz, and D. F. Mosher: Plasma vitronectin polymorphism in normal subjects and patients with disseminated intravascular coagulation. *Blood*, 72, 185-90 (1988)

95. K. T. Preissner and D. Seiffert: Role of vitronectin and its receptors in haemostasis and vascular remodeling. *Thromb Res*, 89, 1-21 (1998)

96. A. Zhou, J. A. Huntington, N. S. Pannu, R. W. Carrell, and R. J. Read: How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat Struct Biol*, 10, 541-4 (2003)

97. Y. Kamikubo, R. De Guzman, G. Kroon, S. Curriden, J. G. Neels, M. J. Churchill, P. Dawson, S. Oldziej, A. Jagielska, H. A. Scheraga, D. J. Loskutoff, and H. J. Dyson: Disulfide bonding arrangements in active forms of the somatomedin B domain of human vitronectin. *Biochemistry*, 43, 6519-34 (2004)

98. M. Kjaergaard, H. Gardsvoll, D. Hirschberg, S. Nielbo, A. Mayasundari, C. B. Peterson, A. Jansson, T. J.

Jorgensen, F. M. Poulsen, and M. Ploug: Solution structure of recombinant somatomedin B domain from vitronectin produced in Pichia pastoris. *Protein Sci*, 16, 1934-45 (2007)

99. X. Li, G. Zou, W. Yuan, and W. Lu: Defining the native disulfide topology in the somatomedin B domain of human vitronectin. *J Biol Chem*, 282, 5318-26 (2007)

100. A. Zhou: Functional structure of the somatomedin B domain of vitronectin. *Protein Sci*, 16, 1502-8 (2007)

101. E. M. Salonen, A. Vaheri, J. Pollanen, R. Stephens, P. Andreasen, M. Mayer, K. Dano, J. Gailit, and E. Ruoslahti: Interaction of plasminogen activator inhibitor (PAI-1) with vitronectin. *J Biol Chem*, 264, 6339-43 (1989)

102. T. Lindahl and B. Wiman: Purification of high and low molecular weight plasminogen activator inhibitor 1 from fibrosarcoma cell-line HT 1080 conditioned medium. *Biochim Biophys Acta*, 994, 253-7 (1989)

103. B. Wiman, A. Almquist, O. Sigurdardottir, and T. Lindahl: Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma. *FEBS Lett*, 242, 125-8 (1988)

104. J. Mimuro and D. J. Loskutoff: Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. Evidence that the protein is vitronectin. *J Biol Chem*, 264, 936-9 (1989)

105. D. A. Lawrence, S. Palaniappan, S. Stefansson, S. T. Olson, A. M. Francis-Chmura, J. D. Shore, and D. Ginsburg: Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J Biol Chem*, 272, 7676-80 (1997)

106. D. Seiffert and D. J. Loskutoff: Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. *J Biol Chem*, 266, 2824-30 (1991)

107. C.R. Schar, Blouse, G.E., Minor, K.H., and Peterson, C.B.: A deletion mutant of vitronectin lacking the somatomedin B domain exhibits residual PAI-1 binding activity. *JBC, in press* (2008)

108. D. A. Lawrence, M. B. Berkenpas, S. Palaniappan, and D. Ginsburg: Localization of vitronectin binding domain in plasminogen activator inhibitor-1. *J Biol Chem*, 269, 15223-8 (1994)

109. N. Arroyo De Prada, F. Schroeck, E. K. Sinner, B. Muehlenweg, J. Twellmeyer, S. Sperl, O. G. Wilhelm, M. Schmitt, and V. Magdolen: Interaction of plasminogen activator inhibitor type-1 (PAI-1) with vitronectin. *Eur J Biochem*, 269, 184-92 (2002)

110. M. van Meijer, R. K. Gebbink, K. T. Preissner, and H. Pannekoek: Determination of the vitronectin binding site on plasminogen activator inhibitor 1 (PAI-1). *FEBS Lett*, 352, 342-6 (1994)

111. J. Padmanabhan and D. C. Sane: Localization of a vitronectin binding region of plasminogen activator inhibitor-1. *Thromb Haemost*, 73, 829-34 (1995)

112. J. K. Jensen, T. Wind, and P. A. Andreasen: The vitronectin binding area of plasminogen activator inhibitor-1, mapped by mutagenesis and protection against an inactivating organochemical ligand. *FEBS Lett*, 521, 91-4 (2002)

113. J. K. Jensen, M. K. Durand, S. Skeldal, D. M. Dupont, J. S. Bodker, T. Wind, and P. A. Andreasen: Construction of a plasminogen activator inhibitor-1 variant without measurable affinity to vitronectin but otherwise normal. *FEBS Lett*, 556, 175-9 (2004)

114. G. Deng, G. Royle, S. Wang, K. Crain, and D. J. Loskutoff: Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin. *J Biol Chem*, 271, 12716-23 (1996)

115. C. D. Madsen, G. M. Ferraris, A. Andolfo, O. Cunningham, and N. Sidenius: uPAR-induced cell adhesion and migration: vitronectin provides the key. *J Cell Biol*, 177, 927-39 (2007)

116. H. Gardsvoll and M. Ploug: Mapping of the vitronectin-binding site on the urokinase receptor: involvement of a coherent receptor interface consisting of residues from both domain I and the flanking interdomain linker region. *J Biol Chem*, 282, 13561-72 (2007)

117. S. Stefansson and D. A. Lawrence: The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. *Nature*, 383, 441-3 (1996)

118. L. Kjoller, S. M. Kanse, T. Kirkegaard, K. W. Rodenburg, E. Ronne, S. L. Goodman, K. T. Preissner, L. Ossowski, and P. A. Andreasen: Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp Cell Res*, 232, 420-9 (1997)

119. D. Seiffert and D. J. Loskutoff: Type 1 plasminogen activator inhibitor induces multimerization of plasma vitronectin. A suggested mechanism for the generation of the tissue form of vitronectin *in vivo*. *J Biol Chem*, 271, 29644-51 (1996)

120. A. M. Sharp, P. E. Stein, N. S. Pannu, R. W. Carrell, M. B. Berkenpas, D. Ginsburg, D. A. Lawrence, and R. J. Read: The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. *Structure*, 7, 111-8 (1999)

121. H. Nar, M. Bauer, J. M. Stassen, D. Lang, A. Gils, and P. J. Declerck: Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and

implications for serpin inactivation. *J Mol Biol*, 297, 683-95 (2000)

122. P. Stein and C. Chothia: Serpin tertiary structure transformation. *J Mol Biol*, 221, 615-21 (1991)

123. J. C. Whisstock, R. Skinner, R. W. Carrell, and A. M. Lesk: Conformational changes in serpins: I. The native and cleaved conformations of alpha (1)-antitrypsin. *J Mol Biol*, 296, 685-99 (2000)

124. K. Aertgeerts, H. L. De Bondt, C. De Ranter, and P. J. Declerck: Crystallization and X-ray diffraction data of the cleaved form of plasminogen activator inhibitor-1. *Proteins*, 23, 118-21 (1995)

125. T. J. Stout, H. Graham, D. I. Buckley, and D. J. Matthews: Structures of active and latent PAI-1: a possible stabilizing role for chloride ions. *Biochemistry*, 39, 8460-9 (2000)

126. R. Egelund, S. L. Schousboe, L. Sottrup-Jensen, K. W. Rodenburg, and P. A. Andreasen: Type-1 plasminogen-activator inhibitor -- conformational differences between latent, active, reactive-centre-cleaved and plasminogen-activator-complexed forms, as probed by proteolytic susceptibility. *Eur J Biochem*, 248, 775-85 (1997)

127. P. Bjorquist, J. Ehnebom, T. Inghardt, L. Hansson, M. Lindberg, M. Linschoten, M. Stromqvist, and J. Deinum: Identification of the binding site for a low-molecular-weight inhibitor of plasminogen activator inhibitor type 1 by site-directed mutagenesis. *Biochemistry*, 37, 1227-34 (1998)

128. R. Egelund, A. P. Einholm, K. E. Pedersen, R. W. Nielsen, A. Christensen, J. Deinum, and P. A. Andreasen: A regulatory hydrophobic area in the flexible joint region of plasminogen activator inhibitor-1, defined with fluorescent activity-neutralizing ligands. Ligand-induced serpin polymerization. *J Biol Chem*, 276, 13077-86 (2001)

129. A. A. Komissarov, P. A. Andreasen, J. S. Bodker, P. J. Declerck, J. Y. Anagli, and J. D. Shore: Additivity in effects of vitronectin and monoclonal antibodies against alpha-helix F of plasminogen activator inhibitor-1 on its reactions with target proteinases. *J Biol Chem*, 280, 1482-9 (2005)

130. A. A. Komissarov, A. Zhou, and P. J. Declerck: Modulation of serpin reaction through stabilization of transient intermediate by ligands bound to alpha-helix F. *J Biol Chem*, 282, 26306-15 (2007)

131. A.K. Komissarov, Andreasen, P.A., Declerck, P.J., Kamikubo, Y., Zhou, A.W., and Gruber, A.: Redirection of the reaction between activated protein C and a serpin to the substrate pathway. *Thromb. Res., in press* (2008)

132. M. Fa, J. Karolin, S. Aleshkov, L. Strandberg, L. B. Johansson, and T. Ny: Time-resolved polarized fluorescence spectroscopy studies of plasminogen activator inhibitor type 1: conformational changes of the reactive center upon interactions with target proteases, vitronectin and heparin. *Biochemistry*, 34, 13833-40 (1995)

133. A. Gibson, K. Baburaj, D. E. Day, I. Verhamme, J. D. Shore, and C. B. Peterson: The use of fluorescent probes to characterize conformational changes in the interaction between vitronectin and plasminogen activator inhibitor-1. *J Biol Chem*, 272, 5112-21 (1997)

134. T. Kirkegaard, S. Jensen, S. L. Schousboe, H. H. Petersen, R. Egelund, P. A. Andreasen, and K. W. Rodenburg: Engineering of conformations of plasminogen activator inhibitor-1. A crucial role of beta-strand 5A residues in the transition of active form to latent and substrate forms. *Eur J Biochem*, 263, 577-86 (1999)

135. H. Im, E. J. Seo, and M. H. Yu: Metastability in the inhibitory mechanism of human alpha1-antitrypsin. *J Biol Chem*, 274, 11072-7 (1999)

136. H. Im and M. H. Yu: Role of Lys335 in the metastability and function of inhibitory serpins. *Protein Sci*, 9, 934-41 (2000)

137. M. Hansen, M. N. Busse, and P. A. Andreasen: Importance of the amino-acid composition of the shutter region of plasminogen activator inhibitor-1 for its transitions to latent and substrate forms. *Eur J Biochem*, 268, 6274-83 (2001)

138. S. J. Harrop, L. Jankova, M. Coles, D. Jardine, J. S. Whittaker, A. R. Gould, A. Meister, G. C. King, B. C. Mabbutt, and P. M. Curmi: The crystal structure of plasminogen activator inhibitor 2 at 2.0 A resolution: implications for serpin function. *Structure*, 7, 43-54 (1999)

139. E. Matsunaga, S. Shiokawa, H. Nakamura, T. Maruyama, K. Tsuda, and Y. Fukumaki: Molecular analysis of the gene of the alpha 1-antitrypsin deficiency variant, Mnichinan. *Am J Hum Genet*, 46, 602-12 (1990)

140. K. Seyama, T. Nukiwa, K. Takabe, H. Takahashi, K. Miyake, and S. Kira: Siiyama (serine 53 (TCC) to phenylalanine 53 (TTC)). A new alpha 1-antitrypsin-deficient variant with mutation on a predicted conserved residue of the serpin backbone. *J Biol Chem*, 266, 12627-32 (1991)

141. W. Poller, J. P. Faber, S. Scholz, S. Weidinger, K. Bartholome, K. Olek, and S. Eriksson: Mis-sense mutation of alpha 1-antichymotrypsin gene associated with chronic lung disease. *Lancet*, 339, 1538 (1992)

142. D. A. Lomas, J. T. Finch, K. Seyama, T. Nukiwa, and R. W. Carrell: Alpha 1-antitrypsin Siiyama (Ser53-->Phe). Further evidence for intracellular loop-sheet polymerization. *J Biol Chem*, 268, 15333-5 (1993)

143. C. Sergi, G. G. Consalez, G. Fabbretti, M. Brisigotti, G. Faa, V. Costa, G. Romeo, and F. Callea: Immunohistochemical and genetic characterization of the

M Cagliari alpha-1-antitrypsin molecule (M-like alpha-1-antitrypsin deficiency). *Lab Invest*, 70, 130-3 (1994)

144. D. A. Lomas, P. R. Elliott, S. K. Sidhar, R. C. Foreman, J. T. Finch, D. W. Cox, J. C. Whisstock, and R. W. Carrell: alpha 1-Antitrypsin Mmalton (Phe52-deleted) forms loop-sheet polymers *in vivo*. Evidence for the C sheet mechanism of polymerization. *J Biol Chem*, 270, 16864-70 (1995)

145. R. L. Davis, A. E. Shrimpton, P. D. Holohan, C. Bradshaw, D. Feiglin, G. H. Collins, P. Sonderegger, J. Kinter, L. M. Becker, F. Lacbawan, D. Krasnewich, M. Muenke, D. A. Lawrence, M. S. Yerby, C. M. Shaw, B. Gooptu, P. R. Elliott, J. T. Finch, R. W. Carrell, and D. A. Lomas: Familial dementia caused by polymerization of mutant neuroserpin. *Nature*, 401, 376-9 (1999)

146. T. Wind, J. K. Jensen, D. M. Dupont, P. Kulig, and P. A. Andreasen: Mutational analysis of plasminogen activator inhibitor-1. *Eur J Biochem*, 270, 1680-8 (2003)

147. A. P. Bijnens, A. Gils, I. Knockaert, J. M. Stassen, and P. J. Declerck: Importance of the hinge region between alpha-helix F and the main part of serpins, based upon identification of the epitope of plasminogen activator inhibitor type 1 neutralizing antibodies. *J Biol Chem*, 275, 6375-80 (2000)

148. T. Wind, M. A. Jensen, and P. A. Andreasen: Epitope mapping for four monoclonal antibodies against human plasminogen activator inhibitor type-1: implications for antibody-mediated PAI-1-neutralization and vitronectin-binding. *Eur J Biochem*, 268, 1095-106 (2001)

149. S. L. Schousboe, R. Egelund, T. Kirkegaard, K. T. Preissner, K. W. Rodenburg, and P. A. Andreasen: Vitronectin and substitution of a beta-strand 5A lysine residue potentiate activity-neutralization of PA inhibitor-1 by monoclonal antibodies against alpha-helix F. *Thromb Haemost*, 83, 742-51 (2000)

150. D. A. Lomas and R. W. Carrell: Serpinopathies and the conformational dementias. *Nat Rev Genet*, 3, 759-68 (2002)

151. S. J. Gardell, J. A. Krueger, T. A. Antrilli, H. Elokdah, S. Mayer, S. J. Orcutt, D. L. Crandall, and G. P. Vlasuk: Neutralization of plasminogen activator inhibitor I (PAI-1) by the synthetic antagonist PAI-749 via a dual mechanism of action. *Mol Pharmacol*, 72, 897-906 (2007)

152. K. E. Pedersen, A. P. Einholm, A. Christensen, L. Schack, T. Wind, J. M. Kenney, and P. A. Andreasen: Plasminogen activator inhibitor-1 polymers, induced by inactivating amphipathic organochemical ligands. *Biochem J*, 372, 747-55 (2003)

153. A. Zhou, R. Faint, P. Charlton, T. R. Dafforn, R. W. Carrell, and D. A. Lomas: Polymerization of plasminogen activator inhibitor-1. *J Biol Chem*, 276, 9115-22 (2001)

154. A. Nykjaer and T. E. Willnow: The low-density lipoprotein receptor gene family: a cellular Swiss army knife? *Trends Cell Biol*, 12, 273-80 (2002)

155. J. Gliemann: Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol Chem*, 379, 951-64 (1998)

156. A. P. Lillis, I. Mikhailenko, and D. K. Strickland: Beyond endocytosis: LRP function in cell migration, proliferation and vascular permeability. *J Thromb Haemost*, 3, 1884-93 (2005)

157. D. K. Strickland and L. Medved: Low-density lipoprotein receptor-related protein (LRP)-mediated clearance of activated blood coagulation co-factors and proteases: clearance mechanism or regulation? *J Thromb Haemost*, 4, 1484-6 (2006)

158. T. Kristensen, S. K. Moestrup, J. Gliemann, L. Bendtsen, O. Sand, and L. Sottrup-Jensen: Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Lett*, 276, 151-5 (1990)

159. D. K. Strickland, J. D. Ashcom, S. Williams, W. H. Burgess, M. Migliorini, and W. S. Argraves: Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J Biol Chem*, 265, 17401-4 (1990)

160. J. Herz, U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K. K. Stanley: Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *Embo J*, 7, 4119-27 (1988)

161. M. V. Cubellis, T. C. Wun, and F. Blasi: Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. *Embo J*, 9, 1079-85 (1990)

162. A. Estreicher, J. Muhlhauser, J. L. Carpentier, L. Orci, and J. D. Vassalli: The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J Cell Biol*, 111, 783-92 (1990)

163. P. H. Jensen, E. I. Christensen, P. Ebbesen, J. Gliemann, and P. A. Andreasen: Lysosomal degradation of receptor-bound urokinase-type plasminogen activator is enhanced by its inhibitors in human trophoblastic choriocarcinoma cells. *Cell Regul*, 1, 1043-56 (1990)

164. K. Orth, E. L. Madison, M. J. Gething, J. F. Sambrook, and J. Herz: Complexes of tissue-type plasminogen activator and its serpin inhibitor plasminogen-activator inhibitor type 1 are internalized by means of the low density lipoprotein receptor-related protein/alpha 2-

macroglobulin receptor. *Proc Natl Acad Sci U S A*, 89, 7422-6 (1992)

165. A. Nykjaer, C. M. Petersen, B. Moller, P. H. Jensen, S. K. Moestrup, T. L. Holtet, M. Etzerodt, H. C. Thogersen, M. Munch, P. A. Andreasen, and *et al.*: Purified alpha 2-macroglobulin receptor/LDL receptor-related protein binds urokinase.plasminogen activator inhibitor type-1 complex. Evidence that the alpha 2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes. *J Biol Chem*, 267, 14543-6 (1992)

166. G. Bu, S. Williams, D. K. Strickland, and A. L. Schwartz: Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proc Natl Acad Sci U S A*, 89, 7427-31 (1992)

167. K. Orth, T. Willnow, J. Herz, M. J. Gething, and J. Sambrook: Low density lipoprotein receptor-related protein is necessary for the internalization of both tissue-type plasminogen activator-inhibitor complexes and free tissue-type plasminogen activator. *J Biol Chem*, 269, 21117-22 (1994)

168. A. Nykjaer, L. Kjoller, R. L. Cohen, D. A. Lawrence, B. A. Garni-Wagner, R. F. Todd, 3rd, A. J. van Zonneveld, J. Gliemann, and P. A. Andreasen: Regions involved in binding of urokinase-type-1 inhibitor complex and pro-urokinase to the endocytic alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Evidence that the urokinase receptor protects pro-urokinase against binding to the endocytic receptor. *J Biol Chem*, 269, 25668-76 (1994)

169. S. K. Moestrup, S. Nielsen, P. Andreasen, K. E. Jorgensen, A. Nykjaer, H. Roigaard, J. Gliemann, and E. I. Christensen: Epithelial glycoprotein-330 mediates endocytosis of plasminogen activator-plasminogen activator inhibitor type-1 complexes. *J Biol Chem*, 268, 16564-70 (1993)

170. T. E. Willnow, J. L. Goldstein, K. Orth, M. S. Brown, and J. Herz: Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J Biol Chem*, 267, 26172-80 (1992)

171. K. M. Argraves, F. D. Battey, C. D. MacCalman, K. R. McCrae, M. Gafvels, K. F. Kozarsky, D. A. Chappell, J. F. Strauss, 3rd, and D. K. Strickland: The very low density lipoprotein receptor mediates the cellular catabolism of lipoprotein lipase and urokinase-plasminogen activator inhibitor type I complexes. *J Biol Chem*, 270, 26550-7 (1995)

172. C. W. Heegaard, A. C. Simonsen, K. Oka, L. Kjoller, A. Christensen, B. Madsen, L. Ellgaard, L. Chan, and P. A. Andreasen: Very low density lipoprotein receptor binds and mediates endocytosis of urokinase-type plasminogen activator-type-1 plasminogen activator inhibitor complex. *J Biol Chem*, 270, 20855-61 (1995)

173. C. X. Liu, Y. Li, L. M. Obermoeller-McCormick, A. L. Schwartz, and G. Bu: The putative tumor suppressor LRP1B, a novel member of the low density lipoprotein (LDL) receptor family, exhibits both overlapping and distinct properties with the LDL receptor-related protein. *J Biol Chem*, 276, 28889-96 (2001)

174. Y. Li, J. M. Knisely, W. Lu, L. M. McCormick, J. Wang, J. Henkin, A. L. Schwartz, and G. Bu: Low density lipoprotein (LDL) receptor-related protein 1B impairs urokinase receptor regeneration on the cell surface and inhibits cell migration. *J Biol Chem*, 277, 42366-71 (2002)

175. J. Gliemann, G. Hermey, A. Nykjaer, C. M. Petersen, C. Jacobsen, and P. A. Andreasen: The mosaic receptor sorLA/LR11 binds components of the plasminogen-activating system and platelet-derived growth factor-BB similarly to LRP1 (low-density lipoprotein receptor-related protein), but mediates slow internalization of bound ligand. *Biochem J*, 381, 203-12 (2004)

176. I. Warshawsky, G. Bu, and A. L. Schwartz: Identification of domains on the 39-kDa protein that inhibit the binding of ligands to the low density lipoprotein receptor-related protein. *J Biol Chem*, 268, 22046-54 (1993)

177. J. C. Zhang, R. Sakthivel, D. Kniss, C. H. Graham, D. K. Strickland, and K. R. McCrae: The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor regulates cell surface plasminogen activator activity on human trophoblast cells. *J Biol Chem*, 273, 32273-80 (1998)

178. M. Conese and F. Blasi: Urokinase/urokinase receptor system: internalization/degradation of urokinase-serpin complexes: mechanism and regulation. *Biol Chem Hoppe Seyler*, 376, 143-55 (1995)

179. A. Nykjaer, M. Conese, E. I. Christensen, D. Olson, O. Cremona, J. Gliemann, and F. Blasi: Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. *Embo J*, 16, 2610-20 (1997)

180. G. Bu and A. L. Schwartz: RAP, a novel type of ER chaperone. *Trends Cell Biol*, 8, 272-6 (1998)

181. G. Bu: The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. *Int Rev Cytol*, 209, 79-116 (2001)

182. J. Herz: The switch on the RAPper's necklace. *Mol Cell*, 23, 451-5 (2006)

183. L. Ellgaard, T. L. Holtet, P. R. Nielsen, M. Etzerodt, J. Gliemann, and H. C. Thogersen: Dissection of the domain architecture of the alpha2macroglobulin-receptor-associated protein. *Eur J Biochem*, 244, 544-51 (1997)

184. P. R. Nielsen, L. Ellgaard, M. Etzerodt, H. C. Thogersen, and F. M. Poulsen: The solution structure of the N-terminal domain of alpha2-macroglobulin receptor-associated protein. *Proc Natl Acad Sci U S A*, 94, 7521-5 (1997)

185. A. Lazic, K. Dolmer, D. K. Strickland, and P. G. Gettins: Structural organization of the receptor associated protein. *Biochemistry*, 42, 14913-20 (2003)

186. D. Lee, J. D. Walsh, I. Mikhailenko, P. Yu, M. Migliorini, Y. Wu, S. Krueger, J. E. Curtis, B. Harris, S. Lockett, S. C. Blacklow, D. K. Strickland, and Y. X. Wang: RAP uses a histidine switch to regulate its interaction with LRP in the ER and Golgi. *Mol Cell*, 22, 423-30 (2006)

187. S. L. Gonias, L. Wu, and A. M. Salicioni: Low density lipoprotein receptor-related protein: regulation of the plasma membrane proteome. *Thromb Haemost*, 91, 1056-64 (2004)

188. D. J. Webb, K. S. Thomas, and S. L. Gonias: Plasminogen activator inhibitor 1 functions as a urokinase response modifier at the level of cell signaling and thereby promotes MCF-7 cell growth. *J Cell Biol*, 152, 741-52 (2001)

189. Y. Li, W. Lu, M. P. Marzolo, and G. Bu: Differential functions of members of the low density lipoprotein receptor family suggested by their distinct endocytosis rates. *J Biol Chem*, 276, 18000-6 (2001)

190. A. Kasza, H. H. Petersen, C. W. Heegaard, K. Oka, A. Christensen, A. Dubin, L. Chan, and P. A. Andreasen: Specificity of serine proteinase/serpin complex binding to very-low-density lipoprotein receptor and alpha2-macroglobulin receptor/low-density-lipoprotein-receptor-related protein. *Eur J Biochem*, 248, 270-81 (1997)

191. P. M. Rettenberger, K. Oka, L. Ellgaard, H. H. Petersen, A. Christensen, P. M. Martensen, D. Monard, M. Etzerodt, L. Chan, and P. A. Andreasen: Ligand binding properties of the very low density lipoprotein receptor. Absence of the third complement-type repeat encoded by exon 4 is associated with reduced binding of Mr 40,000 receptor-associated protein. *J Biol Chem*, 274, 8973-80 (1999)

192. N. Verdaguer, I. Fita, M. Reithmayer, R. Moser, and D. Blaas: X-ray structure of a minor group human rhinovirus bound to a fragment of its cellular receptor protein. *Nat Struct Mol Biol*, 11, 429-34 (2004)

193. C. Fisher, N. Beglova, and S. C. Blacklow: Structure of an LDLR-RAP complex reveals a general mode for ligand recognition by lipoprotein receptors. *Mol Cell*, 22, 277-83 (2006)

194. G. Rudenko, L. Henry, K. Henderson, K. Ichtchenko, M. S. Brown, J. L. Goldstein, and J. Deisenhofer: Structure of the LDL receptor extracellular domain at endosomal pH. *Science*, 298, 2353-8 (2002)

195. G. A. Jensen, O. M. Andersen, A. M. Bonvin, I. Bjerrum-Bohr, M. Etzerodt, H. C. Thogersen, C. O'Shea, F. M. Poulsen, and B. B. Kragelund: Binding site structure of one LRP-RAP complex: implications for a common ligand-receptor binding motif. *J Mol Biol*, 362, 700-16 (2006)

196. N. L. Daly, J. T. Djordjevic, P. A. Kroon, and R. Smith: Three-dimensional structure of the second cysteine-rich repeat from the human low-density lipoprotein receptor. *Biochemistry*, 34, 14474-81 (1995)

197. N. L. Daly, M. J. Scanlon, J. T. Djordjevic, P. A. Kroon, and R. Smith: Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. *Proc Natl Acad Sci U S A*, 92, 6334-8 (1995)

198. D. Fass, S. Blacklow, P. S. Kim, and J. M. Berger: Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature*, 388, 691-3 (1997)

199. W. Huang, K. Dolmer, and P. G. Gettins: NMR solution structure of complement-like repeat CR8 from the low density lipoprotein receptor-related protein. *J Biol Chem*, 274, 14130-6 (1999)

200. K. Dolmer, W. Huang, and P. G. Gettins: NMR solution structure of complement-like repeat CR3 from the low density lipoprotein receptor-related protein. Evidence for specific binding to the receptor binding domain of human alpha (2)-macroglobulin. *J Biol Chem*, 275, 3264-9 (2000)

201. C. L. North and S. C. Blacklow: Solution structure of the sixth LDL-A module of the LDL receptor. *Biochemistry*, 39, 2564-71 (2000)

202. M. Simonovic, K. Dolmer, W. Huang, D. K. Strickland, K. Volz, and P. G. Gettins: Calcium coordination and pH dependence of the calcium affinity of ligand-binding repeat CR7 from the LRP. Comparison with related domains from the LRP and the LDL receptor. *Biochemistry*, 40, 15127-34 (2001)

203. Q. Y. Wang, W. Huang, K. Dolmer, P. G. Gettins, and L. Rong: Solution structure of the viral receptor domain of Tva and its implications in viral entry. *J Virol*, 76, 2848-56 (2002)

204. P. A. Andreasen, L. Sottrup-Jensen, L. Kjoller, A. Nykjaer, S. K. Moestrup, C. M. Petersen, and J. Gliemann: Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. *FEBS Lett*, 338, 239-45 (1994)

205. K. W. Rodenburg, L. Kjoller, H. H. Petersen, and P. A. Andreasen: Binding of urokinase-type plasminogen activator-plasminogen activator inhibitor-1 complex to the endocytosis receptors alpha2-macroglobulin receptor/low-

density lipoprotein receptor-related protein and very-low-density lipoprotein receptor involves basic residues in the inhibitor. *Biochem J*, 329 (Pt 1), 55-63 (1998)

206. I. R. Horn, B. M. van den Berg, S. K. Moestrup, H. Pannekoek, and A. J. van Zonneveld: Plasminogen activator inhibitor 1 contains a cryptic high affinity receptor binding site that is exposed upon complex formation with tissue-type plasminogen activator. *Thromb Haemost*, 80, 822-8 (1998)

207. S. Stefansson, S. Muhammad, X. F. Cheng, F. D. Battey, D. K. Strickland, and D. A. Lawrence: Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J Biol Chem*, 273, 6358-66 (1998)

208. S. Skeldal, J. V. Larsen, K. E. Pedersen, H. H. Petersen, R. Egelund, A. Christensen, J. K. Jensen, J. Gliemann, and P. A. Andreasen: Binding areas of urokinase-type plasminogen activator-plasminogen activator inhibitor-1 complex for endocytosis receptors of the low-density lipoprotein receptor family, determined by site-directed mutagenesis. *Febs J*, 273, 5143-59 (2006)

209. S. K. Moestrup, T. L. Holtet, M. Etzerodt, H. C. Thogersen, A. Nykjaer, P. A. Andreasen, H. H. Rasmussen, L. Sottrup-Jensen, and J. Gliemann: Alpha 2-macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen activator complexes, and receptor-associated protein bind to a region of the alpha 2-macroglobulin receptor containing a cluster of eight complement-type repeats. *J Biol Chem*, 268, 13691-6 (1993)

210. T. E. Willnow, K. Orth, and J. Herz: Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J Biol Chem*, 269, 15827-32 (1994)

211. J. G. Neels, B. M. van Den Berg, A. Lookene, G. Olivecrona, H. Pannekoek, and A. J. van Zonneveld: The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J Biol Chem*, 274, 31305-11 (1999)

212. L. M. Obermoeller-McCormick, Y. Li, H. Osaka, D. J. FitzGerald, A. L. Schwartz, and G. Bu: Dissection of receptor folding and ligand-binding property with functional minireceptors of LDL receptor-related protein. *J Cell Sci*, 114, 899-908 (2001)

213. R. A. Orlando, M. Exner, R. P. Czekay, H. Yamazaki, A. Saito, R. Ullrich, D. Kerjaschki, and M. G. Farquhar: Identification of the second cluster of ligand-binding repeats in megalin as a site for receptor-ligand interactions. *Proc Natl Acad Sci U S A*, 94, 2368-73 (1997)

214. V. Esser, L. E. Limbird, M. S. Brown, J. L. Goldstein, and D. W. Russell: Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J Biol Chem*, 263, 13282-90 (1988)

215. D. W. Russell, M. S. Brown, and J. L. Goldstein: Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J Biol Chem*, 264, 21682-8 (1989)

216. I. Mikhailenko, W. Considine, K. M. Argraves, D. Loukinov, B. T. Hyman, and D. K. Strickland: Functional domains of the very low density lipoprotein receptor: molecular analysis of ligand binding and acid-dependent ligand dissociation mechanisms. *J Cell Sci*, 112 (Pt 19), 3269-81 (1999)

217. I. R. Horn, B. M. van den Berg, P. Z. van der Meijden, H. Pannekoek, and A. J. van Zonneveld: Molecular analysis of ligand binding to the second cluster of complement-type repeats of the low density lipoprotein receptor-related protein. Evidence for an allosteric component in receptor-associated protein-mediated inhibition of ligand binding. *J Biol Chem*, 272, 13608-13 (1997)

218. B. Vash, N. Phung, S. Zein, and D. DeCamp: Three complement-type repeats of the low-density lipoprotein receptor-related protein define a common binding site for RAP, PAI-1, and lactoferrin. *Blood*, 92, 3277-85 (1998)

219. O. M. Andersen, H. H. Petersen, C. Jacobsen, S. K. Moestrup, M. Etzerodt, P. A. Andreasen, and H. C. Thogersen: Analysis of a two-domain binding site for the urokinase-type plasminogen activator-plasminogen activator inhibitor-1 complex in low-density-lipoprotein-receptor-related protein. *Biochem J*, 357, 289-96 (2001)

220. P. A. Andreasen, L. Kjoller, L. Christensen, and M. J. Duffy: The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer*, 72, 1-22 (1997)

221. P. A. Andreasen, R. Egelund, and H. H. Petersen: The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci*, 57, 25-40 (2000)

222. D. E. Vaughan: PAI-1 and atherothrombosis. *J Thromb Haemost*, 3, 1879-83 (2005)

223. W. Bode and R. Huber: Natural protein proteinase inhibitors and their interaction with proteinases. *Eur J Biochem*, 204, 433-51 (1992)

224. S. Thorsen, M. Philips, J. Selmer, I. Lecander, and B. Astedt: Kinetics of inhibition of tissue-type and urokinase-type plasminogen activator by plasminogen-activator inhibitor type 1 and type 2. *Eur J Biochem*, 175, 33-9 (1988)

225. P. Mikus, T. Urano, P. Liljestrom, and T. Ny: Plasminogen-activator inhibitor type 2 (PAI-2) is a spontaneously polymerising SERPIN. Biochemical characterisation of the recombinant intracellular and extracellular forms. *Eur J Biochem*, 218, 1071-82 (1993)

226. A. Zhou, X. Jiang, F. Dou, D. Zhu, and X. Xu: Renaturation, purification, and characterization of human plasminogen activator inhibitor type 2 (PAI-2) accumulated

at high level in Escherichia coli. *J Biochem (Tokyo)*, 121, 930-4 (1997)

227. F. Espana, M. Berrettini, and J. H. Griffin: Purification and characterization of plasma protein C inhibitor. *Thromb Res*, 55, 369-84 (1989)

228. D. L. Eaton, R. W. Scott, and J. B. Baker: Purification of human fibroblast urokinase proenzyme and analysis of its regulation by proteases and protease nexin. *J Biol Chem*, 259, 6241-7 (1984)

229. R. W. Scott, B. L. Bergman, A. Bajpai, R. T. Hersh, H. Rodriguez, B. N. Jones, C. Barreda, S. Watts, and J. B. Baker: Protease nexin. Properties and a modified purification procedure. *J Biol Chem*, 260, 7029-34 (1985)

230. G. A. Hastings, T. A. Coleman, C. C. Haudenschild, S. Stefansson, E. P. Smith, R. Barthlow, S. Cherry, M. Sandkvist, and D. A. Lawrence: Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. *J Biol Chem*, 272, 33062-7 (1997)

231. G. Spraggon, C. Phillips, U. K. Nowak, C. P. Ponting, D. Saunders, C. M. Dobson, D. I. Stuart, and E. Y. Jones: The crystal structure of the catalytic domain of human urokinase-type plasminogen activator. *Structure*, 3, 681-91 (1995)

Abbreviations: $alpha_1$-PI: $alpha_1$-proteinase inhibitor; CTR, complement-type repeat; ECM, extracellular matrix; h: alpha-helix; LDLR: low density lipoprotein receptor; LRP: low density lipoprotein receptor-related protein; PAI-1: plasminogen activator inhibitor-1; PAI-2: plasminogen activator inhibitor-2; PAI-3: plasminogen activator inhibitor-3; PCI: protein C inhibitor; PN-1: protease nexin-1; PPE: porcine pancreatic elastase; RAP: receptor-associated protein; RCL: reactive centre loop; s: strand in a beta-sheet; SMB: somatomedin B domain; sorLA: sorting protein-related receptor; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; uPAR: uPA receptor; VLDLR: very low density lipoprotein receptor

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Send correspondence to: Peter A. Andreasen, Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10C, 8000 Aarhus C, Tel: 45 89425080, Fax: 4586123178, E-mail: pa@mb.au.dk

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