Function-Stabilizing Mechanism of Plasminogen Activator Inhibitor Type 1 Induced upon Binding to α_1-Acid Glycoprotein†

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ABSTRACT: Recently, we found that α_1-acid glycoprotein (AGP), one of the major acute-phase proteins, forms a function-stabilizing complex with plasminogen activator inhibitor 1 (PAI-1). In this study we describe the mechanism by which AGP, as well as its recombinant fragment $AGP_{118-201}$, interacts exclusively with the active form of PAI-1 and stabilizes its conformation. The binding domain of PAI-1 for AGP was initially mapped by antibodies reacting with the well-defined PAI-1 epitopes and then verified in binding assays utilizing a library of PAI-1 mutants. The latter consisted of PAI-1 molecules with individual, tandem, or grouped mutations in the epitope region of MA-55F4C12, MA-33B8, MA-33H1F7, MA-44E4, and MA-8H9D4. Solid-phase binding experiments showed that only MA-8H9D4 did not bind to the PAI-1/AGP complex, indicating that its epitope is hidden upon binding of PAI-1 to AGP. Consistently, only PAI-1 mutants with substitutions in the region of R300−D305, constituting the MA-8H9D4 epitope, showed a lack of binding or severe deficit in both the capacity and affinity of binding to AGP. These results support a location of the binding site close to the epitope within the segment connecting the regions hI with S5A. In conclusion, our present data suggest that AGP binding stabilizes the active conformation of PAI-1 by restricting the movement of β-sheet A and thereby preventing insertion of the reactive center loop.

Plasminogen activator inhibitor type 1 (PAI-1)[1] is the main inhibitor of plasminogen activators (*1*, *2*). It exists in multiple conformational states, including active, latent, and substrate forms, and both latent and substrate PAI-1 are thought to be biologically inactive. PAI-1 also interacts with a number of nonproteinase ligands, including the cell adhesion protein vitronectin (*3*−*5*). PAI-1 binds to vitronectin with a high affinity; however, when in complex with a proteinase, its affinity for vitronectin is markedly decreased (*4*). Recently, by employing the yeast-based two-hybrid system for studying protein−protein interaction, we identified α_1-acid glycoprotein (AGP) that, in addition to vitronectin, can bind to PAI-1 and preserve its inhibitory activity (*6*). AGP, a highly glycosylated polypeptide chain containing 201 amino acid residues, is expressed in human liver (*7*) and in other cells, including human breast epithelial cells (*8*), endothelial cells (*9*), and cultured human granulocytes and monocytes (*10*). Although AGP bound to PAI-1 with lower affinity than vitronectin, this interaction was strong enough to stabilize the active conformation of PAI-1 and prolong its inhibitory activity toward uPA and t-PA (*6*).

AGP is a major acute-phase protein known to increase in concentration during, for instance, inflammatory conditions and cancer (*11*). Although the biological function of AGP remains unknown, it is considered to be a natural antiinflammatory and immunomodulatory agent (*12*, *13*). It binds to basic and neutral drugs as well as steroid hormones. It is also known to be essential for capillary permselectivity in several different capillary beds (*14*, *15*). The AGP plasma concentration increase, as a response to inflammation, is triggered by cytokines (e.g., interleukin 6), and as a consequence, its levels vary in many physiological states (i.e., age and pregnancy) and pathological conditions (i.e., liver cirrhosis, renal disease, and cancer) (*16*). Acute-phase proteins and AGP in particular have long been associated with poor prognostics in many conditions, including active lung and gastrointestinal cancers.

Since the structural requirements for the interaction between PAI-1 and AGP are unknown, in the present study we have attempted to identify the binding site for AGP within the PAI-1 molecule. For this purpose, we performed extensive binding studies using monoclonal antibodies reacting with well-defined PAI-1 epitopes and more than 90 PAI-1 mutants. These studies allowed us to identify a discrete domain on the surface of PAI-1 that interacts with AGP and its fragment $AGP_{118-201}$ and to propose a molecular mechanism by which the active conformation of PAI-1 is maintained in the complex.

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[1] Abbreviations: PAI-1, plasminogen activator inhibitor type 1; AGP, α_1-acid glycoprotein.

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EXPERIMENTAL PROCEDURES

Proteins and Reagents. α_1-Acid glycoprotein (AGP) was purchased from Calbiochem-Novabiochem Co. (San Diego, CA). Antibodies to AGP, mouse monoclonal antibody (Sigma-Aldrich Chemical Co., St. Louis, MO), and rabbit polyclonal antibodies were purchased from Abcam Ltd. (Cambridge, U.K.). Wizard Miniprep for plasmid DNA isolation, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, restriction enzymes, and PCR reagents were purchased from Promega Corp. (Madison, WI). High-binding 96-well microtiter plates were obtained from Costar Science Corp. (Cambridge, MA), *o*-phenylenediamine, bovine serum albumin (fraction V), HRP-conjugated goat anti-rabbit or rabbit anti-mouse IgG, and phenylmethanesulfonyl fluoride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

PAI-1 and Its Mutants. Expression and purification of wild-type PAI-1 and PAI-1 variants were performed as described previously, with minor modifications (*17*). Mutagenesis was carried out by standard procedures, basically as described before (*18*). The presence of the mutation together with the absence of any unwanted mutation was verified by nucleotide sequencing. Purified proteins were subjected to SDS−PAGE for evaluation of their purity, pooled, and stored in aliquots at −20 °C until use. The amount of PAI-1 protein in purified preparations was determined spectrophotometrically at 280 nm (absorbance coefficient $A^{1\%} = 10$). After evaluation of PAI-1 content and purity, PAI-1-containing elution fractions were examined for their inhibitory activity toward t-PA by SDS−PAGE (*17*). Fractions containing the highest proportion of functionally active PAI-1 were pooled and used for further experiments. The substrate PAI-1 is derived from wild-type PAI-1 and isolated as described previously (*19*).

Monoclonal Antibodies. MA-55F4C12, MA-33B8, MA-33H1F7, MA-35A5, MA-44E4, *and* MA-8H9D4 were raised against the tPA/PAI-1 complex as described (*20*), and their epitopes and function were characterized previously. The IgG fraction was purified from ascitic fluid by affinity chromatography on protein A−Sepharose (*21*). The antibody protein concentration was determined at 280 nm (absorbance coefficient $A^{1\%} = 13$) and a M_r of 150000.

AGP Fragment. To obtain $AGP_{118-201}$, a 249 bp cDNA fragment corresponding to 369−618 bp was generated from pGAD-10 containing the entire cDNA for AGP, using the following primers: 5′ GAG CAT TTC GGA TCC TTG CTG ATC CTC 3′ and 5′ CAA GGC TGC TCG AGC TAG GAT TCC CC 3′. The PCR products were digested with *Bam*HI and *Xho*I restriction enzymes and inserted into pRSET plasmid (Invitrogen) for expression in *Escherichia coli* (DH5α; GIBCO-BRL) as a His-tag fusion protein containing 12 residues (MRGSHHHHHHGS) at their N-termini. To purify the recombinant $AGP_{118-201}$ fragment, inclusion bodies were prepared, dissolved in 6 M urea, and separated by chromatography on chelating Sepharose (Pharmacia), loaded with Ni ions according to the manufacturer's instructions. Recombinant $AGP_{118-201}$ fragment was eluted with 0.02 M Tris-HCl, pH 7.9, containing 1.0 M imidazole, 0.5 M NaCl, and 6 M urea. Refolding of the $AGP_{118-201}$ fragment was performed by dialysis of dilute protein solutions (100−200 μg/mL) against 1000 mL of the elution buffer at 4 °C. Then, the urea concentration was reduced by a continuous slow drip of 1000 mL of 20 mM Tris buffer, pH 8.0, into the dialysis solution. A final dialysis was then performed against 0.01 M Tris-HCl, pH 8.0, containing 0.14 M NaCl and NaN_3 (1 mg/mL). Then, the recombinant fragments were concentrated to ∼2 mg/mL by ultrafiltration. The $AGP_{118-201}$ fragment migrated as a single band on PAGE performed both in the presence of SDS and under native conditions.

Solid-Phase Binding Assays. Binding of PAI-1-wt and mutants to immobilized AGP or the $AGP_{118-201}$ fragment was measured by enzyme-linked immunosorbent assay. The wells of 96-well microtiter plates were coated with AGP or the recombinant fragment at 4 μg/mL in PBS. Nonspecific binding sites were blocked by incubation with 1% BSA in PBS for 2 h at room temperature. Direct binding assays were performed by adding increasing concentrations of PAI-1-wt and mutants in PBS, pH 7.4, containing 0.1% BSA and 0.002% Tween 80. The plates were incubated overnight at 4 °C followed by detection of bound PAI-1 using MA-55F4C12−HRP. The background binding to BSA was subtracted from all samples before data analysis. Similarly, interaction of other anti-PAI-1 monoclonal antibodies (MA-33B8, MA-33H1F7, MA-35A5, MA-44E4, MA-8H9D4) bound to AGP or its fragment was evaluated.

Surface Plasmon Resonance. The kinetic parameters (association and dissociation rate constants, k_{on} and k_{off}, respectively) and the affinity constant (K_A) for binding of PAI-1 to $AGP_{118-201}$ were measured by surface plasmon resonance (SPR) using a BIAcore 3000 analytical system. Briefly, the $AGP_{118-201}$ fragment was covalently attached to carboxymethyl-dextran (CM5) chips (BIAcore) to 2000 resonance units (using a concentration of 5 μg/mL in 10 mM acetate buffer, pH 4.5). Subsequently, PAI-1 variants, diluted in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 to a final concentration of 1500 nM, were injected at a flow rate of 30 μL/min (injection volume is 180 μL). After each cycle the chip was regenerated using 3 × 10 μL of 50 mM NaOH. The analyses of the association and dissociation phases were made with the software of the BIAcore 3000. Results were expressed in resonance units (RU), an arbitrary unit specific for the BIAcore instrument (1000 RU corresponds to approximately 1 ng of bound protein/mm^2).

PAI-1 Activity Assay. Samples of PAI-1 or its mutant Q303-D305AA (6.0 nM) dissolved in PBS, pH 7.4, containing 0.1% BSA and 0.01% Tween 20 were incubated at 37 °C in the absence or presence of α_1-acid glycoprotein (0.6 μM). At different time points, aliquots were withdrawn to measure PAI-1 activity toward uPA using chromogenic assays. Briefly, the activity of PAI-1 was evaluated after incubation with high molecular weight uPA (4.8 nM, 25 IU/mL; American Diagnostica) for 30 min at 25 °C. The chromogenic substrate S-2444 (0.5 mM; Molndal, Sweden) was used for uPA. Residual uPA activity was determined by quantifying the change in absorbance at 410 nm. Rates of substrate hydrolysis were calculated and expressed as a percentage of the maximum rate of uPA substrate hydrolysis in the absence of PAI-1.

RESULTS AND DISCUSSION

Several studies have indicated that active PAI-1 is very labile and within 1−2 h converts into an inactive form, unless

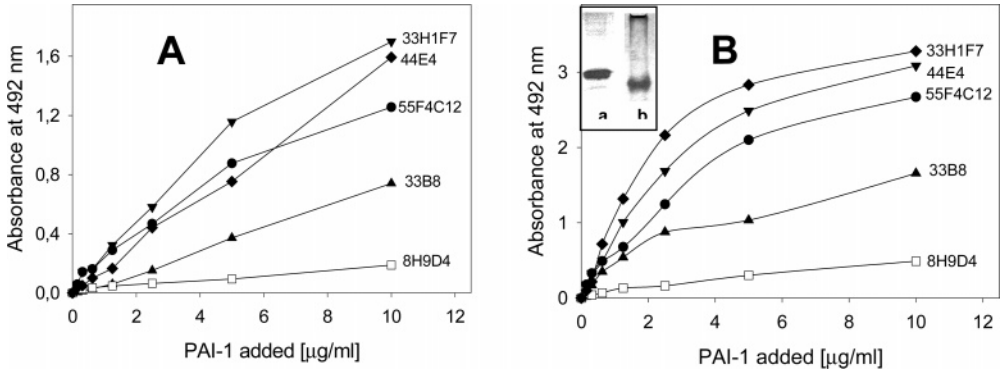

FIGURE 1: Binding of anti-PAI-1 monoclonal antibodies to the PAI-1/AGP complex. Serial 2-fold dilutions of active PAI-1 were applied to the wells of a microplate coated with either AGP (panel A) or its fragment $AGP_{118-201}$ (panel B). Then unbound PAI-1 was washed away, and the PAI-1/AGP complex was titrated with different monoclonal antibodies to PAI-1. Insert (panel B): Gels show the purity of $AGP_{118-201}$ analyzed by SDS−PAGE (lane a) and its homogeneity after refolding as evidenced by electrophoresis under nondenaturing conditions (lane b).

Table 1: Recognition of PAI-1 in Complex with AGP or Its Recombinant Fragment $AGP_{118-201}$ by Anti-PAI-1 Monoclonal Antibodies in a Solid-Phase Binding Assay[a]

		binding to		
MA	epitope	PAI-1[a] (K_A)	PAI-1/AGP	PAI-1/$AGP_{118-201}$
55F4C12	E128-V129-E130-R131,K154	2.7×10^9	+	+
33B8	K88-D89,K176, H229	2.1×10^9	+	+
33H1F7	E130-R131,K154	5.4×10^9	+	+
44E4	H185-R186-R187	4.2×10^8	+	+
8H9D4	R300,Q303,D305	2.6×10^9	−	−

[a] Affinity constants describing the interaction of monoclonal antibodies with PAI-1 were determined in previous work (*18*, *29*).

its active conformation becomes stabilized via interaction with some other proteins, particularly with vitronectin (*22*−*24*). Recently, we found that AGP, similar to vitronectin, can bind PAI-1 with high affinity and serve as a reservoir of the active PAI-1 in circulation (*6*). Our present studies show for the first time that AGP binds exclusively the active form of PAI-1 and provide molecular-based explanations as to why both latent and substrate PAI-1 do not interact with this protein.

Binding of Anti-PAI-1 Monoclonal Antibodies to the PAI-1/AGP Complex. In preliminary experiments to identify the general region of PAI-1 which directly interacts with AGP, we performed binding studies using monoclonal antibodies recognizing well-defined epitopes of the PAI-1 molecule (Table 1).

These antibodies were used previously in studies focused on explaining mechanisms of intermolecular PAI-1 inactivation (*18*, *25*−*29*). Upon binding they are known to neutralize PAI-1 activity via different mechanisms: (a) by conversion of PAI-1 from a suicide inhibitor to a substrate for target proteinases (MA-8H9D4, MA-55F4C12, and MA-33H1F7), (b) by acceleration of the active-to-latent transformation (MA-33B8), and (c) by prevention of Michaelis−Menten complex formation (MA-44E4). Though raised against the PAI-1/tPA complex (*20*), all monoclonal antibodies bound to active PAI-1 with high affinity (Table 1). To search for antibody epitopes that are masked upon binding of PAI-1 to AGP, we performed a solid-phase binding assay. For this purpose, the ELISA plate wells were coated with AGP and incubated with increasing concentrations of the active wild-type PAI-1. The wild-type PAI-1 used in this and subsequent experiments contained 72%, 26%, and less than 2% of active, substrate, and latent conformations, respectively. Binding of monoclonal antibodies to the PAI-1/AGP complex was then evaluated by ELISA. Figure 1A shows that, among antibodies used, MA-55F4C12, MA-44E4, and MA-33H1F7 were the most efficiently bound, indicating that their epitopes are entirely exposed on the PAI/AGP complex. MA-33B8 bound to a lesser extent, while 8H9D4 did not recognize the PAI-1/AGP complex. The difference in binding efficiency of MA-33B8 to the PAI/AGP complex may result from its decreased binding affinity toward active PAI-1 (*28*, *30*, *31*). These data suggest that the epitope of MA-8H9D4 is hidden in the PAI-1 complexed with AGP either by steric hindrance or becomes buried inside the PAI-1 molecule due to the conformational changes induced by the complex formation.

To narrow down a region that binds PAI-1, we cloned several fragments of AGP, including $AGP_{118-201}$. This fragment was identified as interacting with PAI-1 by the yeast two-hybrid system in our previous work (*6*). The recombinant $AGP_{118-201}$ fragment was expressed in *E. coli* as a His-tag fusion protein, purified on nickel chelating columns in 6 M urea, and refolded by sequential dialysis to remove the denaturant. The final product was soluble in aqueous buffers and was homogeneous as assessed by electrophoresis performed in polyacrylamide gels under nondenaturing or denaturing (SDS) conditions (Figure 1B, insert). When this fragment was used in binding experiments, it showed the same ability as intact AGP to form a complex with active PAI-1 which could be recognized by MA-55F4C12, MA-44E4, MA-33H1F7, and MA-33B8, but not by MA-8H9D4, as evidenced by solid-phase binding assays.

AGP and $AGP_{118-201}$ Bind Exclusively the Active Form of PAI-1. In our next experiments we used MA-55F4C12 to evaluate the interactions of different molecular forms of PAI-1 with AGP and its fragment $AGP_{118-201}$. Figure 2A (closed symbols) shows that AGP binds exclusively active PAI-1 in a dose-dependent manner and does not interact with either substrate or latent PAI-1. This indicates that the PAI-1 binding sites are sensitive to conformational changes associated with rapid insertion of the reactive center loop (RCL), cleaved or intact, as strand 4 into β-sheet A. The C-terminal fragment of AGP retains the same binding characteristics as

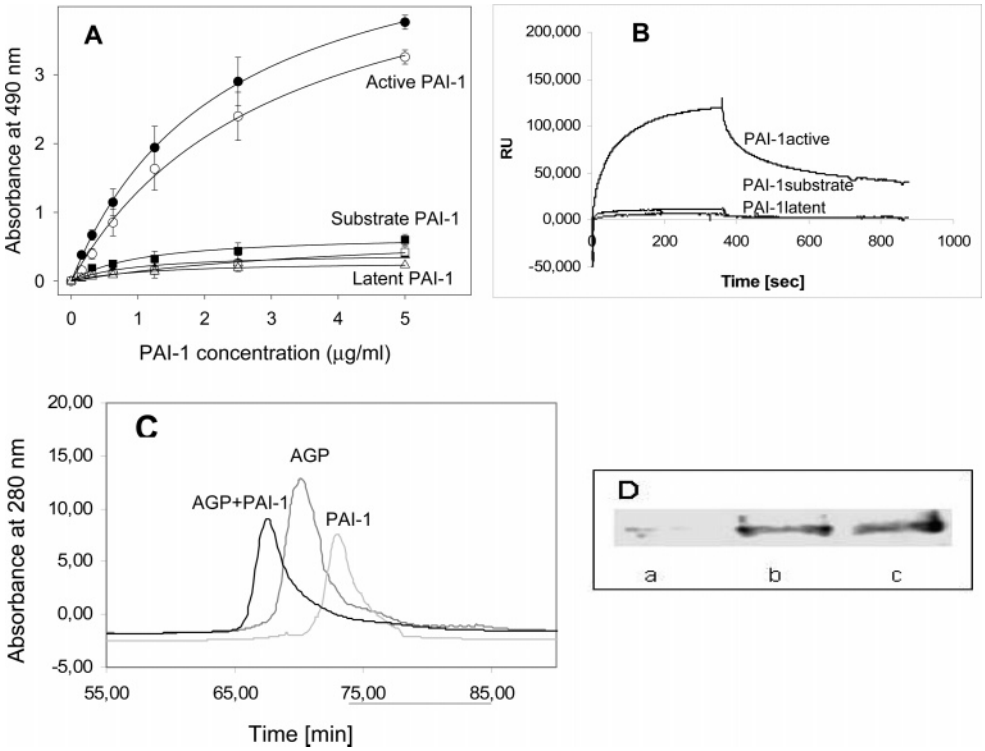

FIGURE 2: Interaction of different PAI-1 forms with AGP. Increasing doses of active, latent, or substrate PAI-1 were incubated in wells coated with either AGP (panel A, closed symbols) or its fragment $AGP_{118-201}$ (panel A, open symbols). The unbound PAI-1 was washed away, and the bound PAI-1 was evaluated using MA-55F4C12 conjugated with horseradish peroxidase by enzyme imunoassay. Panel B shows the interaction of active PAI-1 with $AGP_{118-201}$ analyzed by a surface plasmon resonance. Sensorgrams were recorded during interactions of different forms of PAI-1 (active, latent, substrate) used at 500 nM with $AGP_{118-201}$ immobilized on a sensor chip, CM5. Panel C shows the binding of both components in solution. Equimolar concentrations of AGP and active PAI-1 were incubated for 30 min at room temperature and then applied to a Sephacryl column equilibrated with 0.14 M NaCl buffered with 0.01 M sodium phosphate, pH 7.3, and separated using FPLC. The column was calibrated with AGP and PAI-1 separated under the same conditions. Panel D shows the presence of PAI-1 detected by western immunoblotting in immunoprecipitates obtained after incubation of blood samples of a normal donor (lane a) and diabetic patients (lanes b and c) with antibodies specific to AGP.

Table 2: Rate Constants for Binding of Different Forms of PAI-1 to $AGP_{118-201}$[a]

	PAI-1	k_{on} (M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_A (M)
1	PAI-1 active			
	wild type	$1.6 \pm 0.3 \times 10^4$	$2.0 \pm 0.4 \times 10^{-3}$	$1.1 \pm 0.5 \times 10^7$
	stable mutant[b]	$2.5 \pm 0.2 \times 10^4$	$1.2 \pm 0.3 \times 10^{-3}$	$2.2 \pm 0.4 \times 10^7$
	Q123K	$1.1 \pm 0.5 \times 10^4$	$1.6 \pm 0.6 \times 10^{-3}$	$6.8 \pm 2.1 \times 10^6$
2	PAI-1 latent	NB	NB	NB
3	PAI-1 substrate	NB	NB	NB

[a] $AGP_{118-201}$ was covalently immobilized on a BIAcore CM5 sensor chip. The analyte was injected at 25 °C, and the binding was followed over time by the change in RU. The k_{on} and k_{off} were determined from the association and dissociation phases, respectively. Apparent K_A corresponds to k_{on}/k_{off}. Different molecular forms of wild-type PAI-1 and the constitutively active PAI-1 mutant (N150H, K154T, Q301P, Q319L, M354I) were used. [b] Stable mutant = N150H, K154T, Q301P, Q319L, M354I.

the intact molecule and does not recognize latent or substrate PAI-1 but only its active form (Figure 2A, open symbols). Then, to better characterize the binding of different conformations of PAI-1 to $AGP_{118-201}$, SPR experiments were performed. Figure 2B shows the binding of purified active wild-type PAI-1 to $AGP_{118-201}$ and the lack of binding of both the latent and the substrate form of PAI-1.

Furthermore, binding of both active wild-type PAI-1 and constitutively active PAI-1 mutants (N150H, K154T, Q301P, Q319L, and M354I) to $AGP_{118-201}$ was described by almost identical analytical parameters showing the same binding affinity (Table 2). However, the affinity of PAI-1 binding to $AGP_{118-201}$ appears to be lower than that describing interaction of PAI-1 with intact AGP (*6*). To reassure that the binding of both components occurs also in solution, AGP, PAI-1, and a mixture of AGP and active PAI-1 were separated on a Sephacryl column using FPLC (Figure 2C). Incubation of equimolar concentrations of AGP and PAI-1 resulted in a complex formation which was eluted under neutral pH from the column after 67.5 min, while AGP and PAI-1 alone were eluted after 70 and 73 min, respectively. To identify PAI-1/AGP complexes in human plasma, we next performed co-immunoprecipitation experiments using samples of blood obtained from normal healthy donors and diabetic patients with chronic inflammation known to have increased concentrations of acute-phase proteins. For this purpose, 10 μL of rabbit polyclonal antibodies to AGP was added to 500 μL of plasma aliquots and incubated for 4 h at 4 °C, and immunoprecipitates were isolated using a 50% slurry of protein A/G−agarose (Santa Cruz Biotechnology) overnight at 4 °C. Specifically bound proteins were solubilized in 40 μL of Laemmli sample buffer and separated by SDS−PAGE using a 10% running gel. Then, PAI-1 bound to AGP was identified by western immunoblotting using antibodies specific to PAI-1. Figure 2D shows PAI-1 coprecipitated with AGP when blood plasma samples were taken from patients known to be affected by chronic inflammation.

Binding of PAI-1 Mutants to AGP and Its Fragment. To identify amino acid residues of PAI-1 that contribute to binding of AGP, we used a library of PAI-1 mutants in

Table 3: Rate Constants for Binding of PAI-1 Mutants to $AGP_{118-201}$[a]

MA epitope	PAI-1 mutant	k_{on} (M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_A (M)
MA-55F4C12,	PAI-1-R131A	$(2.3 \pm 0.7) \times 10^4$	$(1.9 \pm 1.3) \times 10^{-3}$	$(3.1 \pm 1.6) \times 10^7$
MA-33H1F7	PAI-1-K154A	$(7.4 \pm 5.2) \times 10^4$	$(6.6 \pm 6.5) \times 10^{-3}$	$(2.0 \pm 1.5) \times 10^7$
E128,V129,E130,R131,K154	PAI-1-154/131AA PAI-1-E128A	$(7.6 \pm 1.2) \times 10^4$	$(2.1 \pm 0.9) \times 10^{-4}$	$(1.2 \pm 0.5) \times 10^8$
	PAI-1-V129A	$(3.0 \pm 2.1) \times 10^4$	$(6.2 \pm 1.8) \times 10^{-4}$	$(4.9 \pm 0.6) \times 10^7$
	PAI-1-E130A	$(2.7 \pm 0.9) \times 10^4$	$(6.6 \pm 2.1) \times 10^{-4}$	$(4.2 \pm 1.5) \times 10^7$
	PAI-1-154/128AA	$(1.1 \pm 0.8) \times 10^4$	$(1.5 \pm 0.06) \times 10^{-3}$	$(7.4 \pm 1.2) \times 10^6$
	PAI-1-154/129AA	$(1.1 \pm 1.1) \times 10^4$	$(1.4 \pm 0.7) \times 10^{-3}$	$(8.0 \pm 0.9) \times 10^6$
MA-33B8	PAI-1-K88A	$(3.8 \pm 1.2) \times 10^4$	$(3.7 \pm 0.9) \times 10^{-3}$	$(6.7 \pm 3.1) \times 10^7$
K88-D89,K176,H229	PAI-1-D89A	$(3.8 \pm 2.3) \times 10^4$	$(1.1 \pm 0.3) \times 10^{-3}$	$(2.7 \pm 1.5) \times 10^7$
	PAI-1-N87-K88-90AAA	$(1.8 \pm 0.5) \times 10^4$	$(2.3 \pm 1.1) \times 10^{-3}$	$(3.7 \pm 2.4) \times 10^7$
	PAI-1-K88-D89AA	$(1.4 \pm 0.6) \times 10^3$	$(2.1 \pm 0.6) \times 10^{-3}$	$(7.9 \pm 4.5) \times 10^6$
	PAI-1-K176A	$(3.0 \pm 0.7) \times 10^4$	$(1.3 \pm 0.1) \times 10^{-3}$	$(2.2 \pm 0.3) \times 10^7$
	PAI-1-H229A	$(4.3 \pm 3.5) \times 10^4$	$(5.2 \pm 2.7) \times 10^{-3}$	$(1.4 \pm 0.3) \times 10^7$
	PAI-1-K88-K176AA	$(3.2 \pm 1.0) \times 10^4$	$(1.7 \pm 0.2) \times 10^{-3}$	$(2.2 \pm 0.3) \times 10^6$
	PAI-1K88-H229AA	$(7.5 \pm 1.0) \times 10^3$	$(1.6 \pm 0.2) \times 10^{-3}$	$(4.7 \pm 1.0) \times 10^6$
MA-8H9D4	PAI-1(R300A)	$(2.1 \pm 0.3) \times 10^4$	$(1.9 \pm 0.1) \times 10\text{-}^4$	$(1.1 \pm 0.2) \times 10^7$
R300,Q303,D305	PAI-1(Q301A)	$(2.3 \pm \mathbf{1.9}) \times \mathbf{10}^3$	$(4.3 \pm \mathbf{4.7}) \times \mathbf{10}^-3$	$(6.9 \pm \mathbf{2.8}) \times \mathbf{10}^5$
	PAI-1(Q303A)	$24.7 \pm \mathbf{15}$	$(1.9 \pm \mathbf{1.9}) \times \mathbf{10}^-3$	$(1.3 \pm \mathbf{1.2}) \times \mathbf{10}^4$
	PAI-1(D305A)	$(1.4 \pm \mathbf{0.5}) \times \mathbf{10}^3$	$(2.5 \pm \mathbf{0.6}) \times \mathbf{10}^-2$	$(5.6 \pm \mathbf{1.9}) \times \mathbf{10}^4$
	PAI-1(R300-301AA)	NB	NB	NB
	PAI-1(Q303-305AA)	NB	NB	NB
MA-44E4	PAI-1-H185A	$(6.2 \pm 0.6) \times 10^4$	$(1.5 \pm 0.4) \times 10^{-3}$	$(4.0 \pm 0.3) \times 10^6$
H185-R186-R187	PAI-1-R186A	$(2.0 \pm 0.4) \times 10^4$	$(1.1 \pm 0.3) \times 10^{-3}$	$(1.8 \pm 0.3) \times 10^7$
	PAI-1-R187A	$(9.7 \pm 0.8) \times 10^4$	$(1.4 \pm 0.4) \times 10^{-3}$	$(6.7 \pm 0.7) \times 10^6$
	PAI-1-H185/R186AA	$(1.1 \pm 0.7) \times 10^4$	$(1.7 \pm 0.1) \times 10^{-3}$	$(6.5 \pm 0.5) \times 10^6$
	PAI-1-R186/R187AA	$(1.7 \pm 1.0) \times 10^4$	$(1.5 \pm 0.2) \times 10^{-3}$	$(1.1 \pm 1.1) \times 10^7$

[a] $AGP_{118-201}$ was covalently immobilized on a BIAcore CM5 sensor chip. The analyte was injected at 25 °C, and the binding was followed over time by the change in RU. The k_{on} and k_{off} were determined from the association and dissociation phases, respectively. Apparent K_A corresponds to the k_{on}/k_{off} ratio and represents the mean ± SD calculated on the basis of three separate measurements ($n = 9-12$).

binding assays. Initially, SPR was used to screen binding of numerous PAI-1 mutants containing individual or grouped amino acid residues substituted by Ala in epitope regions. For this purpose, $AGP_{118-201}$ was immobilized on a CM5 sensor chip, and mutants were used at concentrations ranging from 250 to 1500 nM. Table 3 summarizes the binding characteristics of PAI-1 mutants grouped according to being a part of the epitopes.

Four antibodies, MA-55F4C12, MA-44E4, MA-33H1F7, and MA-33B8, bound to the PAI-1/AGP complex. PAI-1 variants harboring mutations in their epitope region did not show differences in binding characteristics, demonstrating that those epitopes are not located at the PAI-1/AGP interface. Some of them bound to a lower extent than wild-type PAI-1 and reacted with slightly changed binding affinity, indicating that they might contain a less active conformation. This seems to be the case particularly with mutants PAI-1-K88-D89AA, PAI-1-K88-K176AA, PAI-1-K88-H229AA, PAI-1-KE154/128AA, PAI-1-KV154/129AA, PAI-1-H185A, PAI-1-R187A, and PAI-1-H185/R186AA. Of the PAI-1 mutants tested, only those with an impaired MA-8H9D4 epitope showed a dramatic and specific deficit in binding to $AGP_{118-201}$. Single mutations Q301A, Q303A, and D305A significantly reduced the extent of binding to AGP and severely decreased the binding affinity described by the apparent K_As of $(6.9 \pm 2.8) \times 10^5$ M, $(1.3 \pm 1.2) \times 10^4$ M, and $(5.6 \pm 1.9) \times 10^4$ M, respectively. It appears that these three mutations must act together to disrupt AGP binding. Therefore, to confirm this concept, we used two mutants with double substitutions, i.e., R300-Q301AA and Q303-D305AA. Figure 3 shows that mutation Q303A and particularly Q303-D305AA resulted in damaging the PAI-1 binding site for AGP: both mutants did not interact with AGP, indicating that their binding site is destroyed (Table 3).

Functional analysis of the inhibitory activity of these mutants toward t-PA was also performed revealing 60% activity, therefore providing evidence that the lack of binding was not due to rapid conversion of these PAI-1 mutants into the latent form. To confirm this observation, binding of PAI-1 mutants with damaged MA-8H9D4 epitopes to AGP and its C-terminal fragment was further evaluated by solid-phase immunoassay. Figure 3 shows that PAI-1 variants with single mutations (Q301A, Q303A, D305A) and double substitutions (R300-Q301AA and Q303-D305AA) within the MA-8H9D4 epitope do not bind to AGP (Figure 3A) and show severely reduced binding to $AGP_{118-201}$ (Figure 3B,C).

In previous work we showed that the interaction between PAI-1 and α_1-acid stabilizes the active conformation of PAI-1 and prolongs its activity toward uPA and t-PA (*6*). To test functional consequences of the double mutations removing the contended AGP binding site, we compared the effect of α_1-acid glycoprotein on the rate of latency transition in wild-type PAI-1 and its mutant Q303-D305AA. For this purpose they were incubated in the absence or presence of α_1-acid glycoprotein (molar ratio 1:100) at 37 °C. The ability of PAI-1 to inhibit uPA activity was then analyzed at different time points as indicated in Figure 4. In contrast to wild-type PAI-1, α_1-acid glycoprotein had no effect on Q303-D305AA inhibitory activity, indicating that its effect on the rate of latency transition is abolished in the mutant.

To summarize, these experiments show that the PAI-1 binding site for AGP is overlapping the epitope of MA-8H9D4 and consists of amino acid residues between Arg300 and Asp305 located at the bottom of β-sheet A (Figure 5).

This binding site is in the proper configuration exclusively in an active molecule of PAI-1, since only such a form binds to AGP, followed by stabilization of its active conformation. This conclusion is consistent with our previous data showing

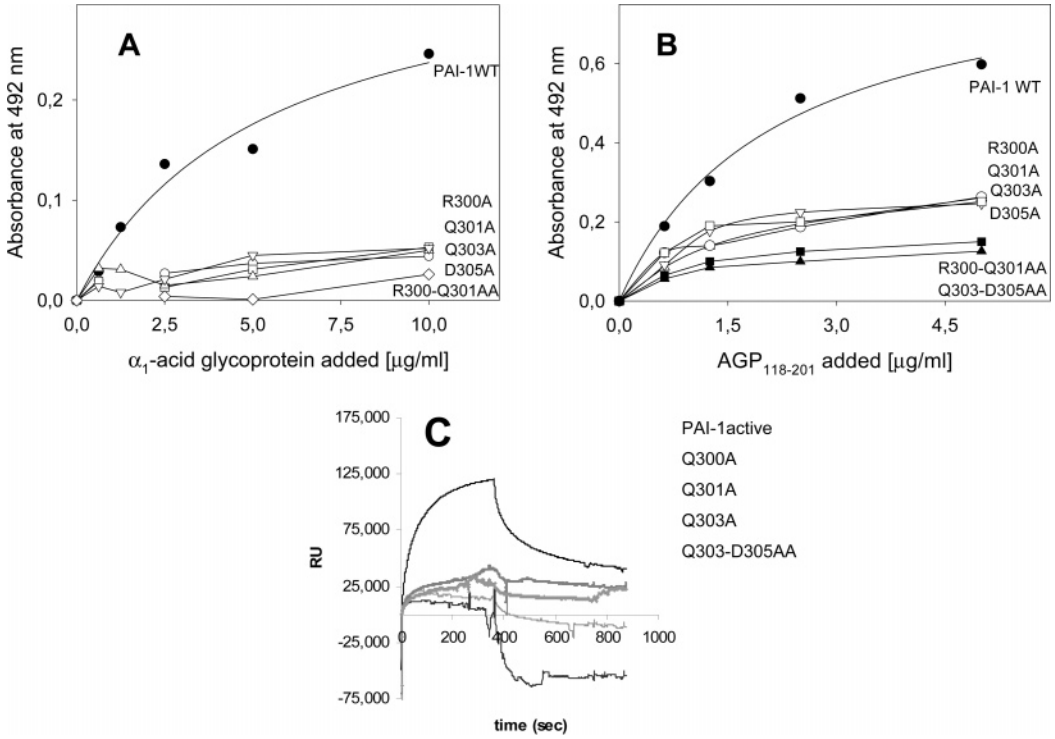

FIGURE 3: Binding of active PAI-1 and its mutants with the impaired MA-8H9D4 epitope to AGP. Panels A and B show the interaction of PAI-1 variants harboring with mutations in the MA-8H9D4 epitope region with AGP and its recombinant fragment $AGP_{118-201}$, respectively, as analyzed by a solid-phase binding assay. Panel C shows sensorgrams recorded during interactions of wild-type active PAI-1 and mutants used at 500 nM with $AGP_{118-201}$ immobilized on a sensor chip, CM5.

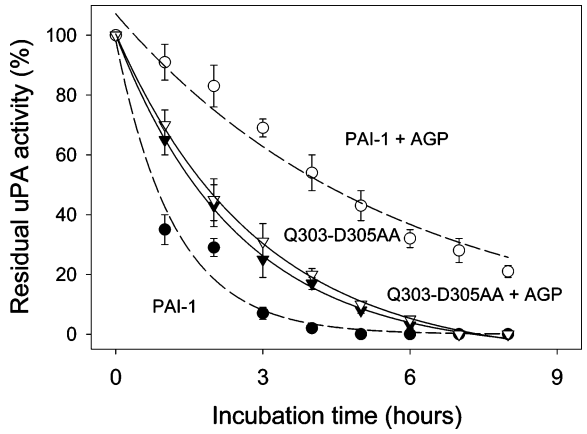

FIGURE 4: Stabilization of PAI-1 but not its mutant Q303-305AA inhibitory activity by α_1-acid glycoprotein. Samples of PAI-1 (circles) or its mutant Q303-D305 (triangles) were incubated at 37 °C in the absence (closed symbols) or presence (open symbols) of α_1-acid glycoprotein added in the molar ratio of 1 to 100. At different time points, aliquots were withdrawn to measure PAI-1 inhibitory activity toward uPA using the chromogenic substrate S-2444. Residual uPA activity was determined by quantifying the change in absorbance at 410 nm. Rates of substrate hydrolysis were calculated and expressed as a percentage of the maximum rate of uPA substrate hydrolysis in the absence of PAI-1.

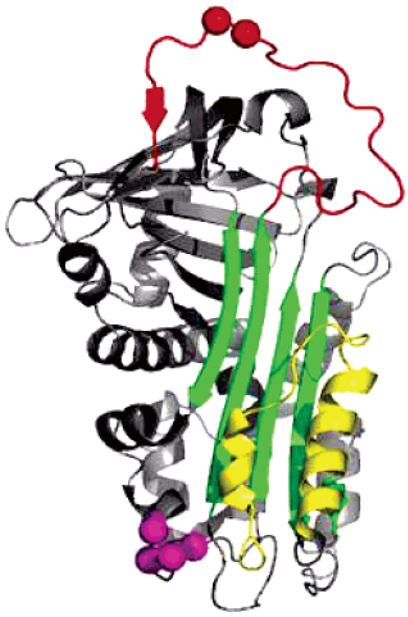

FIGURE 5: Location of the PAI-1 binding site for AGP. By employing PAI-1 mutants in direct binding experiments, the binding site could be located within the sequence R300−D305. These results were consistent with a lack of MA-8H9D4 binding to the PAI-1/AGP complex, suggesting that the location of the binding site is close to its epitope within the segment connecting regions hI with S5A. β-Sheet A is indicated in green, α-helix F and the turn connecting helix F with s3A are in yellow, the reactive site loop containing P1 and P1′ is shown in red, and the four residues (i.e., R300, Q301, Q303, D305) are shown as spheres in magenta.

that binding to AGP results in a delay in spontaneous inactivation of PAI-1 (*6*). The PAI-1 binding site for AGP is distinct from that which interacts with vitronectin, though they are both in close vicinity. This is supported by the observation that PAI-1 Q123K, which has dramatically reduced binding affinity to vitronectin, showed unchanged binding properties toward AGP (Table 2). In the case of vitronectin a number of studies, including X-ray crystal structure analysis (*32*) and mutagenesis (*33*−*36*), have demonstrated that it interacts with amino acid residues of α-helices F and E as well as strand 1 of β-sheet A. The complete RCL insertion associated with transition of active to latent form results in deformation of the PAI-1 binding

site for AGP, explaining why the latent form does not bind to this protein. Finally, our results demonstrate that PAI-1 interacts with AGP via a limited sequence and upon binding its active conformation is stabilized via a novel mechanism involving the segment connecting the regions hI with S5A.

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