The Exon 3 Encoded Sequence of the Intracellular Serine Proteinase Inhibitor Plasminogen Activator Inhibitor 2 Is a Protein Binding Domain*

Poul H. Jensen‡‡, Thomas G. Jensen¶, Walter E. Laug§, Henrik Hager‡, J. Gliemann¶, and Blake Pepinsky**

From the Departments of §Medical Biochemistry and ¶Human Genetics, University of Aarhus, DK-8000 Aarhus C, Denmark. ‡Division of Hematology/Oncology, Childrens Hospital Los Angeles, Los Angeles, California 90027, and **Biogen, Cambridge, Massachusetts 02142

We have used a combination of biochemical and immunological methods to probe for proteins that interact with the cytoplasmic form of plasminogen activator inhibitor 2 (PAI-2) and to identify the structure in PAI-2 that mediates the binding. By affinity chromatography on immobilized PAI-2, we purified a collection of PAI-2-binding proteins. These proteins bound $^{125}$I-labeled PAI-2 in vitro (IC$_{50}$, 10–100 nM) in a calcium-independent reaction that did not abrogate the proteinase inhibitory function of PAI-2. Annexin I was identified among the eluted proteins, and purified annexins I, II, IV, and V, but not III and VI, possessed $^{125}$I-labeled PAI-2 binding activity. Immune precipitation by anti-PAI-2 monoclonal and polyclonal antibodies of metabolically labeled melanoma cells treated with a cleavable cross-linker prior to analysis revealed three prominent proteins with apparent masses of 100, 70, and 50 kDa. We localized the protein binding domain in PAI-2 between amino acid residues 66 and 98, as determined by using a PAI-2 mutant lacking this domain and a synthetic peptide spanning this region. This region of PAI-2 corresponds to exon 3 of the gene sequence thought to be critical for PAI-2 functions.

Plasminogen activator inhibitor 2 (PAI-2), 1 a member of the serine proteinase inhibitor (serpin) gene family (1), is predominantly an intracellular protein abundantly expressed in placenta and macrophages. However, PAI-2 can be secreted as a glycoprotein by a process of facultative translocation due to the presence of an inefficient internal signal sequence (2, 3). The extracellular form functions as an efficient inhibitor of plasminogen activators leading to the clearance of plasminogen activator/PAI-2 complexes (4, 5). The role of the intracellular PAI-2 is unclear. Recent studies using transfected cells suggest that intracellular PAI-2 is an inhibitor of apoptosis (6, 7). A direct inhibition of apoptogenic proteinases by PAI-2 seems likely because cells transfected with a PAI-2 mutant with changed proteinase inhibitory specificity failed to protect the cells against TNF cytotoxicity (7).

Some serpins have developed structures that make them bind ligands other than proteinases, e.g. the hormone binding to the noninhibitory carrier serpins, corticosteroid binding globulin and thyroxine binding globulin (8). However, ligand binding to some inhibitory serpins is important for their functional state, e.g. the increased antiproteolytic activity of antithrombin III upon heparin binding (8) and the changed specificity and increased stability of PAI-1 when bound to vitronectin (9). We have previously demonstrated Glu$_{83,84,86}$ in PAI-2 to be substrate sites for the intracellular enzyme transglutaminase (10), thus forming the basis for the covalent cross-linking of PAI-2 in trophoblast membranes and during keratinocyte differentiation (11–13). Glu$_{83,84,86}$ are localized in a 33-amino acid residue sequence (residue nos. 66 to 98) putatively localized between helices C and D (14, 15). This sequence, which is poorly conserved in the serpins (8), largely corresponds to exon 3 in the PAI-2 gene (16).

Hypothesizing that the intracellular functions of PAI-2 are subject to modulation by binding to cytosolic nonproteinase ligands, we have probed the existence of such binding sites and localized the protein binding domain in PAI-2 to the exon 3 coded segment.

EXPERIMENTAL PROCEDURES

Materials—$^{[125]}$I[Labeled and $^{[35]}$S]methionine were purchased from Amersham Corp. Wild-type recombinant human PAI-2, the deletion mutant lacking amino acids 66–98 (Δ66–98-PAI-2), and human urokinase plasminogen activator (uPA) were described previously (10). The 33-amino acid residue synthetic peptide Asn-Ala-Val-Thr-Pro-Met- Thr-Pro-Glu-Asn-Phe-Thr-Ser-Cys-Gly-Phe-Met-Gln-Gln-Ile-Gln-Lys-Gly-Ser-Tyr-Pro-Asp-Ala-Ile-Leu-Gln-Ala-Gln (66–98-PAI-2), corresponding to amino acid residues 66–98 in PAI-2, was purchased from Schaefer-N A/S, Copenhagen, Denmark. The annexins were purified essentially as described previously; recombinant human annexin I, C-terminal annexin I fragment, and recombinant human annexin V (17, 18); human annexins III, IV, and VI from human placenta, and the bovine C-terminal annexin II fragment from intestinal mucosa (19). Purified human placental (annexin II/p10) was a gift from Dr. V. Gerke, University of Münster, Germany. The monoclonal anti-human PAI-2 IgG$_{2a}$ (No. 3750) was obtained from American Diagnostica, Greenwich, CT. The rabbit polyclonal PAI-2 antisera was raised against recombinant human PAI-2 expressed in Escherichia coli. The rabbit polyclonal anti-human annexin I was described previously (20, 21). The rhodamine-labeled phalloidin and fluorescein isothiocyanate-concanavalin A were from Sigma, and the monoclonal anti-tubulin antibody was from Amersham Corp. All other chemicals and reagents were purchased from commercial sources.
were from Sigma and are of analytical grade unless stated otherwise.

**PAI-2 Affinity Purification**—Ten mg of recombinant PAI-2 were immobilized on 1 ml of CNBr-activated Sepharose 4B according to the manufacturer’s recommendations (Pharmacia Biotech Inc.). Crude placental cytosol was prepared from term placentae obtained after normal deliveries at the Department of Obstetrics and Gynecology, Aarhus University Hospital. The placental tissue was cut in approximately 4-g pieces, rinsed in ice-cold PBS, frozen in liquid nitrogen, and stored at −80 °C. All of the following procedures were performed at 4 °C. The placental tissue was homogenized using a conventional kitchen-type Waring blender for 3 × 15 s in 250 mM sucrose, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 3 mM iodoacetamide, 0.05% polyvinylpyrrolidone 40000, and 10 mM Hepes, pH 7.8. The homogenate was cleared by a two-speed centrifugation at 4°C at 3000 rpm for 4 min and 5000 rpm for 60 min, discarding the pellet after each centrifugation. The cytosol preparation was stored at −80 °C or directly passed over the PAI-2 affinity column. The latter was performed at a flow rate of 0.5 ml/min. Prior to elution, the column was washed in 30 ml of wash buffer (150 mM KCl, 2 mM MgCl₂, and 20 mM Hepes, pH 7.2). PAI-2-binding proteins (PBPs) were eluted from the column by increasing the concentration of KCl to 1 M. The column was washed in 10 ml of 4 M urea followed by 20 ml of wash buffer supplemented with 0.01% Triton X-100 and 0.05% NaN₃. The samples designated for ¹²⁵I-labeled PAI-2 binding assays were made of 45% glycerol prior to storage at −20 °C. The protein concentration was determined by the bicinchoninic acid protein assay (Sigma).

**Binding Assay**—The PBPs, approximately 14 μg/ml, were coated on Polyorb microparticle plates (NUNC, Copenhagen, Denmark) in 0.2 M NaHCO₃, pH 9.6, for 2 h on ice. The residual protein binding capacity was quenched by the addition of 100 μl of 10% bovine serum albumin (BSA) for 1 h. The plates were rinsed twice in binding buffer (150 mM KCl, 2 mM MgCl₂, and 20 mM Hepes, pH 7.0) prior to incubation with 100 μl of ¹²⁵I-tracer (100 000 cpm/ml equivalent to approximately 50 pmol) in binding buffer. All measurements were performed in four replicates with background values (wells were coated only with BSA) measured for each condition. The intrassay coefficient of variation was 2.5% on replicates.

**Ligand Blotting Assay**—¹²⁵I-labeled PAI-2 ligand blotting was performed on material resolved either by nondenaturing SDS-PAGE or 1% agarose electrophoresis. The samples for the SDS-PAGE, diluted 1:1 in 1% SDS-20 mM Tris, pH 6.8, 20% glycerol, were neither heated nor reduced. The proteins were resolved by 8–16% gradient SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore) for 16 h at 200 mA in a tank electroblotting apparatus ( Hoefer). The membranes were blocked in 20 ml Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, plus 2% Tween-20 for 2 h at ambient temperature and then treated with 100 μl ¹²⁵I-labeled PAI-2 in the same buffer with 0.05% Tween 20 overnight at 4 °C and washed extensively with the 0.05% Tween containing buffer. The filters were dried, and bound PAI-2 was detected by autoradiography using Amersham MP films at −80 °C for 16 to 72 h. By this approach, 10 ng of urokinase plasminogen activator, used as a positive control, could be detected after autoradiography for 16 h. The samples for thin layer electrophoresis were diluted 1:1 in 75 mM barbital, 20% glycerol, pH 8.6, and resolved in 1-mm thick 1% agarose gels on a Pharmacia MultiPhor, essentially as described (12). The electroblotting on nitrocellulose membranes (Sartorius, Göttingen, Germany) was performed as described above, and the filter was quenched by 5% BSA for 2 h at 4 °C prior to incubation with 1 μM ¹²⁵I-labeled PAI-2. Bound PAI-2 was detected by autoradiography.

**Cell Culture**—The human melanoma cell line M24met was cultured in RPMI 1640, 10% fetal bovine serum, and 2 mM glutamine as described previously (22). All culture surfaces were preincubated in the conditioned medium from the M24met cell line for 3 h at room temperature prior to seeding of the cells to improve the adhesion and spreading of the transfected cells. Cells were tested for Mycoplasma contamination by Hoechst staining (Boehringer Mannheim). For the induction of the PAI-2 expression, M24met cells were incubated in the presence of human recombinant TNF (Amersham Corp.) for 24 h prior to fixation for immunofluorescence studies.

**Metabolic Labeling, in Situ Cross-linking, and Immunoprecipitation**—M24met cells were grown in four-well tissue culture chamber slides (Nunc). The cells were fixed in 100% methanol for 2 min at 4 °C, rinsed in PBS, and incubated with (20 μg/ml) monomeric avian anti-PAI-2 IgG₄ or 5 μg of rabbit anti-human recombinant PAI-2 IgG for 16 h at 4 °C. The following agents were used as controls: (a) the equivalent amounts of nonimmune mouse IgG₂a (Dako, Copenhagen, Denmark) or preimmune rabbit IgG; and (b) saturation of the antibody with 10 μg of recombinant human PAI-2 prior to incubation with the cell lysate. The IgG was collected by treating the lysates with 40 μl of GammaBind G-Sepharose (Pharmacia Biotech Inc.) for 1 h followed by centrifugation. The GammaBind-G Sepharose was washed seven times in 0.1 M NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, and 0.5% Nonidet P-40, and bound IgG was released by heating samples to 95 °C for 3 min in SDS-sample buffer (62.5 mM Tris, pH 6.8, 2% (w/v) SDS, and 10% glycerol). Some samples were supplemented with 20 mM di-thioerythritol to cleave the disulfide bridge in the cross-linker. All samples were resolved by 10–20% gradient SDS-PAGE, and the proteins were visualized by fluorography after the gels had been impregnated in Amplify (Amersham Corp.).

**Immunofluorescence Studies**—M24met cells were grown in four-well tissue culture chamber slides (Nunc). The cells were fixed in 100% methanol for 2 min at 4 °C, rinsed in PBS, and incubated with (20 μg/ml) monomeric anti-PAI-2 IgG in PBS and 1% bovine serum albumin for 30 min at 20 °C. The primary antibody was detected by incubation with fluorescein isothiocyanate-labeled swine anti-rabbit (Dako, Denmark) for 30 min at 20 °C. The culture slides were washed and mounted. Control labeling was performed without the primary antibody. The cells were examined using an Olympus BX50 immunofluorescence microscope (Olympus, Tokyo, Japan).

**RESULTS**

**Affinity Purification of PAI-2-binding Proteins**—We have used a crude placental cytosol preparation as a source for putative PAI-2-binding proteins because PAI-2 is abundantly expressed in this tissue. For the detection of PAI-2-binding proteins, the binding of ¹²⁵I-PAI-2 was analyzed using a solid phase microtiter plate binding assay. The placental cytosolic
preparation was passed over an affinity column containing immobilized recombinant human PAI-2. Fig. 1 demonstrates the elution of the proteins, designated PAI-2-binding proteins (PBP), from the PAI-2 Sepharose column by increasing the ionic strength to 1 M KCl. The protein concentration of the eluted fractions correlated directly with the 125I-labeled PAI-2 binding activity. The binding of 50 pm 125I-labeled PAI-2 to immobilized PBP was specific because it could be saturated by the presence of 1 nM PAI-2 (compare Fig. 2). Although elution of the affinity column with 50 mM glycine, pH 2.5, or 1 M urea produced a similar set of eluted proteins, no binding activity could be detected; despite neutralization or dilution of the eluate, respectively (data not shown). The binding activity of the proteins in 1 M KCl eluate was also demonstrated by a ligand blotting technique after the samples were resolved by native electrophoresis, blotted onto a nitrocellulose filter, and incubated with 125I-labeled PAI-2 after a blocking step in BSA (data not shown). From 0.55 liter of the placental cytosol containing 11.6 mg/ml of protein, we recovered 3 mg of PBP, representing approximately a 2000-fold purification.

Characterization of the PAI-2 Binding—Fig. 2 demonstrates that the association of 50 pm 125I-labeled PAI-2 to immobilized PBP reached a plateau by about 6 h at 4 °C. The nonspecific absorption to the BSA-coated control wells was rapid and accounted for about 25% of the total binding. Dissociation experiments were performed by removing nonbound ligand in the wells and continuing the incubation in fresh buffer (data not shown). By 24 h, approximately 30% of the label had dissociated, while the amount of bound tracer remained unchanged when the nonbound tracer was left in the well. This demonstrates that the dissociation was not due to “leakage” of bound PBP from the solid phase surface. The rate of dissociation was its sensitivity toward nonionic detergents as inclusion of 0.001% Triton X-100 in the assay buffer completely abrogated the binding (data not shown). The zwitterionic detergent CHAPS was tolerated at a concentration of 0.01% with only a 60% reduction in the specific binding activity (data not shown). Competition experiments demonstrated half saturation of the binding of 20 pm 125I-labeled PAI-2 to PBP by 100 μM nonimmune rabbit IgG (column B), IgG raised against the synthetic (66–98)PAI-2 peptide (column C), and IgG raised against recombinant PAI-2 (column D). The ordinate shows the ratio of bound tracer (% Bound). Each point represents the mean of four replicates in one of three experiments; bars, S.D.

Structural Determinants in PAI-2 Responsible for Binding—To identify the protein binding domain of PAI-2, we used three PAI-2 constructs (Fig. 3A): a recombinant full-length human PAI-2 molecule; a recombinant deletion mutant lacking residues 66–98 (Δ(66–98)PAI-2); and a synthetic peptide corresponding to residues 66–98 (66–98)PAI-2. In a separate experiment, immobilized PBP was incubated with 20 pm 125I-labeled (66–98)PAI-2 peptide in the presence of unlabeled PAI-2 or (66–98)PAI-2 as indicated. The ordinate shows the concentration of unlabeled competitor (pM). The ordinate shows the ratio of bound versus free (B/F) tracer. Points represent the mean of four replicates; bars, S.D. One of five representative experiments; the error bars, S.D. One of five representative experiments for the PAI-2 saturation and one of three for the (66–98)PAI-2 peptide saturation are presented. B, right panel, the concentration dependence of ligand binding to PBP. 125I-Labeled PAI-2 (20 pm) was incubated with immobilized PBP in the presence of the indicated concentrations of PAI-2, the synthetic (66–98)PAI-2 peptide, and the deletion mutant Δ(66–98)PAI-2 (A). In a separate experiment, immobilized PBP was incubated with 20 pm 125I-labeled (66–98)PAI-2 peptide in the presence of unlabeled PAI-2 (B) or (66–98)PAI-2 (C) as indicated. The abscissa shows the concentration of unlabeled competitor (pM). The ordinate shows the ratio of bound versus free (B/F) tracer. Points represent the mean of four replicates; bars, S.D.
PAI-2-binding Proteins

FIG. 4. PBP-bound PAI-2 retains proteinase binding activity. Microtiter wells were either coated with PBP (14 μg/ml, ●) 1 μg/ml, △) prior to blocking of excess proteins binding capacity by BSA or BSA alone (□) and incubated with the indicated concentration of PAI-2 overnight at 4°C. The binding of 20 ps of the 125I-labeled uPA to the PBP-bound PAI-2 was subsequently determined. □, 125I-labeled uPA binding to wells coated with 14 μg/ml PBP in the presence of 200 nm unlabeled uPA. Points, means of four replicates. The S.E. for the uPA binding was less than 5%. One of two experiments is presented.

In order to test the possibility further, we evaluated the synthetic peptide (66–98)PAI-2 as a competitor for the binding of wild-type PAI-2. A complete inhibition of the 125I-labeled PAI-2 tracer binding was observed, although about 100-fold higher competitor concentration of the peptide was needed as compared to the wild-type molecule (Fig. 3B, left panel). The number of PAI-2-binding sites was estimated to 1 x 10^{-13} mol/well, assuming a 1:1 stoichiometry in the binding of 10% of the 10 nm PAI-2. This corresponds to 4 ng of protein, assuming a molecular mass of 40 kDa, and is in accordance with the amount of protein that can be immobilized in microtiter wells. Furthermore, direct binding of 125I-labeled (66–98)PAI-2 peptide to PBP was saturable by PAI-2 or (66–98)PAI-2 (Fig. 3B, left panel). Finally, the importance of this domain for the binding of PAI-2 to PBP was supported by studies using a rabbit serum raised against the (66–98)PAI-2 peptide (Fig. 3B, right panel). As compared to the control samples incubated with 100 μg/ml nonimmune rabbit IgG, the anti-(66–98)PAI-2 peptide IgG inhibited the binding by about 55% to about a level obtained by IgG raised against the recombinant PAI-2. Thus, the exon 3 encoded PAI-2 structure, making up the CD interhelical region, represents a protein binding domain with specificity for cytosolic PAI-2-binding proteins.

PBP-bound PAI-2 Retains Proteinase Binding Activity—Serpins inhibit their target proteinases by forming tight complexes with the catalytic site of the proteinase. Fig. 4 shows that the binding of PAI-2 to PBP does not abrogate its function as an inhibitor because the PBP-bound PAI-2 still can bind the catalytic domain of the proteinase urokinase plasminogen activator. Experimentally, microtiter wells were coated with PBP followed by quenching of the excess protein binding capacity of the plastic with BSA. Control wells were blocked in BSA alone. The wells were incubated with increasing concentrations of PAI-2, and the antiproteinolytic function of the bound PAI-2 was probed by incubation with 125I-labeled uPA. The binding of 125I-labeled uPA was both dependent on the amount of PBP immobilized and on the amount of added PAI-2. The uPA binding was specific because 100 nm unlabeled uPA completely abrogated the PBP-dependent tracer binding. Maximal PBP-dependent binding was obtained at a PAI-2 concentration about 0.4 μg/ml.

Identification of a Class of Intracellular PAI-2-binding Proteins—Analysis of the placental cytosolic proteins eluted from the PAI-2 affinity column by reducing SDS-PAGE (Fig. 5, left) revealed a complex mixture of proteinases ranging from more than 250 kDa to approximately 35 kDa. The absent binding of the Δ66–98PAI-2 to immobilized PBP suggested that all of the PAI-2 binding exclusively took place via the CD-interhelical region. PAI-2 reactive proteinases reacting with the reactive site loop would, as such, not be expected. Still, the absent detection of proteins binding to the reactive site loop might have been due to the solid phase microtiter plate assay. To address this question, we used a 125I-labeled PAI-2 blotting assay (Fig. 6) capable of demonstrating PAI-2 reactive proteinases. PBP or the proteinases (uPA and tPA) were resolved by nonreducing SDS-PAGE and electrophoresed onto a nitrocellulose membrane; then PAI-2 reactive proteinases were detected after incubation with 125I-labeled PAI-2 followed by autoradiography. We did not anticipate any binding to the detergent sensitive CD-interhelical binding PBPs because the samples were treated with SDS prior to electrophoresis. By this technique, 10 ng of two-chain uPA but not noninhibitory single-chain pro-uPA was readily detected (Fig. 6, lane 1 versus lane 2). Activation of the single-chain uPA to two-chain uPA by in situ plasmin treatment allowed 125I-labeled PAI-2 binding (Fig. 6, lane 5). Similar results were obtained with tissue-type plasminogen activator, where the single-chain form also bound 125I-labeled PAI-2, although as expected to a lower extent than the two-chain form (data not shown). When 15 μg of aliquots of PBP were analyzed, no 125I-labeled PAI-2-binding proteins were detected, even after plasmin treatment (Fig. 6, lanes 3 and 6). Longer exposure of the blot also gave no signal (data not shown). Thus, neither the two-chain nor single-chain PAs are present in the PBP elution fractions at significant levels. Since this technique probably would detect other proteinases with specificity for PAI-2, we infer that they are also absent from the preparation.

To identify PAI-2-binding proteins, we mixed PBPs with [35S]methionine-labeled human keratinocyte extract and re-
solved the sample by two-dimensional gel electrophoresis. The gel was stained with Coomassie Brilliant Blue and then subjected to fluorography. The Coomassie Brilliant Blue stained PBP spots that colocalized with [35S]methionine-labeled keratinocyte proteins in the fluorogram were compared with the two-dimensional gel electrophoresis keratinocyte data base (23). Among the proteins resolved by two-dimensional gel electrophoresis, annexin I was one of the predominant PBP proteins, whereas actin and albumin were absent (data not shown). The latter two are markers of nonspecific absorption of abundant intra- and extracellular proteins. The identity of annexin I in the PBP preparation was verified by immunoblotting with a rabbit anti-human annexin I antibody (Fig. 5, right panel).

Annexin I belongs to a highly conserved gene family representing major intracellular proteins. Structurally, they are composed of a variable short N terminus followed by a larger conserved C terminus, the latter containing four to eight repeated copies of a homologous Ca\(^{2+}\) and phospholipid binding domain. Fig. 7 demonstrates the binding of PAI-2 to selected annexins. When incubated with 50 pmol 125I-labeled PAI-2 in a solid phase binding assay, annexins I and II bound about 30% of the tracer as compared to approximately 10% for annexins IV and V. By contrast, annexin III and VI did not bind PAI-2. The different binding efficiencies were not due to immobilization differences in the microtiter wells because extraction of the immobilized annexins by SDS, followed by reducing SDS-PAGE and silver staining, revealed annexin bands of comparable intensity (data not shown). The characteristics of the PAI-2 binding to isolated annexins I and II were similar to the binding to PBP in terms of affinity, independence of divalent cations, and sensitivity to detergents (data not shown). The PAI-2 binding domain in annexins I and II was localized by testing the PAI-2 binding activity of the conserved C-terminal fragment. These domains were generated by limited cleavage of the annexins I and II, followed by chromatographic purification of the N-terminally truncated proteins (19). Both the C-terminal fragments of annexin I and annexin II, known to bind Ca\(^{2+}\) and phospholipid, bound PAI-2 with similar affinity as intact annexin I (data not shown). Annexin II exists intracellularly in two forms, as a monomer and as a heterotetrameric complex with a 10-kDa protein, p10. When analyzed in the microtiter plate assay, we found that purified human placental (annexin II/p10), also bound labeled PAI-2 to the same extent as annexin I (data not shown).

**Localization of Intracellular PAI-2**—We used immunofluorescence microscopy to investigate whether intracellular PAI-2-binding proteins might localize PAI-2 to certain intracellular structures. Previously, detailed analysis of the intracellular localization of PAI-2 had not been performed, probably due to the low constitutive PAI-2 expression. Accordingly, we found the PAI-2 expression level in the M24met cells too low to permit analysis of the intracellular PAI-2 by immunofluorescence microscopy, even though the cells express significant levels of PAI-2, as reported previously (22) and demonstrated in Fig. 8B and Fig. 9. To overcome this problem, we incubated the cells in the presence of the known inducer of PAI-2, TNF-\(\alpha\), in concentrations ranging from 1–25 ng/ml. As evaluated by immunofluorescence microscopy, we observed a dose-response yielding a strong expression in most cells at the highest concentration (data not shown). Fig. 8A demonstrates the PAI-2 expression in the M24met cell line stimulated by 25 ng/ml TNF-\(\alpha\) using fluorescein isothiocyanate-labeled secondary antibody. Control labeling without the primary antibody completely abrogated the staining (data not shown). The PAI-2 staining reveals a fine reticular fluorescent pattern with no preferential intracellular localization. This staining pattern did not colocalize with the endoplasmic reticulum, actin filaments, or microtubules, as determined by staining using concanavalin A, phalloidin, and a tubulin-specific monoclonal antibody (data not shown). The specificity of the monoclonal PAI-2 antibody was demonstrated by Western blotting of a detergent extract of the M24met cells, showing a single band comigrating with recombinant human nonglycosylated PAI-2 (Fig. 8B, left versus right lane).
PAI-2-binding Proteins

Immunoprecipitation of PAI-2-associated Proteins—We used immunoprecipitation of [35S]methionine-labeled M24met melanoma cells chemically cross-linked by the cell permeable cross-linker DSP, which contains a disulfide bond cleavable by reduction, to determine whether specific proteins could be responsible for the apparent reticular organization of the intracellular PAI-2 (Fig. 8). The antibodies used for the precipitation study were the above-described anti-PAI-2 monoclonal antibody (Fig. 9, lanes 2, 3, and 5) and polyclonal antibody raised against recombinant human PAI-2 (Fig. 9, lanes 6, 7, and 9). Fig. 9 shows an SDS-PAGE analysis of the immunoprecipitates. Three prominent bands with apparent masses of approximately 100, 70, and 50 kDa were detected in the anti-PAI-2 immunoprecipitates, although the bands were only apparent after reduction (Fig. 9, lanes 2 and 6). Without reduction, most of the labeled proteins failed to enter the gel (Fig. 9, lanes 3 and 7). The 100- and 70-kDa bands were seen both with the monoclonal and polyclonal PAI-2 antibodies, whereas the 50-kDa band only was precipitated by the polyclonal antibodies (Fig. 9, lanes 2 and 3 versus lanes 6 and 7). This might be due to a blocking of the epitope for the monoclonal antibody in the complex between PAI-2 and the 50-kDa protein. The small amount of the 100- and 50-kDa proteins, seen without reduction (Fig. 9, lanes 3 and 7), suggests that some had been bound noncovalently to the PAI-2 and had resisted the washing during the immunoprecipitation. This was confirmed by immunoprecipitation by both the monoclonal and polyclonal anti-PAI-2 antibodies of cell lysates prepared without DSP (data not shown). The specificity of the 100-, 70-, and 50-kDa proteins as PAI-2-associated proteins was demonstrated by: (a) their presence in immunoprecipitates with mouse or rabbit anti-PAI-2 IgG (Fig. 9, lanes 2, 3, 6, and 7) and absence in immunoprecipitates with nonimmune rabbit or mouse IgG (Fig. 9, lanes 4 and 8); and (b) their absence when precipitated in the presence of an excess of recombinant human PAI-2 (Fig. 9, lanes 5 and 9). The approximately 55-kDa bands in Fig. 9, lanes 2 and 3, might represent specifically precipitated material. However, the presence of bands with equal size, albeit of less intensity, in the precipitate with nonimmune IgG makes such a conclusion uncertain (Fig. 9, lane 4). Thus, the immunoprecipitation experiments suggest that intracellular PAI-2 in M24met cells is tightly associated with approximately 100- and 50-kDa proteins and weakly associated with a 70-kDa protein.

DISCUSSION

Reversible interaction between PAI-2 and other molecules has not been reported previously. In contrast, covalent binding to different proteins through disulfide bridges, mediated by free cysteine residues (24, 25) and via transglutaminase-catalyzed isopeptide bonds (11–13), has been demonstrated. The transglutaminase amine donor substrate sites Gln53,54,56 (10) are localized in the exon 3-encoded domain, corresponding to amino acid residues 58–96 (16). By comparing the primary sequence of PAI-2 and the crystallized serpins, this domain is localized between helices C and D (8, 26). We now identify this domain as responsible for mediating the reversible binding between PAI-2 and cytosolic proteins. This statement is based on the lack of binding of the deletion mutant Δ66–98PAI-2 and the complete saturable binding of wild-type PAI-2 and of (66–98)PAI-2 peptide to preparations of PBP that had been purified by affinity chromatography on PAI-2. The 100-fold lower apparent affinity of the synthetic peptide, as compared to the native molecule, suggests proper peptide folding to be dependent on interactions or constraints conferred by the parental molecule.

Such CD-interhelical insertions are only observed among the group of ovalbumin-like serpins (8, 27–30), and it is tempting to speculate that these insertions represent protein binding domains that evolved in specific serpins. Protein binding to the CD-interhelical domain, like transglutaminase-catalyzed cross-linking via this structure (10), does not affect the proteinase inhibitory function. This is probably due to the localization of the insertion at the opposite side of the molecule, as compared to the reactive center loop, and close to the known heparin and vitronectin binding sites in other serpins (Fig. 10; Refs. 31–34).

The role of intracellular PAI-2 is not clear. Recent studies have demonstrated that transfection of cells with PAI-2 cDNA...
The annexins constitute a highly conserved gene family of intracellular Ca\(^{2+}\) binding domains of the CD-interhelical loop-mediated interactions between PAI-2 and their functional significance is not yet clear, even though they have been ascribed functions as ion channels and as regulators of membrane trafficking, inflammation, coagulation, proliferation, and differentiation (36). We find that PAI-2 binds in a Ca\(^{2+}\)-independent reaction to the C-terminal Ca\(^{2+}\) binding part of annexin I and II. The Ca\(^{2+}\)-independent binding of PAI-2 to annexins makes its functional significance more likely. We did not observe any annexin-like proteins immunoprecipitating with PAI-2 from the metabolically labeled M24met cells, even though the cells express annexin II. However, attempts to cross-link PAI-2 and annexins in vitro never succeeded using the cross-linkers BS\(_3\) and DSP. Thus, if PAI-2/annexin complexes had been present in the M24met cells, their interaction would have been broken by the detergent in the extraction buffer. The unresolved normal function of the annexins makes the experimental assessment of the role of the high affinity interaction between PAI-2 and their C-terminal domains difficult.

In conclusion, we have shown that the CD-interhelical domain of PAI-2 constitutes a binding domain for intracellular ligands including the annexins. Molecular interactions with this domain have been reported to be required for intracellular functions of PAI-2, e.g. inhibition of apoptosis.

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