Acute Phase Protein $\alpha_1$-Acid Glycoprotein Interacts with Plasminogen Activator Inhibitor Type 1 and Stabilizes Its Inhibitory Activity*

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$\alpha_1$-Acid glycoprotein, one of the major acute phase proteins, was found to interact with plasminogen activator inhibitor type 1 (PAI-1) and to stabilize its inhibitory activity toward plasminogen activators. This conclusion is based on the following observations: (a) $\alpha_1$-acid glycoprotein was identified to bind PAI-1 by a yeast two-hybrid system. Three of 10 positive clones identified by this method to interact with PAI-1 contained almost the entire sequence of $\alpha_1$-acid glycoprotein; (b) this protein formed complexes with PAI-1 that could be immunoprecipitated from both the incubation mixtures and blood plasma by specific antibodies to either PAI-1 or $\alpha_1$-acid glycoprotein. Such complexes could be also detected by a solid phase binding assay; and (c) the real-time bimolecular interactions monitored by surface plasmon resonance indicated that the complex of $\alpha_1$-acid glycoprotein with PAI-1 is less stable than that formed by vitronectin with PAI-1, but in both cases, the apparent $K_D$ values were in the range of strong interactions ($4.51 \pm 1.33$ and $0.58 \pm 0.07$ nM, respectively). The on rate for binding of PAI-1 to $\alpha_1$-glycoprotein or vitronectin differed by 2-fold, indicating much faster complex formation by vitronectin than by $\alpha_1$-acid glycoprotein. On the other hand, dissociation of PAI-1 bound to vitronectin was much slower than that from the $\alpha_1$-acid glycoprotein, as indicated by 4-fold lower $k_{off}$ values. Furthermore, the PAI-1 activity toward urokinase-type plasminogen activator and tissue-type plasminogen activator was significantly prolonged in the presence of $\alpha_1$-acid glycoprotein. These observations suggest that the complex of PAI-1 with $\alpha_1$-acid glycoprotein can play a role as an alternative reservoir of the physiologically active form of the inhibitor, particularly during inflammation or other acute phase reactions.

Plasminogen activator inhibitor type 1 (PAI-1) plays a regulatory role in fibrinolysis and is thought to critically influence many other biological processes involving cell migration or tissue remodeling (1). Under normal physiological conditions, PAI-1 is present in blood plasma at nanomolar concentrations that increase rapidly in response to different stimuli, including cytokines associated with defensive processes such as inflammation (2, 3). Among serine proteinase inhibitors (serpins), PAI-1 is the most efficient in vivo inhibitor of both tissue-type and urokinase-type plasminogen activators (4). In contrast to other serpins, its inhibitory activity is associated with its thermodynamically unstable form, which spontaneously converts to the stable inactive form. The active form of PAI-1 is thought to have an exposed center loop that is available for interaction with proteinases (5, 6). The conversion from active PAI-1 to its latent form involves incorporating this surface-exposed loop containing the reactive center into the central $\beta$-sheet of the protein (7, 8). Thus, self-inactivation of PAI-1 is a crucial regulatory mechanism by which this protein functions in circulation.

Because PAI-1 is inherently unstable and readily converts from an active to a latent form, the interaction with vitronectin is critical for regulation of PAI-1 inhibitory activity (9, 10). Stabilization of the active PAI-1 occurs due to formation of a complex with vitronectin, and PAI-1 circulates in blood vessels in such a complex (9). This inhibitor also appears to be complexed with vitronectin on platelets (11), which are regarded as the main source of PAI-1 found in blood plasma (12). Vitronectin binds PAI-1 with high affinity (13) and maintains the active conformation of the inhibitor (14). The vitronectin-binding site on PAI-1 appears to be located on the central $\beta$-sheet, the one that incorporates a surface-exposed loop containing the reactive center, and within adjacent secondary structures (7, 14–16). Therefore, association of vitronectin with the region in the vicinity of the reactive center interferes with movements of the $\beta$-strands and thus inhibits a conversion of PAI-1 to a latent form. On the other hand, the vitronectin interaction with PAI-1 induces allosteric changes in vitronectin that expose cryptic epitopes (17) and modulates vitronectin-dependent cell adhesion (13, 18).

The physiological implications of the PAI-1-vitronectin interaction are important because they affect the functions of both proteins. However, based on the much higher stability of the active PAI-1 associated with subendothelial matrix as compared with that in the vitronectin complex, it seems reasonable to look for some other mechanisms by which the active conformation of PAI-1 can be stabilized. In fact, PAI-1 was suggested to bind to negatively charged phospholipids such as phosphatidylinerine and phosphatidylinoisitol and thus to be activated, for example, after release from platelets (19). Moreover, PAI-1 secreted from activated platelets bound to fibrin associated...
with platelet membranes and inhibited fibrin degradation (20). This binding required active PAI-1, and the fibrin-bound PAI-1, like the PAI-1-vitronectin complex, was shown to form complexes with tPA and uPA. There are more observations indicating that PAI-1 can bind directly (21, 22) or indirectly via vitronectin (23) to fibrin. Recently, another protein that interacts with PAI-1 was identified in the mammary glands (24). It was suggested that insulin-like growth factor-binding protein 5, in addition to activation of cell death via insulin-like growth factor 1 sequestration, can enhance plasminogen conversion to plasmin by sequestering PAI-1 and thus regulate extracellular matrix remodeling (24).

In this report, by using the yeast-based two-hybrid system for studying protein-protein interaction (25, 26), we attempted to identify proteins other than vitronectin that, via binding to PAI-1, can preserve its inhibitory activity. Screening of the human liver cDNA library revealed α1-acid glycoprotein to be capable of complexing with PAI-1. Next, the interaction of α1-acid glycoprotein with PAI-1 was evidenced by several approaches including solid phase binding assays, co-immunoprecipitation experiments, and BlACORE analysis. These analyses revealed that α1-acid glycoprotein interacted with PAI-1 with lower binding affinity than vitronectin but with high enough affinity to stabilize the active conformation of PAI-1 and prolong its inhibitory activity toward uPA and tPA.

**MATERIALS AND METHODS**

**Proteins and Reagents**—Human PAI-1, vitronectin, and α1-acid glycoprotein were purchased from Calbiochem-Novabiochem Co. (San Diego, CA). Recombinant PAI-1 was also kindly donated by Dr. P. J. Declerck (Louvain, Belgium). Single-chain tPA containing >95% single-chain molecule was from Genentech. Human glu-plasminogen, α-thrombin, and plasminogen-free fibrinogen were purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Antibodies to α1-acid glycoprotein, mouse monoclonal antibody AA02, and rabbit polyclonal antibodies were purchased from ICN Biomedicals, Inc. (Aurora, OH). Mouse monoclonal antibody to vitronectin was from Chemicon International, Inc. (Temecula, CA). Mouse monoclonal antibodies to 20S protasome were from Affinity Research Products Ltd. (Manhean, United Kingdom). Mouse monoclonal antibodies to PAI-1, MAH, and clone 7 were purchased with immunoprecipitation from Calbiochem-Novabiochem Co., respectively. Anti-rabbit IgG horse radish peroxidase conjugate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and Wizard Miniprep and Maxiprep kits for isolation of plasmid DNA were purchased from Promega Corp. (Madison, WI). Protein A/G was from Santa Cruz Biotechnology (Santa Cruz, CA). High binding 96-well microtiter plates were obtained from Costar Science Corp. (Cambridge, MA). 2-Amino-bis-3-ethylthiazoline-6-sulfonic acid, bovine serum albumin (fraction V), nitrocellulose, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were from Sigma-Aldrich Chemical Co.

**Two-hybrid Procedures**—Library Screening, and Evaluation of Protein-Protein Interactions—Yeast strains for two-hybrid experiments, as well as all components of the MATCHMAKER Two-Hybrid System together with cDNA library from human liver, were obtained from CLONTECH. Cloning of plasmids was done in *Escherichia coli* strain DH5α, whereas yeast strain Y190 was used to assay protein-protein interactions and for library screening. PAI-1 cDNA was amplified by polymerase chain reaction using Pfu polymerase (Stratagene, La Jolla, CA) and subcloned into the EcoR I and BamHI restriction sites of pAS2-1 to produce pAS2-1-PAI-1. DNA sequencing of the obtained product by using Sequenterm kits (Epigen) revealed an in-frame fusion of PAI-1 to the 3' end of the GAL4 DNA-binding domain. The GAL4-PAI-1 fusion protein was stably expressed in yeast as evidenced by immunoblotting using monoclonal antibody to PAI-1. Concentrations of PAI-1 were determined by enzyme-linked immunosorbent assay using the Imulysé PAI-1 kit from Biopool. Furthermore, PAI-1 in this complex was able to inhibit tPA-induced plasminogen conversion into plasmin.

The two-hybrid assay using the GAL4 system was performed according to the instructions of the manufacturer (CLONTECH). For library screening, Y190 yeast cells were transformed with a liver two-hybrid library inserted into the activation vector pAD10, and pAS2-1-PAI-1 was used as bait. Doubly transformed cells were plated on Leu−, Try−, and His− plates. Colonies were picked, restreaked onto triple minus plates (Leu−Try− His−), and assayed for the LacZ phenotype. Yeast transformants were permeabilized in liquid nitrogen and transferred on nitrocellulose soaked in 60 mM NaHPO4, 40 mM Na2HPO4, 10 mM MgCl2, and 50 mM β-mercaptoethanol and containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Pierce) to a specific activity of 50 μCi/ml, and the labeled proteins were isolated by gel filtration on Sephadex G-25. All proteins migrated as a single band during Laemmlini (27) SDS-polyacrylamide gel electrophoresis.

**Immunoprecipitation and Co-precipitation Experiments**—Aliquots of 125I-labeled PAI-1 (6 nm) were mixed with increasing concentrations of either vitronectin or α1-acid glycoprotein in 500 μl of 0.1 M phosphate buffer, pH 7.1, containing 0.14 M NaCl, 0.05% Tween 20, and 4% PBS-Tween-polyethylene glycol and incubated for an hour at room temperature. Complexes were made by incubating the proteins at molar ratios (PAI-1:vitronectin and PAI-1:α1-acid glycoprotein) ranging from 1:10 to 1:40. In parallel experiments, samples of 125I-labeled α1-acid glycoprotein (5 and 10 nm; 100,000 or 200,000 cpm) were mixed with different concentrations of PAI-1 (30–150 μM) and incubated under the same conditions. One μg of rabbit polyclonal antibodies to vitronectin or α1-acid glycoprotein was added, and incubation continued for 4 h at 4 °C. To isolate immunoprecipitates, 20 μl of 50% slurry of protein A/G-agarose (Santa Cruz Biotechnology) was added, and the incubation mixture was left overnight at 4 °C. The immunoadsorbent complexes were washed three times with PBS-Tween-polyethylene glycol and twice with PBS and then solubilized in 40 μl of Laemmli sample buffer and separated by SDS-PAGE using a 10% running gel (27). Samples were reduced by heating to 100 °C for 5 min in the presence of 5% 2-mercaptoethanol. The gels were dried and exposed to x-ray Kodak film for a day at −70 °C. To identify PAI-1:α1-acid glycoprotein complexes in human plasma, 125I-labeled α1-acid glycoprotein (50 nm) was added to 500 μl of control blood plasma samples from an Imlysé kit (Biopool) and incubated for 18 h at 4 °C. Positive control plasma (50 μg PAI-1/ml) and negative control plasma (PAI-1-depleted plasma) were used. Next, 1 μg of rabbit polyclonal antibodies to PAI-1 was added and incubated for 4 h at 4 °C, and immunoprecipitates were isolated as described above.

**Solid Phase Binding Assays**—The wells of 96-well microtiter plates were coated with either polyclonal antibodies to vitronectin at 1 μg/ml in PBS. Unbound proteins were washed from the wells, and nonspecific binding sites were blocked by incubation with 3% BSA and 0.05% Tween 20 in PBS for an hour at room temperature. Direct binding assays were performed by adding increasing concentrations of PAI-1 to immobilized α1-acid glycoprotein-, vitronectin-, or BSA-coated plates in Tris-buffered saline, pH 7.5, containing 1% BSA, 0.05% Tween 20, and 1 mM CaCl2, and the plates were then incubated for an hour at 37 °C. The activation of latent PAI-1 by treatment with SDS was performed on all samples before binding assays, as described previously (28). Unbound PAI-1 was aspirated, and the wells were washed three times with PBS containing 0.1% BSA and 0.05% Tween 20. To detect bound PAI-1, the plates were incubated with polyclonal antibodies to PAI-1 for an hour at room temperature and then washed with PBS containing 0.1% BSA and 0.05% Tween 20. The plates were incubated with goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase. The reaction was developed using 2,2'-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) from Sigma at 1 mg/ml in 0.1 M sodium citrate, pH 4.5, and the change in color was determined at 405 nm. To detect nonspecific binding, all assays were done simultaneously on plates coated with BSA alone and processed as described above. The background binding to BSA was subtracted from all samples before data analysis.

**Surface Plasmon Resonance**—The kinetic parameters (association and dissociation rate constants, kon and koff, respectively) and the affinity constant (Kd) of PAI-1 with either α1-acid glycoprotein and PAI-1 were measured by surface plasmon resonance using a BlACORE X (Amersham Pharma Biotech). In parallel, the same interactions using the vitronectin complex with PAI-1 were evaluated. Briefly, PAI-1 was covalently attached to carboxymethyl dextran (CM5) chips (BlACORE) previously activated with N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl carbodiimide according to the manufacturer's instructions. Experiments were performed at 25 °C using Tris-buffered saline/BiAs (150 mM NaCl, 3.4 mM EDTA, 0.005% P20, and 50 mM
Tris-HCl, pH 7.4) as a running buffer. The sensor chip was regenerated with a short pulse of 200 nM glycine-HCl, pH 2.2. The amount of ligands, α1-acid glycoprotein or vitronectin, bound to immobilized PAI-1 was monitored by measuring the variation of the surface plasmon resonance angle as a function of time. Results were expressed in RU, an arbitrary unit specific for the BIAcore instrument (1000 RU correspond to ~1 ng of bound protein/nm²). The association rate constant, kₐ, and dissociation rate constant, kₛ, were determined separately from individual association and dissociation phases, respectively, assuming one-to-one interaction. The overall affinity constant, Kₒ, was derived from kₐ/kₛ.

PAI-1 Activity Assay—Samples of PAI-1 (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 either vitronectin (0.6 μM) or α1-acid glycoprotein (0.6 μM). At different time points, aliquots were withdrawn to measure PAI-1 activity toward uPA and tPA using chromogenic assays.

Briefly, the activity of PAI-1 was evaluated after incubation with high molecular weight uPA (4.8 nM; Molndal) or single-chain tPA (4.6 nM; 150 IU/ml; American Diagnostica) or 20S proteasome tPA (0.5 mM; American Diagnostica) for 30 min at 25 °C. The chromogenic substrate S-2444 (0.5 mM; Molndal) for uPA or Spec was determined separately from individual association and dissociation phases, respectively, assuming one-to-one interaction. The overall affinity constant, Kₒ, was derived from kₐ/kₛ.

RESULTS AND DISCUSSION

Interaction of PAI-1 with α1-Acid Glycoprotein Identified by the Two-hybrid System—Several studies have indicated that active PAI-1 is very labile and converts within 1–2 h into an inactive form, unless its active conformation becomes stabilized via interaction with some other proteins (29–31). Subsequently, vitronectin was found to bind PAI-1 with high affinity and to serve as a reservoir of the active PAI-1 in circulation (9–11, 31–34). Our present studies show for the first time that α1-acid glycoprotein also interacts with PAI-1 and may play a significant role in the regulation of fibrinolysis. This protein has been identified after using the yeast two-hybrid system to screen human liver cDNA libraries cloned into the GAL4 activation domain, with PAI-1 fused to the GALA DNA-binding domain for use as the bait protein. Of 50 His+ LacZ colonies isolated, 10 gave a strong interaction, and after DNA sequence analysis and data bank searches, 3 of them revealed that they encode almost the entire molecule of α1-acid glycoprotein, i.e. without the 7 beginning amino acids from the signal peptide. This major acute phase protein is comprised of 201 amino acid residues, which may form to large extent a lipocalin domain (reviewed in Ref. 35). The specificity and nature of the interaction between PAI-1 and α1-acid glycoprotein were characterized by co-formation of various constructs into the Y190 yeast strain. β-Galactosidase activity was detected only in yeast co-transformed with PAI-1 and α1-acid glycoprotein, ruling out the possibility that single plasmids, either pGAD10-α1-acid glycoprotein or pAS2-1-PAI-1, contain intrinsic transcriptional activity or interact with other proteins nonspecifically.

Human α1-acid glycoprotein is a product of a cluster of three adjacent genes: AGP-A, AGP-B, and AGP-B′ covering 70 kb of the human genome and located on chromosome 9. AGP-A is actively expressed in human liver and codes for the major component of serum α1-acid glycoprotein (ORM1, orosomucoid) (35). In addition to hepatocytes (36), different forms of this protein are expressed in other cells, including human breast and endothelial cells (37), endothelial cells from dermal tissue (38), and cultured human granulocytes and monocytes (39). Under physiological conditions, α1-acid glycoprotein plasma concentration remains stable (about 1 g/liter) but increases severalfold during acute phase reactions caused by systemic tissue injury, inflammation, and infection (37, 39). Although the biological function of α1-acid glycoprotein remains unknown, it is considered to be a natural anti-inflammatory and immunomodulatory agent (40, 41). Interestingly, a vast number of studies have shown that the increased PAI-1 levels and activity are associated with all these pathophysiological processes, particularly under inflammatory conditions.

Complex Formation and Coprecipitation of PAI-1 and α1-Acid Glycoprotein.—To determine whether α1-acid glycoprotein interacts with PAI-1 in vivo, both proteins were immunoprecipitated from the incubation mixture in which one of them was radiolabeled. In the first set of experiments, 125I-PAI-1 was mixed with α1-acid glycoprotein or vitronectin to achieve molar ratios in both cases ranging from 1:10 to 1:50 and incubated overnight. The complexes were then precipitated using antibodies specific to α1-acid glycoprotein or vitronectin, respectively. As illustrated in Fig. 1A, both proteins were associated with PAI-1, and their complexes were detectable by autoradiography after SDS-polyacrylamide gel electrophoresis. However, the intensity of bands differed significantly, suggesting a higher binding affinity of PAI-1 to vitronectin than to α1-acid glycoprotein. Although PAI-1 is known to be very sensitive to oxidative radiolabeling (42, 43), we used PAI-1 radiiodinated with Iodobeads in the preliminary experiments because (a) this preparation contained a population of active PAI-1 molecules (corresponding to ~35% of total PAI-1) that formed SDS-stable complexes with tPA, (b) 125I-PAI-1 bound efficiently to vitronectin, which is known to bind with high affinity only active PAI-1 (12), and (c) 125I-PAI-1 binding to α1-acid glycoprotein tested in fresh human blood plasma was competed by unlabeled active PAI-1, and the complex was not precipitated by nonspecific antibodies (Fig. 1B). Formation of the complex between α1-acid glycoprotein and PAI-1 was further evidenced in the second system, in which α1-acid glycoprotein was labeled, and the complex was precipitated with antibodies specific to PAI-1 (Fig. 2A). To examine whether such a complex can also be formed in blood plasma, two samples of standard human blood plasma provided with the Imula kit (Biopool) representing a positive control for PAI-1 (50 ng PAI-1/ml) and a negative control (PAI-1-depleted plasma) were used. As shown in Fig. 2B, 125I-α1-acid glycoprotein bound to PAI-1 present in blood plasma, and the resulting complex was precipitated by antibodies to PAI-1. Only traces of such a complex could be detected when the experiment was done using the
negative control (PAI-1-depleted plasma), whereas there was a strong interaction between both proteins in the case of the positive control blood plasma. Also in this system, binding of $^{125}$I-α₁-acid glycoprotein to PAI-1 was inhibited by a 100-fold molar excess of unlabeled α₁-acid glycoprotein (Fig. 2C).

Interaction of α₁-acid glycoprotein with PAI-1 was further analyzed by solid phase binding assays. Fig. 3 summarizes results from experiments in which vitronectin and α₁-acid glycoprotein were immobilized on microtiter wells, followed by incubation with varied concentrations of PAI-1. Bound PAI-1 was quantified using rabbit polyclonal anti-PAI-1 and goat anti-rabbit IgG conjugated with horseradish peroxidase as first and second antibodies, respectively. PAI-1 bound to immobilized α₁-acid glycoprotein in a dose-dependent manner but with substantially reduced affinity compared with vitronectin.

Comparison of PAI-1 Binding Affinity to α₁-Acid Glycoprotein and Vitronectin by Surface Plasmon Resonance—The real-time biomolecular interactions in vitro during complex formation between PAI-1 and α₁-acid glycoprotein were next monitored by surface plasmon resonance. For this purpose, limiting amounts of PAI-1, α₁-acid glycoprotein, and vitronectin were immobilized on a separate sensor chips through amine coupling. Injection of active PAI-1 as the analyte to sensor chips containing either immobilized α₁-acid glycoprotein or vitronectin produced a typical surface plasmon resonance binding signal. However, the inhibitor also interacted strongly with dextran on the uncoated sensor chips and remained adsorbed to dextran even after the washing procedure. To avoid this interaction, all following binding studies were performed using PAI-1 immobilized on the sensor chip and α₁-acid glycoprotein or vitronectin as the analytes. Consistent with previous studies (44), after covalent coupling of PAI-1 to the dextran surface, 75% of the molecules remained in the right orientation to react with tPA (data not shown). As shown in Fig. 4, characteristic binding signals were monitored when α₁-acid glycoprotein or vitronectin was brought into contact with immobilized PAI-1. The binding to both α₁-acid glycoprotein and vitronectin was dose-dependent, and the maximum response monitored at the end of the protein injection phase was 248 and 510 RU, respectively, for 5000 RU of initially immobilized PAI-1. The corresponding molar ratio was estimated at 0.65 mol/mol and was consistent with a 1:1 interaction. There was no binding of α₁-acid glycoprotein to the uncoated surface and the immobilized vitronectin. There was also no binding of α₁-acid glycoprotein to the vitronectin-PAI-1 complexes (Fig. 5). In these experi-
PD.1 immobilized on the sensor was first saturated with vitronectin and then treated with α1-acid glycoprotein. As we can see on Fig. 5, further injections of α1-acid glycoprotein to such a sensor did not result in additional binding signals, indicating that binding sites on the PAI-1 molecule for vitronectin and α1-acid glycoprotein may be the same or may be located in the adjacent regions. In these latter control experiments, the rapid change in the resonance signal was due to a dilution buffer-induced, nonspecific alteration in the bulk refractive index. Consistently, vitronectin did not bind to α1-acid glycoprotein or α1-acid glycoprotein-PAI-1 complexes coupled to the sensor chip (data not shown).

The $k_{\text{on}}$ has been determined from the dissociation phase of the binding of α1-acid glycoprotein or vitronectin to immobilized PAI-1, using the sensograms obtained with different concentrations of the soluble component (Table I). Similarly, $k_{\text{on}}$ was evaluated from the association phase and, finally, the apparent $K_d$ of the binding was calculated (Table I). As shown in Table I, the on rate for binding of α1-acid glycoprotein or vitronectin to PAI-1 differed by 2-fold, indicating much faster complex formation by vitronectin than by α1-acid glycoprotein. On the other hand, PAI-1 dissociation was much slower from the vitronectin complex, as indicated by 4-fold lower values of $k_{\text{off}}$ than from the α1-acid glycoprotein complex. Taken together, these data suggested that the vitronectin complex is formed more rapidly and remains more stable than the α1-acid glycoprotein complex with PAI-1, but in both cases, the apparent $K_d$ values are in the range of strong interactions.

Recent studies on the stoichiometry of the complex revealed that it is composed of two vitronectin and four PAI-1 molecules and that there are two PAI-1-binding sites on vitronectin (45). The localization of PAI-1-binding sites on the vitronectin molecule is not clear, and at present there are data indicating that three regions of vitronectin can be involved in interactions with PAI-1. The first one corresponds to the somatomedin B domain formed by the N-terminal 44 amino acid residues of vitronectin (14, 46). The second site was proposed to be located in the positively charged region comprising amino acid residues from 345–379, also known to interact with heparin (47). The third one was suggested to be within the region 115–121 (48). However, in contrast to the first two sites, the last one does not appear to stabilize the active conformation of PAI-1. The presence of several binding sites in vitronectin can explain why its complex with PAI-1 may interact with other molecules including fibrin (21, 22) and heparin (47).

The binding sites involved in the interaction of PAI-1 with α1-acid glycoprotein have not yet been identified. Because neither vitronectin nor α1-acid glycoprotein bound to preformed complexes of PAI-1, namely, to PAI-1-α1-acid glycoprotein or PAI-1-vitronectin, respectively, it may be suggested that binding sites for both proteins may be the same or are located in close proximity on the PAI-1 molecule. On the other hand, human α1-acid glycoprotein consists of ~45% carbohydrates attached in the form of five complex-type N-linked glycans (49, 50) that show tetra-antennary as well as di- and tri-antennary structure. The terminating sugars on the glycan chains are responsible for a great deal of the diversity found on glycans, and neuraminic acid is one of the common sugars among them, giving rise to a very low pI. Therefore, an alternative mechanism explaining the interaction of α1-acid glycoprotein with PAI-1 may involve its binding to a cluster of positively charged amino acid residues of the heparin-binding site. This domain is located in and around helix D of PAI-1, where the amino acid residues Lys-65, Lys-69, Arg-76, Lys-80, and Lys-88 are the major determinants for interaction with heparin (51). Interestingly, in the same region, there is a cryptic high affinity binding site for the low density lipoprotein receptor-related protein as well (52). BIAcore measurements are made under constant flow conditions and do not allow the ability to distinguish between PAI-1 interactions among the several potential binding sites present on both vitronectin and α1-acid glycoprotein. Therefore, dissociation constants given in Table I can represent a mean value, which at present cannot be ascribed to the interaction of particular sites during the complex formation.

**Stabilization of PAI-1 Activity in Complex with α1-Acid Glycoprotein**—To further investigate the nature of the interaction between PAI-1 and α1-acid glycoprotein, we tested whether complex formation between these two proteins stabilizes the active conformation of PAI-1 and prolongs its activity toward uPA and tPA. For this purpose, PAI-1 was incubated in the absence or presence of α1-acid glycoprotein (molar ratio, 1:200) at 37 °C, and the ability of PAI-1 to inhibit uPA and tPA activity was analyzed at different time points as indicated in Fig. 6. Upon binding to PAI-1, α1-acid glycoprotein significantly protected its inhibitory activity, but not to the same extent as vitronectin did. However, after incubation for 2 h at 37 °C, both proteins stabilized PAI-1 activity to the same extent, i.e. ~60% of PAI-1 retained the original inhibitory activity, although with time, the efficiency of α1-acid glycoprotein protection of PAI-1 activity decayed much faster than that of vitronectin. After 4 h, PAI-1 alone was completely latent, whereas in the presence of α1-acid glycoprotein, there was still about 40% inhibitory activity in the incubation mixture. Under the same conditions, there remained ~60% of the original PAI-1 activity in the incubation mixture containing vitronectin. It is noteworthy that more than 20% of PAI-1 was still active after 6 h at 37 °C in the presence of α1-acid glycoprotein. These data indicate that α1-acid glycoprotein has a substantial stabilizing effect on PAI-1 activity.
Because α₁-acid glycoprotein, together with serum albumin and lipoprotein, is the most common drug-binding protein in blood plasma, large variations in its concentration were described to have important pharmacokinetic implications (reviewed in Ref. 53). It seems reasonable to speculate that circulating and cell-associated α₁-acid glycoprotein added in the molar ratio of 1:100. At different time points, aliquots were withdrawn to measure PAI-1 inhibitory activity toward uPA (A) and tPA (B) using chromogenic substrates S-2444 and Spectrozyme TPA, respectively. Residual uPA and tPA activities were determined by quantifying the change in absorbance at 410 nm. Rates of substrate hydrolysis were calculated for each of the various experimental conditions and expressed as a percentage of the maximum rate of uPA substrate hydrolysis in the absence of PAI-1. In B, the residual activity of tPA is shown after treatment with PAI-1 (solid line) and PAI-1 in complex either with vitronectin (dashed line) or α₁-acid glycoprotein (dotted line) as described under "Materials and Methods."

To summarize, this study shows for the first time that α₁-acid glycoprotein interacts with PAI-1 and may play a significant role in the regulation of fibrinolysis. Under a variety of experimental conditions, we have demonstrated that binding of PAI-1 to α₁-acid glycoprotein results in significant stabilization of its inhibitory activity toward plasminogen activators. Therefore, α₁-acid glycoprotein can play a significant role in stabilization of the active conformation of PAI-1 and may be an addition to vitronectin as a reservoir of active PAI-1, particularly during inflammation and other acute phase reactions.

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