Association of Plasminogen Activator Inhibitor Type 2 (PAI-2) with Proteasome within Endothelial Cells Activated with Inflammatory Stimuli

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Quiescent endothelial cells contain low concentrations of plasminogen activator inhibitor type 2 (PAI-2). However, its synthesis can be rapidly stimulated by a variety of inflammatory mediators. In this study, we provide evidence that PAI-2 interacts with proteasome and affects its activity in endothelial cells. To ensure that the PAI-2-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-2 and pCMV-PAI-2 and coinmunoprecipitation of both proteins with anti-PAI-2 antibodies and silencing of the PAI-2 gene using specific small interfering RNA (siRNA). Subsequently, cellular distribution of the PAI-2-proteasome complexes was established by immunogold staining and electron microscopy analyses. As judged by confocal microscopy, both proteins appeared in a diffuse cytosolic pattern, but they also could be found in a dense perinuclear and nuclear location. PAI-2 was not polyubiquitinated, suggesting that it bound to proteasome not as the substrate but rather as its inhibitor. Consistently, increased PAI-2 expression abrogated degradation of degron analyzed after cotransfection of HeLa cells with pCMV-PAI-2 and pd2EGFP-N1, prevented degradation of p53, as evidenced both by confocal microscopy and Western immunoblotting, and inhibited proteasome cleavage of specific fluorogenic substrate. This suggests that PAI-2, in endothelial cells induced with inflammatory stimuli, can inhibit proteasome and thus tilt the balance favoring proapoptotic signaling.

Plasminogen activator inhibitor type-2 (PAI-2) has both extracellular and intracellular functions and acts as a multifunctional protein. It exists predominantly as a 47-kDa nonglycosylated intracellular form. Due to the lack of an N-terminal signal peptide, only a small percentage of PAI-2 is exported from cells, by the nonclassical secretory pathway, as a glycosylated 60-kDa form. PAI-2 contains a unique domain bridging helices C and D, called the C-D loop, which directly interacts with different proteins, including retinoblastoma protein, proteasome subunit member 1/7, interferon response factor 3, ZNF198/FGFR1 fusion kinase, annexins, pre-mRNA processing factor 8, and vitronectin.

The primary physiological function of PAI-2 is thought to be the regulation of plasminogen activators in the extravascular compartment. However, such a role is not supported by studies using PAI-2-/- mice that show no discernable defects in fibrinolysis. Covalent PAI-2-uPA complexes, readily formed in vitro, have also never been unequivocally demonstrated in vivo. On the other hand, PAI-2 has been proposed to play a role in different processes, including tumor metastasis, embryo implantation, and macrophage survival. Consistently, a broad range of activities were ascribed to PAI-2, such as protection of the retinoblastoma protein from degradation, regulation of keratinocyte and monocyte proliferation, and differentiation. Priming interferon α/β responses, inhibition of annexin-1 cleavage, interleukin 1β processing, promotion of adipose tissue development, and the inhibition of apoptosis in some but not all settings.

Recently, we showed that another serpin, PAI-1, decreased proteasome activity in endothelial cells, and thus, it could lead to induction of programmed cell death that is frequently triggered by blocking the ubiquitin proteasome system. Endothelial apoptosis is observed in various physiological and pathological conditions, such as wound healing, scar formation, atherosclerosis, and diabetic eye disease in the adult, as well as in developing capillaries during embryogenesis. In this report, we show that PAI-2 can interact with proteasome in endothelial cells activated with inflammatory stimuli and, upon binding to its β1 subunit, inhibits its activity.

Experimental Procedures

Proteins and Reagents—Mouse monoclonal antibody to 20S proteasome β1 subunit (MCP421), rabbit polyclonal antibody to 20S proteasome β1 subunit (FL241), mouse monoclonal antibody to PAI-2, goat polyclonal antibody to PAI-2 (A-19), rabbit polyclonal antibody to PAI-2 (H-70), mouse monoclonal antibody to p53 (DO-1), HRP-conjugated goat anti-rabbit, rabbit anti-goat, and goat anti-mouse IgG were
from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-β-actin antibodies were from Abcam (Cambridge, UK), AlexaFluor 568-conjugated donkey anti-rabbit antibodies were from Invitrogen, and FITC-conjugated goat anti-mouse antibodies were from Millipore (Billerica, MA). Protein A/G was from ThermoScientific (Rockford, IL), and Mowiol was from Calbiochem/Merck. Epoxomicin and calpain inhibitor II were from Sigma-Aldrich.

**Methods**

**Cell Culture**—Human endothelial cell line EA.hy926 and HeLa cells were obtained from the 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 concentration in cell lysates was measured by the BCA method (ThermoScientific kit).

**RNA Interference of PAI-2 Expression**—For silencing the PAI-2 gene expression, a set of three target-specific 20–25-bp PAI-2 siRNAs (Santa Cruz Biotechnology, Inc.) was used. As a negative control, the functional non-targeting siRNAsc was used, containing four mismatches for any human, mouse, and rat gene. The sequence of its sense strand was 5'-UAGCGACUAACACAUCAAUU-3'. Synthetic siRNAs (30 nM) were transfected to 50–60% confluent HeLa cells with Lipofectamine 2000 (Invitrogen) with detachable chambered upper structures. After an 18-h stimulation with TNF or LPS, they were fixed with ice-cold methanol for 20 min, washed three times with PBS, and incubated with blocking buffer (PBS containing 3% BSA) for 1 h at room temperature. Next, the cells were incubated with mouse monoclonal anti-PAI-2 (American Diagnostica) and rabbit anti-β1 proteasome subunit (FL-241). 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-C1 version 3.6 software.

For degron studies, HeLa cells were transfected with pro2EGFP-N1 or pEGFP-1 and cotransfected with pCMV/HA or pCMV/HA/PAI-2. Cells, 24 h after transfection, 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-2 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-C1 version 3.6 software. In parallel, the same cells were analyzed by Western immunoblotting in terms of the extent of GFP degradation.

**Postembedding 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 three changes (30 min each) of the mixture of LR-White resin (Polysciences, Inc., Warrington, PA) with 100% ethanol (at 1:1, 2:1, and 3:1) on a rotator overnight infiltration with LR-White, and embedding in this resin. After thin sectioning, samples were collected on carbon-Formvar-coated nickel grids (Agar Scientific Ltd., Stansted, UK), wetted in PBS (30 min), and incubated in 1% BSA, 0.1% Tween 20 in PBS for 30 min as a nonspecific blocking agent. Immunostaining was performed on 50-μm droplets in a moist chamber using mAb against human PAI-2 (American Diagnostica Inc., Stamford, CT; at 1:100 dilution) and the rabbit anti-β1 subunit of 20 S proteasome (FI-241, Santa Cruz Biotechnology; at 1:40), which were applied overnight at room temperature. After washing (six times for 5 min each) with 1% BSA, 0.1% Tween 20, PBS (six times for 5 min each), the cells were incubated with the anti-mouse IgG (at 1:20) conjugated to 10-nm colloidal gold particles and anti-rabbit IgG conjugated to 5-nm colloidal gold particles (1:20) for 1 h at room temperature. Immunostaining of PAI-1 and 20 S proteasome α3 subunit was done as described recently (24). After extensive washing (BSA/Tween 20/PBS six times for 5 min each, PBS four times for 5 min each, and double-distilled H₂O for 5 min), specimens were dried overnight. Some specimens were counterstained with 1% uranyl acetate. Ultrastructural analysis of the specimens was carried out with a high-performance biology transmission electron microscope, JEM 1400 (JEOL Co.), equipped with a high resolution digital camera (CCDMORADA, SiS-Olympus) in the Laboratory of
Electron Microscopy (Nencki Institute of Experimental Biology).

**Proteasome Inhibition**—EA.hy926 cells were seeded onto 6-well plates and treated with DMSO (as mock treatment) or proteasome inhibitors: epoxomicin (50 mM) or calpain inhibitor II (25 μM), respectively. After incubation for 4 h, cells were collected and lysed with the Nonidet P-40 buffer, and cellular protein concentrations were measured as described above. Protein aliquots (30 μg) taken from each sample were loaded and separated by SDS-PAGE in 10% gels. After electrophoretic transferring onto PVDF membrane, PAI-2, p53, and control β-actin were detected by polyclonal antibodies A-19, mouse monoclonal DO1, or rabbit polyclonal antibody, respectively.

**Inhibition of 20 S Proteasome Activity by PAI-2**—The activity of purified 20 S proteasome was measured using a 20 S proteasome assay kit (Calbiochem) following the manufacturer's instructions. Briefly, 0.2 μg of purified proteasome was preincubated for 15 min in 100 μl of assay buffer with or without PAI-2 (0.5 μg). Then 5 μM fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC in 100 μl of assay buffer was added, and the fluorescence signal (λ<sub>ex</sub> = 380 nm; λ<sub>em</sub> = 460 nm) was monitored over time.

**Ubiquitination Experiments**—Polyubiquitinated proteins were extracted from cell lysates using the Ubiquitinated Protein Enrichment kit (Calbiochem) according to the manufacturer's instructions. Briefly, EA.hy926 cells were treated with DMSO (as mock treatment) or with epoxomicin (50 μM) for 6 h. Then cells were lysed in the Nonidet P-40-containing buffer, and 500-μg aliquots of protein were incubated with polyubiquitin affinity beads. The extent of PAI-2 ubiquitination was determined by immunoblotting with A-19 polyclonal antibody.

**Statistical Analysis**—All values are expressed as mean ± S.D. compared with controls and among separate experiments.

![Figure 1. Direct interaction of PAI-2 with proteasome in endothelial cells.](image-url)
Paired and unpaired Student’s t tests were employed to determine the significance of changes. A p value of <0.05 was considered statistically significant.

RESULTS

Interaction of PAI-2 with Proteasome—In preliminary experiments, we show, consistent with an earlier observation (27), that in unstimulated human endothelial cells, PAI-2 expression is low or undetectable but markedly increases after inflammatory stimuli (Fig. 1, A, input). It is represented predominantly by the nonglycosylated 43-kDa form. Because PAI-2 was recently described to interact with the β1 protein subunit (7), next, to identify PAI-2/proteasome complexes in endothelial cells, the EA.hy 926 cell lysates were incubated either with monoclonal antibody to the β1 proteasome subunit or to PAI-2. Lysates incubated with nonimmune control IgG were used as control. Immunoprecipitates were then isolated using a 50% slurry of protein A/G-agarose overnight at 4 °C. Specifically bound proteins were solubilized in 40 μl of Laemmli sample buffer and separated by SDS-PAGE in 10% gels under reducing conditions. Indeed, endogenous proteasome co-immunoprecipitated from cell extracts with PAI-2 (Fig. 1). After scanning coimmunoprecipitated PAI-1, proteasome β1 subunit and corresponding inputs, it can be seen that after activation of cells with LPS (20 μg/ml), about 20% of total PAI-2 or proteasome β1 subunit is coimmunoprecipitated. Up-regulation of PAI-2 expression in endothelial cells upon activation with inflammatory stimuli was further evidenced by immunofluorescence (Fig. 1).

FIGURE 2. Ultrastructural visualization of PAI-2/proteasome complexes by immunogold labeling. EA.hy926 cells were stimulated with TNF (20 ng/ml), fixed, and thin sectioned. Samples of cells were treated overnight with anti-PAI-2 monoclonal antibody and rabbit anti-20 S proteasome β1 subunit antibodies (Fl-241). Then the cells were incubated with second antibodies conjugated with colloidal gold particles, 10 and 5 nm, respectively. A and B show a representative distribution of 10 nm (PAI-2) and 5 nm (proteasome) gold particles within control endothelial cells and TNF-stimulated endothelial cells, respectively. Scale bar, 200 nm (50 nm in insets). Arrows show gold particles corresponding to PAI-2. C–G show examples of colocalization of both proteins in TNF-stimulated endothelial cells (C, scale bar, 100 nm; D, E, and G, scale bar, 50 nm) or LPS-stimulated endothelial cells (F, scale bar, 50 nm). H, a representative colocalization of 10- and 5-nm gold particles within control and TNF-activated endothelial cells. I compares the shortest distance that separates 10- and 5-nm gold particles in control and TNF-activated cells. Data are shown as the mean distance measured between 100 large and small gold particles ± S.D. (error bars). **, p < 0.001.

FIGURE 3. Comparison of PAI-2/proteasome and PAI-1/proteasome complexes in TNF-stimulated endothelial cells. Panels A and B and panels C and D show complexes of PAI-1 with 20 S proteasome α3 subunit and PAI-2 with β1 proteasomal subunit, respectively, indicating different stoichiometries (10-nm gold particles correspond to PAI-1 or PAI-2, whereas 5-nm particles correspond to proteasome). These pictures are representative of a number of cells analyzed during three separate experiments. Scale bar, 50 nm. E compares diameters of more than 40 relevant complexes observed in cells analyzed during three different experiments, expressed as the mean ± S.D. (error bars). **, p < 0.001.
Binding of PAI-2 to Proteasome

FIGURE 4. PAI-2 protects p53 against degradation by proteasome in endothelial cells treated with TNF and LPS. EA.hy926 cells were exposed to the epoxomicin for 4 h and lysed, and protein extracts were applied to polyubiquitin affinity beads. Proteins eluted from the beads were analyzed by Western immunoblotting using anti-PAI-2 or anti-p53 antibodies. Polyubiquitin-activation of PAI-2 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-2 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 (A). B, accumulation of p53 and IκBα after activation of cells with LPS and their treatment with proteasome and calpain inhibitors. EA.hy926 cells were incubated with epoxomicin (50 μM) or calpain inhibitor II (100 μM) or stimulated with TNF (20 ng/ml) or LPS (20 μg/ml) for 18 h, and then cells were harvested, lysed in 1% Nonidet P-40, and analyzed by immunoblotting with anti-PAI-2, anti-p53, anti-IκBα, and anti-β-actin antibodies. After immunodetection of PAI-2, p53, and IκBα, accomplished with 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 corresponding to IκBα or p53 was determined. Data were obtained from three separate experiments and normalized to β-actin expression (C and D). **, p < 0.001. E–G, show that down-regulation of PAI-2 expression in EA.hy926 cells by specific siRNA abolishes p53 accumulation induced by TNF or LPS. In E, aliquots of EA.hy926 cells collected before or after activation with TNF (20 ng/ml) or LPS (20 μg/ml), treated either with a set of three siRNAs specific to PAI-2 mRNA (siRNA_PAI2) or control non-targeting siRNAss. Synthetic siRNAs (30 nm) were transfected to 50–60% confluent EA.hy926 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The same siRNAs were analyzed by Western immunoblotting using antibodies to PAI-2, p53, and β-actin. Blotting with nonimmune IgG was also performed (not shown), F, quantitative data presented in E when analyzed as described in the legend to C and D. Error bars, S.D.

low, but it significantly increased upon activation of cells with TNF and LPS. Silencing of the PAI-2 gene with siRNA almost abolished the PAI-2 expression, proving the specificity of the staining. In resting cells, both PAI-2 and proteasome were mostly found in cytoplasmic regions subjacent to the nucleus, showing a strong punctate staining. Upon activation with TNF or LPS, both proteins could be detected in whole cytoplasm and the nucleus.

Thus, the presence of the PAI-2-proteasome complex in cells activated with LPS or TNF was evidenced by (a) blotting with anti-PAI-2, when the immunoprecipitate was pulled down with antibodies to β1 proteasome subunit (Fig. 1A), and (b) blotting with antibodies to β1 proteasome subunit, when the immunoprecipitate was pulled down with mAb to PAI-2 (Fig. 1B).

Identification of PAI-2–Proteasome Complexes within Endothelial Cells—We next turned to immunogold electron microscopy to detect 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-2 were found in entire cells both in the cytoplasm and nucleus after activation with TNF or LPS (Fig. 2), and the majority of them were separated from 5-nm gold particles corresponding to proteasome in control cells. However, there were areas of intense labeling alternating with others quite devoid of a given antigen. Furthermore, they formed clusters of gold particles differing in size, particularly in cells activated with TNF (Fig. 2, C–E and G) or LPS (Fig. 2, B and F). The large and small gold particles were separated by 8.98 ± 5.85 nm in such clusters compared with 92.74 ± 62.95 nm for the shortest distance between 10- and 5-nm gold particles in control cells (Fig. 2, H and I). These clusters showed different composition and sizes of the PAI-2-proteasome complexes. Their diameter varied from 31 to 168 nm with an average of 106.1 ± 12.4 nm (Fig. 3). When compared with those formed by PAI-1, complexes of PAI-2 with proteasome are not so dense, consist of fewer molecules of both proteins, and are characterized by larger diameters. Nonspecific 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, strongly suggest that PAI-2 specifically interacts with proteasome within endothelial cells in all cellular regions.
Effect of PAI-2 on Proteasome Activity in Endothelial Cells—Normally, in resting cells, p53 usually exists in a hypophosphorylated form at low concentrations due to rapid degradation through the ubiquitin-dependent proteasome pathway (28). Fig. 4 showed that, in contrast to p53, PAI-2 was not polyubiquitinated in cells treated with the proteasome inhibitor, epoxomicin. 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-2, polyubiquitination was not detectable. Consistently, PAI-2 expression was not changed in endothelial cells after inhibition of proteasome, whereas p53 and IκBα were greatly accumulated in the same cells (Fig. 4B). This effect is specific and is not observed when calpain inhibitor II is used in place of epoxomicin. The increased PAI-2 expression in EA.hy926 observed upon treatment with TNF or LPS was accompanied by accumulation of both p53 and IκBα (Fig. 4C and D). To confirm that this is via PAI-2 levels, in the next experiments, we silenced the PAI-2 gene with specific siRNA in TNF- or LPS-activated EA.hy926 cells. Then PAI-2 and p53 levels in whole cell lysates, collected 24, 48, and 72 h after transfection, were analyzed by Western immunoblotting. Fig. 4, E and F, shows that the silencing of PAI-2 expression in stimulated cells by TNF and LPS is associated with drastic reduction of the p53 level, confirming a role of PAI-2 in protection of p53 against degradation.

PAI-2 Inhibits in Vivo and in Vitro Proteasome Activity—To further examine whether expression of PAI-2 might affect proteasome function in cultured cells, next we took advantage of HeLa cells because these cells do not express endogenous PAI-2. For this purpose, both PAI-2 and d2EGFP-N1 were cotransfected into HeLa cells. d2EGFP contains 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. 5, the fluorescence of GFP in cells transfected with d2EGFP-N1 (Fig. 5B) was lower than that in cells expressing EGFP (Fig. 5A), indicating its rapid proteolytic degradation, which could be completely blocked by the proteasome inhibitor epoxomicin (Fig. 5C). Cotransfection of HeLa cells with pcMV-PAI-2 significantly increased fluorescence.

FIGURE 5. PAI-2 inhibits d2EGF-N1 degron degradation. HeLa cells grown on coverslips were transfected with pEGFP-N1 (green) (A) or d2EGFP-N1 (green) (B–E) and treated with epoxomicin (C) or cotransfected either with a control vector (pcMV) (D) or the vector expressing PAI-2 (pcMV-PAI-2) (E). Cells were fixed 24 h after transfection and stained with anti-PAI-2 (red) and Hoechst 33342 (blue). In A–C, EGFP expression (pictures a) and nuclei stained with Hoechst (pictures b) were merged and shown in pictures c. Pictures b in D and E show nuclei stained with Hoechst and d2EGFP-N1 expression, whereas pictures c show expression of PAI-2 in HeLa cells after transfection with pcMV and pcMV-PAI-2, 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 or presence either PAI-2 or the proteasome inhibitor epoxomicin (F). The cells were harvested and immunoblot analyses were performed using anti-GFP and anti-PAI-2 antibodies. Blotting with anti-β-actin was used as a loading control. G, quantitative data obtained and analyzed as described in the legend to Fig. 4, C and D. Fluorescent images and Western immunoblotting data shown are representative of those obtained during three separate experiments. Error bars, S.D.
cence intensity in pd2EGFP-N1-transfected cells (Fig. 5E) when compared with cells transfected with empty vector pCMV (Fig. 5D). This indicates that PAI-2 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 Fig. 5, 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. 5, F and G, increased levels of the d2EGFP band were only observed in cells coexpressing d2EGFP and PAI-2 or treated with proteasome inhibitor epoxomicin, indicating that proteasome activity is inhibited effectively by expression of PAI-2. On the other hand, calpain inhibitor II did not inhibit d2EGFP degradation (not shown).

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-2 with the proteasome, in the next experiments, we switched to more specific analysis. HeLa cells were transfected with control pCMV or pCMV-PAI-2 for 24 h, solubilized with 1% Nonidet P-40, and used to investigate the effect of PAI-2 on the expression of p53 targeted for proteasomal degradation. Fig. 6, A–C, shows the presence of PAI-2 in transfected HeLa cells analyzed by confocal microscopy and Western immunoblotting. The increased PAI-2 expression in HeLa cells transfected with pCMV-PAI-2 inhibited degradation of p53, as demonstrated by its significant accumulation within the cells (Fig. 5C). This indicates that the elevated PAI-2 in endothelial cells contributes to protection of natural proteasome substrates against proteolytic degradation. Such an inhibitory effect of PAI-2 on proteasome was further confirmed by in vitro experiments showing that due to direct interaction, PAI-2 inhibits 20 S proteasome chymotrypsin-like activity when tested using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (Fig. 6, D and E). The decrease in proteasome activity observed in vitro in the presence of PAI-2 is statistically significant (p < 0.001).

DISCUSSION

The major finding in this study is that PAI-2 and proteasome can form a complex inside of endothelial cells, and upon binding, PAI-2 affects proteasome activity. Because proteasome inhibition in most cell types has been shown to sway the balance favoring proapoptotic signaling, we believe that the up-regulated PAI-2 in endothelial cells by modulating proteasome activity may promote apoptosis. Specificity of PAI-2 interaction with proteasome in vivo was demonstrated by (a) co-precipitation of both proteins with anti-PAI-2 and anti-proteasome antibodies from endothelial cells and finally proved by (b) colocalization experiments using electron microscopy, which supported an interaction of PAI-2 with proteasome within the cells. Most detected complexes of PAI-2 with proteasome were found in the cytosol diffusing freely within the cells. Because PAI-2 was not ubiquitinated in endothelial cells, we conclude that it bound to proteasome not as a substrate but as its inhibitor. Consistently, the elevated PAI-2 levels abolished degron degradation by proteasome and prevented degradation of p53 in EA.hy926 and HeLa cells transfected with pCMV-PAI-2. This was further supported by observations that PAI-2 inhibited 20 S activity in vitro when incubated with the fluorogenic substrate.

The mechanism by which PAI-2 inhibits proteasome activity is unclear. PAI-2 directly interacts with the catalytic β1 subunit (7), constituting together with other β subunits the inner ring of proteasome, which is accessible only via the gate region. Moreover, in contrast to PAI-1, PAI-2 does not induce formation of aggresomes (24). As judged by immunogold staining and electron microscopy, PAI-2 complexes with proteasome are much larger than those of PAI-1 and contain fewer molecules of both proteins. Because PAI-2 inhibits 20 S proteasome activity tested in vitro using the fluorogenic substrate, it is likely that it is able to interact preferentially with a population of more catalytically active proteasomes located in “proteolysis centers” of cells (29).

Disregulated PAI-2 expression or PAI-2 polymorphisms have been associated with a number of diseases involving inflammation. Decreased PAI-2 expression was associated with pre-eclampsia (30), whereas expression was up-regulated in
asthma (31), periodontal disease (32), and hyperkeratotic corn tissue (33). PAI-2 polymorphisms have also been associated with antiphospholipid syndrome (34), lupus (35), and myocardial infarction in some studies (36) but not others (37), and all of these diseases have been associated with increased Th1 responses (29, 38). The ability of PAI-2 to inhibit the proteolytic activity of proteasome may explain the significance of this serpin for controlling the Th1-promoting cytokine production by macrophages. The phenotype of PAI-2 characterizes macrophages, was mapped to enhanced CD40 and NF-κB signaling (39). Because both CD40 and IκBα have natural substrates of proteasome, it is reasonable to predict that PAI-2, by interaction with proteasome, can protect them from degradation, thereby promoting signaling pathways in which these proteins participate. It can be assumed that PAI-2, after up-regulation of its expression, induces apoptosis by inhibiting proteasome and thus preventing degradation and/or processing of specific regulatory proteins.

In summary, PAI-2 is distributed between the cytosol and the nucleus of endothelial cells. In some cellular compartments, PAI-2 interacts with proteasome and inhibits its activity. Thus, PAI-2 can influence intracellular anti- and proapoptotic balance, promoting signaling and leading to apoptosis.

Acknowledgments—We acknowledge the expertise of R. Bartosiewicz in acquisition of digital images in electron microscopy and M. Osinska in specimen counterstaining.

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