Plasminogen Activator Inhibitor Type 1 Interacts with α3 Subunit of Proteasome and Modulates Its Activity*

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Plasminogen activator inhibitor type 1 (PAI-1), a multifunctional protein, is an important physiological regulator of fibrinolysis, extracellular matrix homeostasis, and cell motility. Recent observations show that PAI-1 may also be implicated in maintaining integrity of cells, especially with respect to cellular proliferation or apoptosis. In the present study we provide evidence that PAI-1 interacts with proteasome and affects its activity. First, by using the yeast two-hybrid system, we found that the α3 subunit of proteasome directly interacts with PAI-1. Then, to ensure that the PAI-1-proteasome complex is formed in vivo, both proteins were coimmunoprecipitated from endothelial cells and identified with specific antibodies. The specificity of this interaction was evidenced after transfection of HeLa cells with pCMV-PAI-1 and coimmunoprecipitation of both proteins with anti-PAI-1 antibodies. Subsequently, cellular distribution of the PAI-1-proteasome complexes was established by immunogold staining and electron microscopy analyses. Both proteins appeared in a diffuse cytosolic pattern but also could be found in a dense perinuclear and nuclear location. Furthermore, PAI-1 induced formation of aggresomes freely located in endothelial cytoplasm. Increased PAI-1 expression abrogated degradation of degron analyzed after cotransfection of HeLa cells with pCMV-PAI-1 and pd2EGFP-N1 and prevented degradation of p53 as well as IkBα, as evidenced by both confocal microscopy and Western immunoblotting.

Plasminogen activator inhibitor type 1 (PAI-1) plays a regulatory role in fibrinolysis and many other biological processes involving tissue remodeling, cell proliferation, migration, and apoptosis (1–5). Its synthesis and activity are tightly regulated, and normally PAI-1 is present as a trace protein in plasma. However, under pathological conditions such as cancer, atherosclerosis, diabetes, and severe obesity, PAI-1 levels are dramatically increased both inside of cells and in the extracellular matrix or in blood plasma (6). Interestingly, a number of observations show that either an exogenous source of PAI-1 or cancer cells overexpressing PAI-1 is effectively able to induce apoptosis and control growth of tumor vasculature. Furthermore, the aggressiveness of tumor growth and metastasis can be diminished after transfection with PAI-1 of tumor cell lines such as HT-1080 fibrosarcoma cells (7), murine melanoma (8), PC-3 prostate cancer cell lines (9), or malignant keratinocytes (10). Proapoptotic activity of PAI-1 was also evidenced using endothelial cells isolated from the aortas of PAI-1−/− mice. Such cells showed decreased spontaneous apoptosis relative to WT cells, but they recovered the proliferative phenotype associated with hyperactivation of Akt after treatment with recombinant PAI-1 (3, 11). Inhibition of apoptosis by PAI-1 appears to be independent of the PAI-1/uPA complex, signaling through uPAR or binding to vitronectin, but has been directly linked with the inhibitory effect of PAI-1 on the activity of caspase-3 but not caspase-8 (12, 13). On the other hand, PAI-1 deficiency has been associated with activated caspase-3 in atherosclerotic plaques from apoE−/− mice (13). Indeed, PAI-1 forms a high affinity complex with caspase-3, thereby directly inhibiting caspase-3 activity. However, PAI-1 has pleiotropic effects, and the exact mechanism underlying PAI-1 apoptosis regulating function is still not known.

This study was designed to search for novel intracellular proteins that interact with PAI-1 in endothelial cells and for alternative mechanisms by which PAI-1 may modulate cellular proliferation or apoptosis. We provide evidence that PAI-1, when up-regulated in cells, interacts with proteasome α3 subunit and modulates its proteolytic activity, thus promoting apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Wizard Miniprep for plasmid DNA isolation and restriction enzymes was purchased from Promega Corp. (Madison, WI). Rabbit polyclonal antibodies to 20 S proteasome α3 (H-125), mouse monoclonal antibody to 20 S proteasome α3 (MCP257), rabbit polyclonal antibody to β1 (FL-241), mouse monoclonal antibody to PAI-1 (C-9), rabbit anti-IκBα antibodies (C-21), mouse monoclonal antibody to green fluorescent protein (GFP; B-2), and HRP-conjugated goat anti-rabbit and goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). 20 S proteasome was from Enzo Life Sciences (Plymouth Meeting, PA). Constitutively active PAI-1 mutant was from American Dignostica.
(Stamford, CT). Rabbit polyclonal antibodies to PAI-1 (for ELISA) and rabbit anti-26 S proteasome S4 subunit were from Calbiochem/Merck, rabbit anti-β actin polyclonal antibodies were from Abcam (Cambridge, UK), AlexaFluor 568-conjugated donkey anti-rabbit antibodies were from Molecular Probes/Invitrogen, and FITC-conjugated goat anti-mouse antibodies were from Chemicon/Millipore (Billerica, MA). Protein A/G was from Pierce/Thermo Scientific (Rockford, IL). Mowiol was from Calbiochem/Merck. High binding 96-well microtiter plates were obtained from Costar Science Corp. (Cambridge, MA). o-Phenylenediamine, bovine serum albumin (fraction V), epoxomicin, and calpain inhibitor II were from Sigma. Plasmids pd2EGFP-N1 with a destabilized variant of the enhanced GFP and pCMV/HA, yeast strains for two-hybrid experiments, and all components of the MATCHMAKER two-hybrid system together with the cDNA library from human liver were from Clontech/Takara Bio (Mountain View, CA).

Two-hybrid Procedures, Library Screening, and Evaluation of Protein-Protein Interactions—The two-hybrid assay using the GAL4 system was performed as described previously (14). Briefly, plasmids were propagated in Escherichia coli strain DH5α, whereas yeast strain Y190 was used to assay protein-protein interactions. cDNA library from human liver was cloned in GAD10 vector in EcoRI restriction site. PAI-1 cDNA was amplified by polymerase chain reaction using Pfu polymerase (Stratagene, La Jolla, CA) and subcloned to produce pAS2–1–PAI-1, which was used as bait.

Solid Phase Binding Assay—The wells of 96-well microtiter plates were coated overnight at 4 °C with either proteasome or vitronectin at 1 μg/ml in PBS, and the assay was performed as described previously (14). Direct binding assays were performed by adding increasing concentrations of PAI-1 to immobilized proteasome-, vitronectin-, or BSA-coated plates in Tris-buffered saline, pH 7.5, containing 1% BSA, 0.01% Tween 20, and 1 mM CaCl2. To detect bound PAI-1, the plates were incubated with polyclonal antibodies to PAI-1 for 1 h 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-ethylbenzthiazidine-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.

Cell Culture—Human endothelial cell line EA.hy926 and HeLa were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM with high glucose supplemented with 10% FBS and antibiotics in a 90–95% humidified atmosphere of 5% CO2. The cells, at 85% confluence, were washed extensively with PBS, cultured in serum-free medium supplemented with 1% BSA, and incubated with TNFα or LPS (Sigma). After 18 h, cells were washed with PBS and resuspended in Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, containing 1% Nonidet-Igepal, 150 mM NaCl, 5 mM EDTA), and the soluble protein fraction was collected by centrifugation. Protein concentrations in cell lysates were measured with the BCA method (Pierce/Thermo Scientific kit). For transfection experiments, cells (2×105 cells/ml) were seeded onto a cell chamber or 25-cm2 culture flask. Lipofectamine 2000 (Invitrogen) was used for DNA transfer into the cells according to the manufacturer’s instruction. Cells were collected at 24 or 48 h post-transfection and lysed as described above.

Pull Down Assay—The α3 proteasomal subunit was expressed in the E. coli strain BL21(DE3). A PRESET (Invitrogen) construct containing cDNA encoding the proteasome α3 subunit was prepared. EA.hy926 cells were used for mRNA isolation, and cDNA was amplified using SuperScript™ III One-Step RT-PCR System (Invitrogen). Recombinant α3 subunit was purified from bacterial water-soluble fraction on a chelating Sepharose fast flow column (GE Healthcare). His6-tagged α3 subunit was subjected to SDS/PAGE and Western blot analysis, then probed with mouse monoclonal antibody MCP257 directed against α3 proteins (Santa Cruz Biotechnology). Finally, purified recombinant proteins immobilized on NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare) were incubated with the cell lysates for 18 h at 4 °C (500 μg/ml). The beads were then washed with PBS buffer three times and treated with sample buffer containing 2% SDS and 5% β-mercaptoethanol. The protein samples were analyzed by Western blotting using mouse monoclonal antibody to PAI-1. The beads without immobilized α3 were used as a negative control for antibody specificity.

Immunoprecipitation and Co-precipitation Experiments—Aliquots of125I-labeled PAI-1 (6 nM) were mixed with either vitronectin or proteasome in 500 μl of 0.1 M phosphate buffer, pH 7.1, containing 0.14 M NaCl, 0.05% Tween 20, and 4% PEG (PBS-Tween-polyethylene glycol buffer) and incubated for 1 h at room temperature. One μg of rabbit polyclonal antibodies to vitronectin or proteasome was added, and incubation was continued for 4 h at 4 °C. To isolate immunoprecipitates, 20 μl of 50% slurry of protein A/G-agarose (Pierce/Thermo Scientific) was added, and the incubation mixture was left overnight at 4 °C with orbital rotation. The immunosorbent 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 in 10% running gel. Samples were reduced by boiling for 5 min in the presence of 5% 2-mercaptoethanol. The gels were dried and exposed to Kodak x-ray film. For coimmunoprecipitation experiments, cells were lysed with Nonidet P-40 lysis buffer for 20 min on ice and centrifuged at 14,000 rpm for 30 min at 4 °C. Next, protein concentration was measured with BCA methods. 500 μg of protein from each lysate was incubated with 2.5 μg of anti-PAI-1 or anti-α3 proteasome subunit antibodies on a rotator overnight at 4 °C. Subsequently, 100 μl of protein A/G-agarose bead slurry was added to each cell extract, and the incubation was continued for another 3 h. The beads were washed 3 times with PBS, suspended in a 5× concentration of SDS-PAGE loading buffer, and boiled for 5 min. Proteins released from the resin were separated by electrophoresis and immunodetected by anti-α3 proteasome sub-
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unit, anti-β1 proteasome subunit, anti-S4 proteasome subunit, or anti-PAI-1 antibodies.

Confocal Immunofluorescence Microscopy—For microscopic examination, cells were plated at a density 5 × 10⁵ cells/ml on Permanox Cover slips in eight-well tissue chamber slides (Nunc) with detachable chambered upper structures. After 18 h of stimulation with TNFα or LPS, they were fixed with ice-cold methanol for 20 min, washed 3 times with PBS, and incubated with blocking buffer (PBS containing 3% BSA) for 1 h at room temperature. Next, the cells were incubated with monoclonal C-9 antibody to PAI-1 and polyclonal H-125 antibodies to α3 proteasome subunit. After washing, FITC- or AlexaFluor 568-conjugated secondary antibodies were used as required. The cells were then visualized using a confocal microscope (Nikon D-Eclipse C1) and analyzed with EZ-C1v.3.6 software.

For degron studies, HeLa cells were transfected with d2EGFP-N1, pEGFP-1, and cotransfected with pCMV/HA or pCMV/HA/PAI-1. 24 h after transfection cells were fixed with 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.2% saponin containing 0.1% BSA in PBS for 30 min and then blocked with 5% BSA in PBS for 30 min. Cells were incubated with anti-PAI-1 antibody (1:100 in 0.1% saponin, 0.1% BSA in PBS) followed by secondary antibodies labeled with AlexaFluor 568. Mowiol was used as a mounting medium. The cells were then visualized using a confocal microscope (Nikon D-Eclipse C1) and analyzed with EZ-C1v.3.6 software. In parallel, the same cells were analyzed by Western immunoblotting in terms of the extent of GFP degradation.

Post-embedding Double Immunolabeling in Electron Microscopy—Cells were fixed for 30 min at room temperature in freshly prepared 4% paraformaldehyde, 0.5% glutaraldehyde in PBS buffer. Next, cells were rinsed in PBS four times (each for 5 min) and dehydrated in a graded series of ethanol solutions followed by 3 changes (30 min each) of the mixture of LR-White resin (Polysciences, Inc., Warrington, PA) with 4% paraformaldehyde, 0.5% glutaraldehyde, 30 min, and then blocked with 5% BSA in PBS for 30 min. Cells were incubated with anti-PAI-1 antibody (1:100 in 0.1% saponin, 0.1% BSA in PBS) followed by secondary antibodies labeled with AlexaFluor 568. The cells were then visualized using a confocal microscope (Nikon D-Eclipse C1) and analyzed with EZ-C1v.3.6 software. In parallel, the same cells were analyzed by Western immunoblotting in terms of the extent of GFP degradation.

RESULTS

Interaction of PAI-1 with Proteasome—As a first step to identifying intracellular proteins that interact with PAI-1, we employed the yeast two-hybrid system. To screen the human liver cDNA library cloned into the GAL4 activation domain, PAI-1 was fused to the GAL4 DNA binding domain for use as the bait protein. Of 50 His⁺ LacZ⁺ colonies isolated, 5 gave a strong interaction and, after DNA sequence analysis and database searches, revealed that they encode the entire molecule of α3 subunit of proteasome (Fig. 1A). The specificity and nature of the interaction between PAI-1 and α3 subunit were characterized by co-transformation of various constructs into
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FIGURE 1. Direct interaction of PAI-1 with proteasome. Panel A shows restriction analysis of five pGAD10 plasmids isolated from His + LacZ colonies after yeast two-hybrid system screening. The encoded cDNA sequence, cloned into an EcoRI site, corresponded to the entire a3 subunit of human proteasome. Direct binding of PAI-1 to the proteasome was then confirmed by immunoprecipitation of the 125I-PAI-1-proteasome complexes (panel B). Immunoprecipitated complexes of 125I-labeled PAI-1 with proteasome were separated by SDS-PAGE (10% gels) under reducing conditions. The first and third lanes show complexes of 125I-labeled PAI-1 with vitronectin (Vn) and proteasome, respectively. The second and fourth lanes show corresponding control immunoprecipitates. Panel C shows direct interaction of PAI-1 with proteasome analyzed by solid phase binding assay. Increasing doses of PAI-1 were incubated for 1 h at 37 °C in wells coated with proteasome. The unbound PAI-1 was washed away, and the bound PAI-1 was evaluated by ELISA using antibodies to PAI-1. Nonspecific binding observed in the absence of proteasome was subtracted. Data represent the mean ± S.D. obtained during three separate experiments. Panel D shows pulldown experiments using the His-tagged a3 proteasome subunit immobilized on NHS-Sepharose beads. EA.hy926 cells, control, or stimulated either with TNF (5 ng/ml and 20 ng/ml) or LPS (5 μg/ml and 20 μg/ml) were used as a source of PAI-1. PAI-1 was identified by Western immunoblotting with monoclonal anti-PAI-1 antibodies. Inputs of PAI-1 and β actin are shown. Direct interaction of both proteins in endothelial cells was further demonstrated by coinmunoprecipitation (panel E). EA.hy926 cells, control and stimulated with TNF (5 ng/ml and 20 ng/ml) or LPS (5 μg/ml and 20 μg/ml), were lysed with 1% Nonidet P-40 lysis buffer and precipitated with anti-PAI-1 (left) or anti-a3 proteasome subunit (right) antibodies. Next, protein-protein complexes were captured by 100 μl of protein A/G-agarose beads. Bound proteins were solubilized by SDS sample buffer and subjected to SDS-PAGE and Western immunoblotting (I8) analysis. Coinmunoprecipitated (IP) proteins were detected with specific anti-a3 or anti-PAI-1 monoclonal antibodies. Inputs of a3 subunit and PAI-1 as well as blotting with nonimmune IgG and anti-actin antibodies are shown as well.

The Y190 yeast strain. β-Galactosidase activity was detected only in yeast co-transformed with PAI-1 and a3 subunit, ruling out the possibility that single plasmids, either pGAD10-a3 subunit or pAS2-a1-PAI-1, contain intrinsic transcriptional activity or interact with other proteins nonspecifically.

To determine whether proteasome interacts with PAI-1 in vitro, both proteins were immunoprecipitated from the incubation mixture in which one of them was radiolabeled. After overnight incubation of 125I-PAI-1 with proteasome or vitronectin at the molar ratio of 1:10 to 1:50, the complexes were precipitated using specific antibodies. As illustrated in Fig. 1B, both proteins were associated with PAI-1, and their complexes were detectable by autoradiography after SDS-PAGE. However, the intensity of bands differed significantly, suggesting a higher binding affinity of PAI-1 to vitronectin than to proteasome.

Interaction of proteasome with PAI-1 was further analyzed by solid phase binding assays. In this experiment proteasome was 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 the first and second antibodies, respectively. As seen in Fig. 1C, PAI-1 bound to immobilized proteasome in a dose-dependent manner. To demonstrate that both proteins can directly interact and form a complex, we expressed the a3 proteasome subunit in bacteria. Then, the recombinant His6-tagged fusion protein was immobilized on NHS-activated Sepharose and incubated with cell lysates of control EA.hy926 cells and those activated with TNF or LPS. The Sepharose beads were then washed, and the bound protein was analyzed by Western immunoblotting with mouse monoclonal antibodies specific to PAI-1 (C-9) or to a3 proteasome subunit (right). The presence of PAI-1 in a
high molecular weight complex with proteasome, particularly in cells activated with LPS or TNF, was evidenced by (a) blotting with anti-\( \alpha \)-H9251\( \beta \)-proteasome subunit, when the immunoprecipitate was pulled down with antibodies to PAI-1 and (b) blotting with antibodies to PAI-1, when the immunoprecipitate was pulled down with mAb to \( \alpha \)-H9251\( \beta \)-proteasome subunit (Fig. 1E).

To determine the subcellular distribution of PAI-1 and a possible co-localization with proteasome, subconfluent EA.hy926 cells were stained for both proteins and examined by immunofluorescence. To detect proteasome and PAI-1, cells were treated with polyclonal antibodies to \( \alpha \)-H9251\( \beta \)-proteasome subunit and mAb C-9 to PAI-1. Then the cells were stained with second antibodies labeled with Texas Red rhodamine or fluorescein, respectively. Proteasome was found in whole cytoplasm and the nucleus, showing a strong punctate staining together with a diffuse cytoplasmic localization. PAI-1 was focally enriched in cytoplasmic regions located subjacent to the nucleus, particularly in cells activated with TNF and LPS. Average fluorescence derived from proteasome and PAI-1 when merged revealed perinuclear regions with colocalization of both proteins (Fig. 2).

**Identification of PAI-1-Proteasome Complexes within Endothelial Cells**—We next turned to immunogold electron microscopy to detect direct association of both proteins in endothelial cells. Whole mounts of EA.hy926 cells, control or activated with TNF, and their ultrathin sections were studied using transmission electron microscopy. Numerous 10-nm gold particles representing immunoreactive PAI-1 were found in entire cells both in the cytoplasm and nucleus (Fig. 3 and 4). However, the gold label bound to a particular anti-PAI-1 antibody was quite unequally distributed, with areas of intense labeling alternating with others quite exempt of a given antigen. Furthermore, they formed clusters of gold particles differing in size, particularly in cells activated with TNF. Such clusters were localized in different cellular regions, including those subjacent to nucleus (Fig. 3A), in the vicinity of vesicles and within vesicles (Fig. 3B). Such clusters were not seen in the nucleus (Fig. 3C). Occasionally, gold grains could be seen on heavily staining material at, apparently, intersections of stress fibers or various kind of filaments (Fig. 3D). Clusters of 10-nm gold particles corresponding to anti-PAI-1 co-existed almost always with the smaller size 5-nm gold particles representing immunoreactive proteasome (Fig. 3). These clusters showed different composition and sizes of the PAI-1-proteasome complexes. Their diameter varied from 42 to 115 nm with the average of 89.4 ± 15.4 nm and an average distance of 7 nm (Fig. 3, panels E–J). Unspecific staining was excluded in control experiments without primary antibodies (not shown).

To sum up, these data in situ taken together with the coimmunoprecipitation from cell homogenates (preceding section) strongly suggest that PAI-1 specifically interacts with proteasome within endothelial cells in all cellular regions but the nucleus.

To estimate the specificity of PAI-1-proteasome association, the intermolecular distances between PAI-1 and TGN46, a protein that shuttles between endosomes and the Golgi complex, were determined similarly in EA.hy926 cells ultrathin sections. Fig. 4 displays electron micrographs obtained after immunoreaction with anti-PAI-1 and anti-TGN46 using gold-labeled second antibodies and counterstained with uranyl acetate. In contrast to data shown in Fig. 3, in this experiment PAI-1 was immunolabeled with 5-nm, gold particles, and TGN46 was immunolabeled with 10-nm gold particles.
Double immunogold labeling of both proteins demonstrated that the 5- and 10-nm gold particles were totally separated or at distances >80 nm from each other, indicating no association between PAI-1 and TGN46 (Fig. 4). However, due to the high preservation of cell ultrastructure and the absence of spurious background, these data show that both proteins can be found in the same cellular compartment, i.e. in trans Golgi network.

**Effect of PAI-1 on Proteasome Activity in Endothelial Cells**—To examine whether PAI-1 is involved in the proteasomal degradation of cellular proteins, we took advantage of HeLa cells, as these cells do not express endogenous PAI-1. HeLa cells were transfected with control pCMV or pCMV-PAI-1 for 24 or 48 h, solubilized with 1% Nonidet P-40, and used to investigate the effect of PAI-1 on the expression of proteins targeted for proteasomal degradation. Fig. 5A shows the presence of proteasome and PAI-1 in the co-precipitating proteins by anti-PAI-1 antibody. As evidenced by immunoblotting with anti-α3 and anti-PAI-1 antibodies, both proteins can form a complex also in HeLa cells. Furthermore, this complex reacted with anti-β1 antibody, indicating that PAI-1 bound to the assembled 20 S proteasome. The negative reaction with anti-S4 antibody suggests that 26 S proteasome does not co-immunoprecipitate with PAI-1. Fig. 5B shows expression of proteasome subunits α3, β1, and S4 in lysates of HeLa cells transfected with pCMV and pCMV-PAI-1, which were taken for co-immunoprecipitation experiments.

In unstressed normal cells, p53 usually exists in a hypophosphorylated form at only low levels due to rapid degradation through the ubiquitin-dependent proteasome pathway (15). Fig. 6A shows that in contrast to p53, PAI-1 is not polyubiquitinated in cells treated with proteasome inhibitor, ep-
oxomicin. This was further confirmed after fractionation of cellular proteins by affinity chromatography on the polyubiquitin affinity beads. The majority of p53 appeared to be polyubiquitinated, whereas in the case of PAI-1, polyubiquitination was not detectable. Consistently, PAI-1 expression is not changed in endothelial cells after inhibition of proteasome, whereas p53 is being highly accumulated in the same cells (Fig. 6, B and C). This effect is specific and is not observed when calpain inhibitor II is used in place of epoxomicin.

To further investigate whether expression of PAI-1 might affect proteasome function in cultured cells, we cotransfected both PAI-1 and d2EGFP-N1 into HeLa cells. d2EGFP-N1 is a destabilized variant of the enhanced green fluorescent protein (EGFP). At its C terminus, d2EGFP contains a fragment of mouse ornithine decarboxylase (residues 422–461) with a PEST amino acid sequence that targets the protein for degradation and results in rapid protein turnover. As shown in Fig. 7 the fluorescence of GFP in cells transfected with d2EGFP-N1 (panel B) was lower than that in cells expressing EGFP (panel A), indicating its rapid proteolytic degradation, which can be completely blocked by proteasome inhibitor epoxomicin (panel C). Cotransfection of HeLa cells with pCMV-PAI-1 significantly increased fluorescence intensity in pd2EGFP-N1-transfected cells (panel E) when compared with cells transfected with empty vector pCMV (panel D). This indicates that PAI-1 expressed in HeLa cells prevented targeting of the d2EGFP-N1 sequence for efficient degradation by proteasome. To reconfirm that d2EGFP is effectively degraded by proteasome, the same cells as those shown in panels A–E, which were transfected with pEGFP or pd2EGFP-N1, were analyzed by SDS-PAGE and immunoblotted with anti-EGFP antibodies. As shown in Fig. 7F, increased levels of d2EGFP band were only observed in cells coexpressing d2EGFP and PAI-1 or treated with proteasome inhibitor epoxomicin, indicating that proteasome activity is inhibited effectively by expression of PAI-1. Similar results were obtained when we treated cells expressing d2EGFP with MG132. On the other hand, calpain inhibitor II did not inhibit d2EGFP degradation.

Because this assay suffers from its non-physiological foundation and a failure to demonstrate that the change in the degron is directly related to the interaction of PAI-1 with the...
proteasome in the next experiments, we switched to a more specific analysis. Among well known targets for proteasomal degradation there are p53 and IκBα. Consistent with an earlier observation (20), our present study showed that intracellular PAI-1 protein in endothelial cells activated with TNF or LPS was abundant and appeared in both a diffuse cytosolic pattern and a dense perinuclear location. The latter represented a Golgi apparatus confirming that this structure is an important site for a temporary storage of PAI-1 in endothelial cells. Diffused PAI-1 antigen detected in the cytoplasm of endothelial cells represented the transportation of PAI-1 in undefined vesicles between the Golgi apparatus and plasma membrane. Here we also demonstrate by electron microscopy experiments that PAI-1 is present in the nuclei of endothelial cells. Similar to other serpins such as protein inhibitor 6, protein inhibitor 8, monocyte neutrophil elastase inhibitor, and the viral serpin CrmA, PAI-1 lacks classical nuclear localization signals and is small enough to diffuse through nuclear pores (21). Although most activities of PAI-1 are expressed in extracellular compartments, recent evidence indicates that it is also implicated in different intracellular processes including cell apoptosis. There are conflicting observations showing that, depending upon the type of cells and existing conditions, PAI-1 may act as a pro- or anti-apoptotic factor. In endothelial cells, elevated PAI-1 expression has been associated with increased cell apoptosis (21). In contrast, in vascular smooth muscle cells it has been linked with increased proliferation, probably due to a direct inhibitory effect on the activity of caspase-3 (12, 13). In fact, PAI-1 has been shown to form a high affinity complex with caspase-3, thereby directly inhibiting caspase-3 activity (13). PAI-1 was also associated with increased activity of the FLICE-like inhibitory protein (FLIP), which can disrupt apoptosis mediated by death receptors. This indicates another mechanism by which PAI-1 can protect against apoptosis, thus, promoting proliferation and

**FIGURE 6. PAI-1 is not polyubiquitinated in endothelial cells.** EA.hy926 cells were exposed to epoxomicin for 4 h, then lysed, and protein extracts were applied to polyubiquitin (Ub) affinity beads. Proteins eluted from the beads were analyzed by Western immunoblotting using anti-PAI-1 and anti-p53 antibodies (panel A). Polyubiquitination of PAI-1 is not detectable in total cell extracts obtained from cells treated with proteasome inhibitor or from control cells exposed only to DMSO (mock treatment). Polyubiquitinated PAI-1 is also not seen after separation of these two extracts by affinity chromatography. In contrast, p53 was found in both types of cell extracts and in fractions isolated from the polyubiquitin affinity beads. The presented data are representative of three independent experiments. Panel B shows accumulation of p53 after treatment of cells with proteasome and calpain inhibitors. EA.hy926 cells were incubated with epoxomicin (50 μM) or calpain inhibitor II (100 μM), then cells were harvested after 4 h, lysed in 1% Nonidet P-40, and analyzed by immunoblotting with anti-PAI-1, anti-p53, and anti-β actin antibodies. After immunodetection of PAI-1 and p53, accomplished using an enhanced chemiluminescence kit, films were scanned, and protein bands were quantitated using the Gel Doc 2000 gel documentation system (Bio-Rad). To quantify the densitometric scans, the background was subtracted, and the area for each protein peak was determined. Data were obtained from three separate experiments and normalized to β actin expression (panel C). **, indicates p < 0.001.

**DISCUSSION**

Because its discovery as an inhibitor controlling generation of plasmin (16, 17), PAI-1 has been attributed roles in an array of cellular processes in morphologically and functionally different cells, such as hepatocytes, adipocytes, endothelial cells, smooth muscle cells, and neuronal cells (18). Positive staining of PAI-1 has also been detected in stroma cells at the front of different invasive cancers, indicating its role in metastasis (19). Although PAI-1 is a secreted protein, any intracellular events occurring after it is overexpressed or internalized and is able to interact with intracellular proteins should not be discounted.

Consistent with an earlier observation (20), our present study showed that intracellular PAI-1 protein in endothelial cells activated with TNF or LPS was abundant and appeared in both a diffuse cytosolic pattern and a dense perinuclear location. The latter represented a Golgi apparatus confirming that this structure is an important site for temporary storage of PAI-1 in endothelial cells. Diffused PAI-1 antigen detected in the cytoplasm of endothelial cells represented the transportation of PAI-1 in undefined vesicles between the Golgi apparatus and plasma membrane. Here we also demonstrate by electron microscopy experiments that PAI-1 is present in the nuclei of endothelial cells. Similar to other serpins such as protein inhibitor 6, protein inhibitor 8, monocyte neutrophil elastase inhibitor, and the viral serpin CrmA, PAI-1 lacks classical nuclear localization signals and is small enough to diffuse through nuclear pores (21). Although most activities of PAI-1 are expressed in extracellular compartments, recent evidence indicates that it is also implicated in different intracellular processes including cell apoptosis. There are conflicting observations showing that, depending upon the type of cells and existing conditions, PAI-1 may act as a pro- or anti-apoptotic factor. In endothelial cells, elevated PAI-1 expression has been associated with increased cell apoptosis (21). In contrast, in vascular smooth muscle cells it has been linked with increased proliferation, probably due to a direct inhibitory effect on the activity of caspase-3 (12, 13). In fact, PAI-1 has been shown to form a high affinity complex with caspase-3, thereby directly inhibiting caspase-3 activity (13). PAI-1 was also associated with increased activity of the FLICE-like inhibitory protein (FLIP), which can disrupt apoptosis mediated by death receptors. This indicates another mechanism by which PAI-1 can protect against apoptosis, thus, promoting proliferation and
FIGURE 7. PAI-1 affects proteasome activity in HeLa cells. HeLa cells grown on coverslips were transfected with pEGFP-N1 (panel A) or d2EGFP-N1 (panels B–E) and treated with epoxomicin (panel C) or cotransfected either with a control vector (pCMV, panel D) or the vector expressing PAI-1 (pCMV-PAI-1, panel E). Cells were fixed 24 h after transfection and stained with anti PAI-1 (red) and Hoechst 33342 (blue). In panels A–C, EGFP expression (a) and nuclei stained with Hoechst (b) were merged and shown in c. In panels D and E show expression of PAI-1 in HeLa cells after transfection with pCMV and pCMV-PAI-1, respectively. The same cells were analyzed by Western immunoblotting to detect EGFP degradation in cells transfected with pEGFP-N1 or d2EGFP-N1 in the absence and presence of PAI-1 or the proteasome inhibitor epoxomicin (panel F). The cells were harvested, and immunoblot analyses were performed using anti-GFP and anti-PAI-1 antibodies. Blotting with anti-β actin was used as a loading control. Fluorescent images and Western immunoblotting data shown are representative of those obtained during three separate experiments.
other vascular remodeling processes. The complexity of PAI-1 actions results from its various activities, namely the ability to inhibit \( (a) \) generation of plasmin (22), \( (b) \) cell adhesion mediated by vitronectin (23), and \( (c) \) caspase 3 activity (24) and by \( (d) \) inducing the internalization of urokinase plasminogen activator/receptor and integrin \( \alpha V/\beta 3 \) from the cell surface (25, 26). This variety of activities may be responsible for dual cell-specific biological functions of PAI-1, especially with respect to cellular proliferation or apoptosis. Thus, due to various interactions outside and inside of the cells, PAI-1 can divert intracellular signaling from induction of apoptosis to induction of proliferation (27).

Here, we report an additional mechanism by which PAI-1 may be implicated in affecting the apoptosis proliferation balance in endothelial cells. By yeast two-hybrid screening the cDNA library with the entire PAI-1 molecule as bait, we found the \( \alpha 3 \) subunit of 20 S proteasome, suggesting that both proteins may interact. Then, we confirmed their interaction \( \text{in vivo} \) by \( (a) \) co-precipitation of both proteins with anti-PAI-1 and anti-proteasome antibodies from endothelial cells.

**Figure 8. Effect of PAI-1 overexpression on proteasome activity.** Panel A shows that PAI-1 inhibits degradation of p53 by 20 S proteasome in an \textit{in vitro} assay. In this experiment, recombinant p53 (120 ng) was incubated with 20 S proteasome (120 ng) at 37 °C for 60 min in the presence of the recombinant constitutively active mutant of PAI-1 (120 ng) or epoxomicin (50 µM). Then samples were mixed with Laemmli denaturation buffer, heated at 96 °C for 5 min, and analyzed by Western blotting (IB) using monoclonal antibodies C-9 to PAI-1 and DO1 to p53 or rabbit polyclonal antibodies to \( \alpha 3 \) proteasome subunit. In panel B, to evaluate the inhibitory effect of PAI-1 on proteasome activity \( \text{in vivo} \), EA.hy926 and HeLa cells were transfected with either pCMV or pCMV-PAI-1 for 24 h, lysed, and analyzed by Western blotting using antibodies to PAI-1, p53, \( \alpha 3 \beta 6 \), and \( \beta \) actin. This experiment was repeated three times with similar results. Immunodetection of proteins was accomplished using an enhanced chemiluminescence kit, and films were scanned, and protein bands corresponding to p53 (panel C) and \( \alpha 3 \beta 6 \) (panel D) were quantitated using the Gel Doc 2000 gel documentation system (Bio-Rad). To quantify the densitometric scans, the background was subtracted, and the area for each protein peak was determined and normalized to \( \beta \) actin expression. Results are expressed as the mean ± S.D. of three different experiments performed in duplicate. ** indicates \( p < 0.001 \). Panel E shows that degradation of polyubiquitinated p53 protein is inhibited in cells enriched in PAI-1 as compared with control ones. In this experiment EA.hy.926 cells were transfected with control pCMV or pCMVPAI-1 for 24 h and lysed, and protein extracts were applied to polyubiquitin affinity beads. Proteins eluted from the beads were analyzed by Western immunoblotting using anti-p53 antibodies. Blotting with anti-\( \beta \) actin was used as a loading control.
Binding of PAI-1 to Proteasome

The specificity of this interaction was further supported after (b) transfection of HeLa cells with pCMV-PAI-1 and coimmunoprecipitation of both proteins with anti-PAI-1 antibodies and (c) colocalization experiments using confocal microscopy and, particularly, electron microscopy, which supported an interaction of PAI-1 with proteasome within the cells. Most detected complexes of PAI-1 with proteasome were found in the cytosol diffusing freely within the cells. However, PAI-1 could also be seen to be associated with proteasome tightly bound to cytoskeletal elements, probably to intermediate filaments and actin-myosin complexes previously identified to interact with proteasome (28, 29). Although proteasomes are present throughout the cell, there are indications that at some locations they are more catalytically active, and at certain regions of the cells they form “proteolysis centers” (30, 31). Furthermore, in some regions they form aggregates containing proteasomes and ubiquitinated proteins, termed “aggresomes” (32). Although proteasomes degrade substrates at the proteolytic center under physiological conditions, it is unlikely that they can degrade proteins once they are aggregated and trapped within fully formed aggresomes (31). Formation of aggresomes can be induced by the inhibition of the proteasome but also by overexpression of proteins normally degraded by the proteasome (33). Because PAI-1 was not ubiquitinated in endothelial cells, we can conclude that it induced formation of aggresomes due to inhibition of proteasome activity.

It is noteworthy, that binding of PAI-1 to 20 S proteasome inhibits its activity in vitro when incubated with purified p53. Consistently, the elevated PAI-1 levels abolished degron degradation by proteasome and prevented degradation of p53 and IkBα in EA.hy926 and HeLa cells transfected with pCMV-PAI-1.

The α3 subunit plays a crucial role during proteasome formation as it assembles into a heptameric ring-like structure by itself and, thus, may initiate proteasome complex formation (34). Eukaryotic proteasomes are made up by four rings, each of which contains seven different subunits occurring at specific regions of the cells they form “proteolysis centers” (30, 31). Furthermore, in some regions they form aggregates containing proteasomes and ubiquitinated proteins, termed “aggresomes” (32). Although proteasomes degrade substrates at the proteolytic center under physiological conditions, it is unlikely that they can degrade proteins once they are aggregated and trapped within fully formed aggresomes (31). Formation of aggresomes can be induced by the inhibition of the proteasome but also by overexpression of proteins normally degraded by the proteasome (33). Because PAI-1 was not ubiquitinated in endothelial cells, we can conclude that it induced formation of aggresomes due to inhibition of proteasome activity.

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