Proteinase Inhibitor 6 Cannot Be Secreted, Which Suggests It Is a New Type of Cellular Serpin

(Received for publication, September 29, 1995)

Fiona L. Scott, Paul B. Coughlin, Catherina Bird, Loretta Cerruti, John A. Hayman, and Phillip Bird

From the Department of Medicine, Monash Medical School, Clive Ward Centre, Box Hill Hospital, Box Hill 3128, Australia and the Department of Pathology, Box Hill Hospital, Box Hill 3128, Australia

We have recently described a new serine proteinase inhibitor, proteinase inhibitor 6 (PI-6). This serpin has features that suggest it may function intracellularly, but its close resemblance to ovalbumin serpins like plasminogen activator inhibitor 2 (PAI-2) raises the possibility that it is secreted to regulate an extracellular proteinase. To determine whether PI-6 is secreted, we have examined its cellular distribution by immunohistochemistry and have attempted to induce its release from platelets and from cultured cells. We find that PI-6 is present in endothelial and epithelial cells, but it is apparently cytoplasmic and it is not released from cells in response to phorbol ester, dibutyryl cAMP or tumor necrosis factor-α treatment. It is also not released from activated platelets. The addition of a conventional signal peptide to the amino terminus of PI-6 directed its translocation into the endoplasmic reticulum (ER), resulting in glycosylation but not secretion of the molecule. By contrast, the addition of the same signal peptide to PAI-2 markedly enhanced its translocation and secretion. Glycosylated PI-6 was sequestered in the ER and was incapable of interacting with thrombin. The failure of PI-6 to move along the secretory pathway, and the loss of inhibitory function of ER-localized PI-6, demonstrates that unlike PAI-2, PI-6 is not naturally secreted. Taken together, these results suggest that PI-6 has evolved to fulfil an intracellular role and that it represents a new type of cellular serpin.

Serine proteinase inhibitors (serpins) are a family of structurally related proteins that regulate the activity of serine proteinases involved in extracellular processes such as coagulation, fibrinolysis, complement fixation, and embryo implantation. Several members of the family have lost proteinase inhibitory function and have evolved extracellular functions such as serving as lipophilic molecule transporters and peptide hormone precursors (1).

Recently, we and others have identified a new Arg-serpin known as proteinase inhibitor 6 (PI-6), 1 the placental thrombin inhibitor, or the cytoplasmic anti-proteinase (2, 3). Although PI-6 efficiently inhibits the extracellular proteinases plasmin, trypsin, thrombin, and urokinase in vitro (4), it is unusual because it is present in cytosolic extracts, it is not found in the medium of cultured cells, it lacks a conventional signal sequence, and it is sensitive to oxidation (2, 5). These properties suggest that PI-6 may have an intracellular function. At present, the only serpin with a clearly defined intracellular role is the viral protein crmA, which is an inhibitor of granzyme B and the interleukin-1β-converting enzyme (6, 7).

PI-6 closely resembles the ovalbumin serpins. This group of proteins includes ovalbumin, plasminogen activator inhibitor 2 (PAI-2), the squamous cell carcinoma antigens (SCCA-1 and SCCA-2), maspin, and the monocyte neutrophil elastase inhibitor (8, 9). All of the ovalbumin serpins lack conventional signal sequences, yet they are found as extracellular glycoproteins. At least two of these ovalbumin serpins, PAI-2 and SCCA, appear to exist mainly as cytosolic proteins but are efficiently secreted and glycosylated in response to specific stimuli. For example, glycosylated PAI-2 is released from monocytes in response to tumor necrosis factor-α and phorbol ester treatment (10), and SCCA is released from transformed squamous epithelial cells (11). Thus it cannot be inferred from the lack of a conventional signal sequence and an apparent cytosolic location that PI-6 is confined intracellularly or that it has an intracellular function.

To determine if PI-6 is released to function in the extracellular milieu, we have examined its cellular distribution using immunohistochemistry and have attempted to induce its secretion from cultured cells and platelets. Furthermore, we have provided it with a conventional signal sequence to assess whether it can be efficiently glycosylated and released if directed into the secretory pathway. We find that PI-6 is located in endothelial cells, in platelets, and in a subset of epithelial cells but that it is not released from activated platelets nor from cultured cells in response to tumor necrosis factor-α, phorbol ester, or cAMP analogues. PI-6 directed into the secretory pathway is glycosylated but loses inhibitory activity and is retained in the endoplasmic reticulum. On the basis of these studies, we conclude that PI-6 is not naturally secreted and that it is a true intracellular serpin.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 and K562 cells were maintained as described previously (5). U937 cells were maintained as for K562 cells. COS-7 cells were transfected using the DEAE-dextran/chloroquine method as described (12).

Antibodies and Histology—Rabbit anti-aminoglycoside 3'-phosphotransferase (NEO) antibodies were purchased from 5 Prime-3 Prime, Inc. The anti-PAI-2 monoclonal antibody was from the American Diagnostics. The anti-PI-6 antisera used in the secretion, pulse-chase and immunofluorescence experiments has been described previously (2). For the histological experiments, new rabbit anti-PI-6 antibodies were prepared as before, except that the antigen was recombinant PI-6.
produced in a yeast expression system (4). Preparation of 4-μm paraffin-embedded tissue sections and immunohistochemical staining was as described previously (13), except that 3-amino-9-ethylcarbazole (stock solution 0.4% (v/v) in formamide) was used as the developing reagent (DAKO). Briefly, sections were dewaxed and blocked in 3% (v/v) hydrogen peroxide followed by 10% (v/v) horse serum in phosphate-buffered saline (PBS). Sections were then incubated for 1 h in an empirically determined dilution of the primary antibody (typically 1:200), washed in PBS, and then incubated for 20 min in a 1:100 dilution of biotinylated swine anti-rabbit immunoglobulins (DAKO E353). Following a further wash in PBS, streptavidin-horseradish peroxidase (DAKO K377) was added for 30 min. Sections were washed again in PBS and developed using diaminobenzidine freshly diluted to 6% (w/v) in PBS with 0.05% (v/v) formamide stock solution into 0.1× acetate buffer (pH 5.2). Slides were counterstained and mounted in Crystal/Mount (Biomeda Corp.).

To control, serial sections were incubated with a similar dilution of non-immune rabbit serum as primary antibody.

Thrombin—Thrombin was prepared from prothrombin purified from human plasma (14). Iodinations, and estimations of its concentration and activity were performed exactly as described previously (2).

Assay for Secretion of PI-6 from Cultured Cells—Approximately 1 × 10⁹ COS-7, K562, or U937 cells were treated for 24 h with 25 ng/ml of phorbol 12-myristate 13-acetate (Sigma), 50 ng/ml of human tumor necrosis factor α (Boehringer Mannheim), or 1 ml of dibutyl cAMP (Sigma). Cells were separated from the medium by centrifugation at 500 g for 10 min, then washed twice in PBS, pH 7.4, and resuspended in 1 ml of 10% (v/v) protein A-Sepharose (Pharmacia Biotech Inc.). After incubation overnight at 4°C, the immunocomplexes were collected, washed twice in NETGEL containing 250 mM NaCl, 0.025% (w/v) SDS, and once in 10 mM Tris, pH 8.0. Samples were resuspended in 30 μl of 20 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1 M dithiothreitol (reducing buffer), and then analyzed by SDS-PAGE according to Laemmli (15) followed by autoradiography.

Immunoprecipitation and Activation of Platelets—Human platelet-rich plasma from 40 ml of blood were prepared and washed as described previously (16). 5 ml of a platelet suspension containing 4 × 10⁹ cells/ml was divided into three parts and treated as follows. (i) 250 μl were activated by the addition of iodinated thrombin (5 ng/ml) for 10 min at 37°C. (ii) 25 μl of the suspension were pelleted, resuspended in 2.5 ml Tyrode's buffer (140 mM NaCl, 1.3 mM KCl, 0.2 mM MgCl₂, 24 mM NaHCO₃, 5 mM Heps, 5.5 mM glucose, and 0.025% (w/v) SDS), and then analyzed by SDS-PAGE. The disrupted platelets were centrifuged at 100,000 × g for 1 h, and 250 μl of the supernatant were incubated with 5 μl iodinated thrombin for 10 min at 37°C. (iii) 2.25 ml of platelets were treated as in (ii) but were not activated with thrombin (5 ng/ml) for 10 min at 37°C and then treated with 1 μM dithiothreitol (reducing buffer). The samples were then loaded onto a 10% (v/v) polyacrylamide gel directly to analyze immunoprecipitated and activity were performed exactly as described previously (13), except that 3-amino-9-ethylcarbazole was used as the developing reagent (DAKO). Briefly, sections were dewaxed and blocked in 3% (v/v) hydrogen peroxide followed by 10% (v/v) horse serum in phosphate-buffered saline (PBS). Sections were then incubated for 1 h in an empirically determined dilution of the primary antibody (typically 1:200), washed in PBS, and then incubated for 20 min in a 1:100 dilution of biotinylated swine anti-rabbit immunoglobulins (DAKO E353). Following a further wash in PBS, streptavidin-horseradish peroxidase (DAKOC377) was added for 30 min. Sections were washed again in PBS and developed using diaminobenzidine freshly diluted to 6% (w/v) in PBS with 0.05% (v/v) formamide stock solution into 0.1× acetate buffer (pH 5.2). Slides were counterstained and mounted in Crystal/Mount (Biomeda Corp.).

Intracellular Location of PI-6

The demonstration of PI-6 staining in endothelial and epithelial cells was performed and activity were performed exactly as described previously (2).

RESULTS

Tissue Distribution of PI-6—Our previous studies of human and mouse tissue sections have shown that PI-6 mRNA is present in many embryonic and adult organs (5, 20). To identify cells that synthesize PI-6, we carried out an immunohistochemical survey of human adult tissues. Affinity-purified anti-PI-6 antibodies were used with standard methods to probe sections of a variety of tissues including skin, breast, uterus, placenta, testes, skeletal muscle, bone marrow, lung, bowel, and liver. From this analysis, it appeared that PI-6 is synthesized predominantly in capillary endothelial cells and in epithelial cells such as those forming the spinous layer of the epidermis, forming hair follicles, sweat gland secretory ducts, endometrial glands, mammary intralobular ducts, testicular seminiferous tubules, and liver bile ducts. It was also observed in the syncytiotrophoblast of placenta. In all of these cells, PI-6 staining appeared to be cytoplasmic, with no staining of membranes or intercellular bridges. The pattern of PI-6 expression is illustrated in Fig. 1, which shows a section of human dermis. Here PI-6 is evident in the small blood vessels and in the differentiated epithelial cells of the sweat gland ducts but not in the gland itself.

The demonstration of PI-6 staining in endothelial and epithelial cells accounts for the wide distribution of PI-6 previously observed by RNA analysis. The presence of PI-6 in these cells is also consistent with a model for PI-6 function in which it is released by epithelial or endothelial cells to participate in the regulation of extracellular proteinases. In this respect, it might resemble the closely related serpin, PAI-2, which is released to regulate urokinase (10). To test if PI-6 is normally released following synthesis or on stimulation of particular cells, we examined its production in a number of systems in which regulated or constitutive release might occur.

Pulse-Chase Experiments—At 48 h posttransfection, 2 × 10⁶ COS cells were washed once in PBS and placed in warm serum-free RPMI 1640 medium lacking methionine. After 30 min, the medium was replaced with warm serum- and methionine-free RPMI 1640 medium containing 100 μCi of [³⁵S]methionine and cysteine (Exper 253PS protein labeling mix, DuPont NEN). The labeling was terminated after 30 min by changing the medium, by either collecting the medium and lysing the cells or by replacing the labeling medium with warm Dulbecco's modified Eagle's medium containing 10% (v/v) NuSerum (Collaborative Research Inc.). In the latter case, the chase (chase phase) was continued for a specified time then terminated by collecting the medium and lysing the cells. Medium and cell extracts were prepared and immunoprecipitated using the appropriate anti-serum and protein A-Sepharose as described above. 1 μg of thrombin was added to some samples immediately prior to immunoprecipitation. Immunoprecipitates were analyzed by reducing SDS-PAGE. Gels were enhanced in Amplify (Amersham Corp.), and the samples were visualized by fluorography.

Endoglycosidase and Tunicamycin Treatments—Immunoprecipitates from transfected, labeled COS cells were resuspended in 34 μl of 0.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol and boiled for 10 min. The sample was split in two, 2 μl of 0.5 mM sodium citrate pH 5.5 was added to each portion followed by 1 μl (1000 units) of endoglycosidase H, (New England Biolabs) to one portion only. After 1 h at 37°C, the samples were analyzed by SDS-PAGE and fluorography.

Tunicamycin (10 μg/ml, Boehringer Mannheim) was added to the medium of transfected COS cells 18 h before labeling commenced and was included throughout the labeling procedure.

Indirect Immunofluorescence—Transfected COS cells were prepared for analysis by indirect immunofluorescence as described previously (19).

Plasmids—The PI-6 expression vector pSVTII/PT/P is described in Coughlin et al. (5). The plasmids pSVHA/NEO and pSVH/NEO are described in Ref. 17. The plasmid pSVHA/PI-6 was constructed as follows. A mutagenic oligonucleotide 5′-GCCATGATCATCTTTCGCGTCT-3′ was synthesized that removes the initiation codon of PI-6 to form a BglII site and substitutes Val2 with Leu (Bresatec, Australia). 20 pmol of this oligonucleotide and 20 pmol of a T3 primer (Promega) were used in a PCR, which also included 5 ng of a PI-6 DNA template (PTI/P cDNA 5 cloned into Bluescript II KS (Strategene)). Amplification was performed using the proof-reading Vent polymerase (1 unit) under the reaction conditions (New England Biolabs). 30 cycles of 95°C for 90 s, 45°C for 60 s, 70°C for 180 s were performed. The amplified fragment was cloned into pCR/TA (Invitrogen) and sequenced completely to verify the presence of the desired alteration and to rule out second site mutations. A BglII-XbaI fragment containing the modified PI-6 DNA was then separated from the pCR/TA vector fragment by digestion with BglII and XbaI. The resulting plasmid was sequenced to verify an in-frame fusion of the pSHT HA signal sequence and the PI-6 cDNA formed by ligation of the compatible BamHI and BglII ends.

The PAI-2 expression plasmid pEUKPAI-2 (a gift of Dr. R. Medcalf) consists of the human PAI-2 cDNA cloned into pEUK-C1 (Clontech). A PAI-2 derivative containing the HA signal sequence was similarly constructed as a similar manner to pSVHA/PI-6, PCR primers 5′-ATGAGCATTCT-TGTGTG-3′ (sense) and 5′-GGACTGTAGGTGGACGCAAATCT-3′ (antisense) were designed to amplify the coding sequences of PAI-2. The sense primer inserts a BamHI site near the initiation codon and substitutes Leu4 with Pro. The antisense primer inserts an SpeI site just after the termination codon. Following amplification with Vent polymerase, the fragment was cloned into pCR/TA for verification, released by BamHI-SpeI digestion and ligated to pSHT deawed with BamHI and SpeI.


We have previously noted the presence of PI-6 mRNA in the megakaryoblastic cell line, MEG-01 (5), and we have shown that these cells contain an SDS-resistant thrombin-complexing activity that is immunoprecipitable with anti-PI-6 antibodies. The presence of PI-6 in MEG-01 cells suggests that it may also be present in platelets. To test this, we lysed human platelets by freeze-thawing, incubated aliquots of the lysate with iodinated thrombin, and tested for the presence of an SDS-resistant complex by reducing SDS-PAGE and autoradiography. As shown in Fig. 2 (lane 2), platelet lysate contains two SDS-resistant thrombin complexes, the smaller of which is immunoprecipitable by anti-PI-6 antibodies (Fig. 2, lane 5). The larger complex was not immunoprecipitable (Fig. 2, lane 4), and almost certainly consists of thrombin bound to protease nexin I, which is a well characterized and potent thrombin inhibitor contained in platelet α-granules (21–23).

To test whether PI-6 is released on platelet activation, we stimulated platelets with iodinated thrombin to cause release of the granule contents and then separated the platelets from the releasate. The activated platelets were then subjected to lysis by freeze-thawing to prepare cytosolic extracts, which were incubated with a fresh aliquot of iodinated thrombin. All of the samples were then reduced and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2 (lane 1), the releasate from activated platelets contained the larger complex, which was not immunoprecipitable using anti-PI-6 antibodies (lane 4). By contrast, the cytosol of the activated platelets contained the smaller complex (lane 3), which was immunoprecipitable (lane 6). These results demonstrate that PI-6 is present in platelet cytosol but that its secretion is not induced on platelet activation.

PI-6 Is Not Released by Resting or Stimulated Cultured Cells—We have examined a number of cultured cell lines by RNA analysis, indirect immunofluorescence, or thrombin-com-
platelets generated a smaller complex (lane 6). Species was immunoprecipitable (lane 4). Cytosol from activated platelets generated a complex (lane 3) that was immunoprecipitable with PI-6 antibodies (lane 6). Total lysate from unstimulated platelets generated both complexes (lane 2), but only the smaller species was immunoprecipitable (lane 5).

Immunoprecipitation assays for the presence of PI-6. These include primary human umbilical vein endothelial cells; the human lines HeLa, HepG2, HT1080, K562, U937, and THP1; the simian line, COS-7; and the murine lines SP2, Balb/c 3T3, F9, E14, and STO. With the exception of THP1, all of these cells produce PI-6 (data not shown). We have tested conditioned media from most of these cells for PI-6 activity by the thrombin complexing assay or for PI-6 antigen by immunoblotting and have not detected any evidence for PI-6 release into the medium.

By analogy to the situation for PAI-2 or SCCA, we considered the possibility that PI-6 is only released into the medium in response to a specific signal. To test this, we tested K562 cells, U937 cells, or COS-7 cells with inducers of the protein kinase C signal transduction pathway (phorbol 12-myristate 13-acetate) and to the culture supernatant (M) prior to immunoprecipitation with anti-PI-6 antibodies. Immune complexes were collected and analyzed by 10% SDS-PAGE and autoradiography.

Fig. 3. PI-6 activity (thrombin complexing ability) is not detected in media conditioned by agonist-treated cell lines. Cells were untreated (−ve) or treated for 24 h with 25 ng/ml phorbol 12-myristate 13-acetate (PMA), 50 ng/ml tumor necrosis factor α (TNF), or 1 mM dibutyl cAMP. Iodinated thrombin was added to the cell lysates (C) and to the culture supernatant (M) prior to immunoprecipitation with anti-PI-6 antibodies. Immune complexes were collected and analyzed by 10% SDS-PAGE and autoradiography.

Fig. 4. Biosynthesis of human PI-6 in transfected COS cells. COS cells were transfected with pSVTPI/T1/P DNA. 48 h posttransfection, cells were starved for 30 min in media lacking methionine, labeled with 100 μCi of [35S]methionine and then incubated in complete media for the indicated times. Cell extracts and media samples were prepared at each time point and immunoprecipitated with PI-6 antibodies. Immune complexes were collected, reduced, and analyzed by 10% SDS-PAGE and fluorography.

As shown in Fig. 5, a derivative of PI-6 (HA/PI-6) containing the influenza virus HA signal sequence fused to the amino terminus of PI-6 was constructed by PCR-mediated mutagenesis of PI-6 and in-frame cloning into the expression vector, pSV40 (18). This vector provides the SV40 early promoter followed by the HA signal sequence, cloning sites, and termination codons. A similar derivative of PAI-2 (HA/PAI-2) was constructed as a control (Fig. 5). (Although it is predominantly cytosolic, PAI-2 is known to be capable of travelling through the intracellular compartment.)
conventional secretory pathway (25, 26), and the efficiency with which it enters the ER can be enhanced by attaching a heterologous signal sequence (27). The HA/PI-6, PAI-2, and HA/PAI-2 expression plasmids were transfected into COS cells and subjected to pulse-chase analysis as described above. Entry into the ER and travel through the secretory pathway was predicted to result in an apparent increase in the size of both proteins and release into the medium. Since PI-6 (42 kDa) and PAI-2 (47 kDa) each have three N-linked glycosylation sites, increases in size of at least 10–12 kDa were expected for both molecules. As shown in Fig. 5A, proteins approximately 42, 45, 47, and 50 kDa in size were immunoprecipitated from extracts of COS cells expressing HA/PI-6. These probably represent HA/PI-6 glycosylated at 0, 1, 2, or 3 sites, respectively. The number and sizes of these proteins did not alter during a 3-h chase period, and none were detected in the media, suggesting that HA/PI-6 cannot exit the secretory pathway.

By contrast, three forms of PAI-2 were detected in extracts of COS cells immediately after labeling (Fig. 5B). The smallest, most abundant form represents cytosolic, unglycosylated PAI-2 (47 kDa), which is not released into the medium. Three larger forms were present in extracts in much lower amounts and represent glycosylated PAI-2 (50–55 kDa). Slight but increasing amounts of these larger forms were detected in media samples during the chase period (Fig. 5B). This pattern of expression is consistent with the inefficient secretion of PAI-2 that has been described previously (26). Addition of the HA signal sequence to PAI-2 significantly altered the pattern of expression (Fig. 5C). In this case, far less 47-kDa PAI-2 was observed, and significant quantities of the larger forms were present in the cell extracts and were secreted into the medium. This confirmed that the HA signal can markedly increase the efficiency of PAI-2 entry into the ER, leading to a substantial increase in the amount of PAI-2 that exits the secretory pathway.

HA/PI-6 is Glycosylated and Retained in the ER—To confirm that the forms of HA/PI-6 observed in transfected COS cells are glycoproteins, the effect of tunicamycin on HA/PI-6 biosynthesis was examined. Tunicamycin is an inhibitor of N-linked glycosylation that effectively prevents the transfer of precursor oligosaccharides to nascent polypeptides in the ER (28). COS cells producing either PI-6 or HA/PI-6 were labeled in the presence or absence of tunicamycin, and extracts were prepared and immunoprecipitated with anti-PI-6 antisera. As shown in Fig. 6, treatment with tunicamycin had no effect on the production or size of normal PI-6, demonstrating that the molecule is not usually glycosylated. By contrast, tunicamycin abolished the production of the 45-, 47-, and 50-kDa forms of HA/PI-6, showing that these species are glycoproteins and confirming that HA/PI-6 can enter the secretory pathway.

The failure to detect secretion of the HA/PI-6 glycoforms (Fig. 6) suggested that they are trapped somewhere along the secretory pathway. To assess where this block occurs, indirect immunofluorescence experiments were carried out. COS cells producing either PI-6, HA/PI-6, PI-2, or HA/PAI-2 were fixed, permeabilized, and probed with either PI-6 or PAI-2 antibodies. After detection with FITC-conjugated secondary antibodies, cells were examined by fluorescence microscopy (Fig. 7). Cells producing PI-6 and PAI-2 showed the diffuse, intracellular pattern of staining expected for cytosolic proteins, whereas cells producing HA/PAI-2 showed the characteristic Golgi staining observed for secreted glycoproteins. By contrast, cells containing HA/PI-6 showed a reticular pattern of staining usually associated with proteins located in the ER.

To confirm its apparent ER localization, the pattern of HA/PI-6 staining was compared with that seen in COS cells producing HA/NEO, which is a chimeric protein consisting of the HA signal fused to the bacterial enzyme neomycin 3'-phosphotransferase (HA/NEO). It has previously been shown that the HA signal can direct the NEO polypeptide into the ER where it is trapped, whereas mutation of the HA signal sequence results in a protein (mHA/NEO) that is cytosolic (17). The expression
patterns in cells producing HA/NEO and mHA/NEO resembled those of HA/PI-6 and PI-6, respectively (Fig. 7), supporting the proposition that HA/PI-6 is sequestered in the ER.

Glycosylation of nascent proteins is an ordered process that commences in the ER and continues in the Golgi apparatus. Proteins remaining in the ER normally have different oligosaccharide structures compared with those that have travelled to the Golgi and can be distinguished by the effect of endoglycosidase H (endo H). Resident ER proteins or nascent secretory proteins that have not left the ER contain "high mannose" oligosaccharides that can be removed by endo H. Proteins that have entered the Golgi apparatus have their N-linked carbohydrates modified and are resistant to endo H. This basis, it was predicted that HA/PI-6 proteins trapped in the ER would be sensitive to endo H. Proteins that have entered the Golgi apparatus have their N-linked carbohydrates modified and are resistant to endo H. On this basis, it was predicted that HA/PI-6 proteins trapped in the ER would be sensitive to endo H. COS cells producing HA/PI-6 or HA/NEO were metabolically labeled as described above, chased for 0 or 2 h, lysed, and immunoprecipitated using the appropriate antibodies. Immune complexes were split and treated or not treated with endo H prior to SDS-PAGE analysis.

As shown in Fig. 8, endo H treatment of HA/PI-6 immunoprecipitates completely removed the HA/PI-6 glycoforms, and no endo H-resistant proteins were observed 2 h after the labeling was terminated. Similar results were obtained with immunoprecipitates from cells containing the ER-resident HA/NEO protein. These results support the notion that HA/PI-6 is sequestered in the ER, and suggest that little movement of HA/PI-6 from the ER to Golgi occurs.

ER-localized HA/PI-6 is Nonfunctional—Cytosolic and glycosylated forms of PAI-2 do not differ in proteinase inhibitory activity (25, 29). To test whether HA/PI-6 retains inhibitory function, thrombin was added to labeled extracts of mock transfected COS cells, and to those producing PI-6 or HA/PI-6. As described above, the thrombin-PI-6 interaction results in an SDS-resistant complex that can be immunoprecipitated using anti-PI-6 antibodies. It was therefore expected that normal PI-6 bound to thrombin would give rise to a 67-kDa complex (2, 30), whereas HA/PI-6 and thrombin would give rise to a larger complex due to glycosylation of PI-6. Following immunoprecipitation, SDS-resistant complexes were observed in the mock samples (due to low level production of endogenous PI-6 by COS cells (30)) and in those from cells producing normal PI-6 (Fig. 9). By contrast, a larger complex between HA/PI-6 and thrombin did not form, although a species corresponding to thrombin complexed with simian PI-6 was evident in these

**Fig. 7. Intracellular localization of HA/PI-6 by indirect immunofluorescence.** COS cells were transfected with pSVTfPTI/P (A), pSVTfHA/PI-6 (B), pEUkPAI-2 (C), pSVTHA/PAI-2 (D), pSVMHA/NEO (E), or pSVHA/NEO (F). 48 h posttransfection, cells were fixed, permeabilized, and probed with rabbit PI-6 antiserum diluted 1:200 (upper panels), mouse PAI-2 monoclonal antibody diluted 1:50 (middle panels), or rabbit NEO antiserum diluted 1:200 (lower panels). The primary antibodies were detected by the appropriate sheep fluorescein isothiocyanate-conjugated secondary antibodies. Cells were examined by fluorescence microscopy.

**Fig. 8. Effect of endoglycosidase H treatment on HA/PI-6.** COS cells were transfected with pSVTHA/PI-6 (HA/PI-6) or pSVHA/NEO (HA/NEO) DNA. 48 h posttransfection, cells were starved for 30 min in media lacking methionine, labeled for 30 min in media containing 100 μCi [35S]methionine, and then incubated in complete media for the indicated times. Cell extracts and media samples were prepared at each time point and immunoprecipitated with the appropriate antibodies. Immune complexes were collected and treated (+) or not treated (−) with endo H. Samples were then reduced, and analyzed by 10% SDS-PAGE and fluorography.
increase in the signal concentration kinetically favors the formation of the SRP-signal complex. Consequently, if PI-6 possesses a weak signal sequence, it can be predicted that increased PI-6 transcription and the biosynthesis of large quantities of PI-6 might be accompanied by constitutive secretion of the molecule. This is certainly the case for PAI-2 produced in phorbol ester-treated U937 cells; PAI-2 transcription increases markedly and is paralleled by secretion of up to 70% of nascent PAI-2 (25). In this study, we were unable to identify a treatment that increases expression of endogenous PI-6 mRNA or that leads to the release of PI-6 protein. Furthermore, overexpression of human PI-6 in COS cells did not lead to secretion.

An alternative pathway for PI-6 release might be through regulated secretion, in which the molecule is stored in an intracellular compartment and released in response to a specific signal. Although our histological and immunofluorescence experiments provide no evidence for such a compartment, we used platelets to model this situation because they contain PI-6, the regulated release of platelet contents is well-characterized, and they are known to release protease nexin 1 (another serpin) on activation. In addition, we treated several PI-6-producing cell lines with agents designed to activate intracellular signaling pathways likely to trigger regulated secretion. PI-6 was not released from activated platelets, nor was it released from stimulated cell lines, suggesting that regulated secretion of PI-6 does not occur. Another argument against intracellular storage and regulated secretion of PI-6 is that entry of proteins into storage compartments usually occurs via the secretory pathway after movement through the Golgi. Since PI-6 cannot move past the ER, it is unlikely to be stored in a conventional secretory granule.

A number of studies have been performed in which normally cytosolic or nuclear proteins have been introduced into the ER by attaching a heterologous conventional signal sequence (17, 32, 33). In all cases, the proteins successfully entered the ER and were glycosylated but did not move along the secretory pathway. The reason for this is thought to be a failure to fold correctly due to oxidation and formation of inappropriate disulfide bonds. Malfolded proteins in the ER are retained and degraded by a mechanism that remains obscure (34). By contrast, heterologous signal sequences added to normally secreted proteins do not impair processing and secretion (17, 27). On the basis of such studies, we predicted that if PI-6 is a cytosolic serpin, attachment of the HA signal would result in incorrect folding and failure to exit the ER. On the other hand, if PI-6 can be glycosylated and secreted under certain circumstances, attachment of the HA signal is known to enhance the amount appearing in the medium. Our studies clearly support the first prediction and argue strongly that PI-6 is a cytosolic serpin that has evolved to meet an intracellular function. Given that PI-6 is an inhibitory serpin, it is likely that this involves the regulation of an intracellular protease.

Taken with our previous work demonstrating differences between PI-6 and the ovalbumin serpins in gene localization and structure (20, 35), the results of this study show that PI-6 can now be distinguished from the ovalbumin serpins by three criteria: gene structure, gene localization, and the failure to be regulated in intracellular signaling pathways likely to trigger regulated secretion. The recent finding that the MNE1 gene co-localizes with PI-6 on human chromosome 6p25 (36, 37) indicates that MNE1 may not belong to the ovalbumin serpins as suggested previously (8). If this is the case, it is conceivable that MNE1 will have a gene structure similar to PI-6, and will prove to be nonsecreted. Thus PI-6 may be the prototype of a new class of intracellular serpins.

Acknowledgments—We thank Dr. J. Sun for assistance with the...
construction of HA/PI-6 and Dr. R. Medcalf for donating the anti-PAI-2 monoclonal antibody and providing the expression vector, pEUKPAI-2.

REFERENCES
1. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
2. Coughlin, P. B., Tetzl, T., and Salem, H. H. (1993) J. Biol. Chem. 268, 9541–9547
3. Morgenstern, K. A., Henzel, W. J., Baker, J. B., Wong, S., Pastuszyn, A., and Kidz, W. (1993) J. Biol. Chem. 268, 21560–21568
4. Sun, J., Coughlin, P., Salem, H., and Bird, P. (1995) Biochim. Biophys. Acta 1252, 28–34
5. Coughlin, P., Sun, J., Cerruti, L., Salem, H. H., and Bird, P. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9417–9421
6. Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P., and Salvesen, G. (1994) J. Biol. Chem. 269, 19331–19337
7. Quan, L. T., Caputo, A., Bleackley, R. C., Pickup, D. J., and Salvesen, G. S. (1995) J. Biol. Chem. 270, 10377–10379
8. Remold-O'Donnell, E. (1993) FEBS Lett. 315, 105–108
9. Schneider, S. S., Schick, C., Fish, K. E., Miller, E., Pena, J. C., Treter, S. D., Hui, S. M., and Silverman, G. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3147–3151
10. Belin, D. (1993) Thromb. Haemostasis 70, 144–147
11. Suminami, Y., Kishi, F., Sekiguchi, K., and Kato, H. (1991) Biochem. Biophys. Res. Commun. 181, 51–58
12. Teissdale, M. S., Bird, C. H., and Bird, P. (1994) Immund. Cell Biol. 72, 480–488
13. Jackson, D. E., Mitchell, C. A., Bird, P., Salem, H. H., and Hayman, J. A. (1995) J. Pathol. 175, 421–432
14. Thompson, E. A., and Salem, H. H. (1988) Thromb. Haemostasis 59, 415–420
15. Lommi, U. K. (1970) Nature 227, 680–685
16. Baiking, N. L., and Majerus, P. W. (1974) Methods Enzymol. 31, 149–155
17. Bird, P., Gething, M.-J., and Sambrook, J. (1987) Cell Biol. 105, 2905–2914
18. Madison, E. L., and Bird, P. (1992) Gene (Amst.) 121, 179–180
19. Lazaravits, J. J., and Roth, M. (1988) Cell 53, 743–752
20. Sun, J., Rose, J. B., and Bird, P. (1995) J. Biol. Chem. 270, 16089–16096
21. Baker, J. B., and Gronke, R. S. (1986) Semin. Thromb. Hemostasis 12, 216–220
22. Gronke, R. S., Bergman, B. L., and Baker, J. B. (1987) J. Biol. Chem. 262, 3030–3036
23. Gronke, R. S., Knauer, D. J., Veeraraghavan, S., and Baker, J. B. (1989) Blood 73, 472–478
24. Medcalf, R. L., Kruthof, E. K. O., and Schleuning, W. D. (1988) J. Exp. Med. 168, 751–759
25. Gentry, C., Kruthof, E. K. O., and Schleuning, W. D. (1987) J. Biol. Chem. 104, 705–712
26. Ye, R. D., Wun, T. C., and Sadler, J. E. (1988) J. Biol. Chem. 263, 4869–4875
27. von Heijne, G., Liljestrom, P., Mikus, P., Andersson, H., and Ny, T. (1991) J. Biol. Chem. 266, 15240–15243
28. Tkacz, J. S., and Lampen, J. O. (1975) Biochem. Biophys. Res. Commun. 65, 248–257
29. Wohland, A., Belin, D., and Vassalli, J.-D. (1987) J. Biol. Chem. 265, 320–339
30. Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., and Finlayson, J. S. (1977) J. Biol. Chem. 252, 3587–3598
31. Rapoport, T. A., Heinrich, R., Walter, P., and Schulmeister, T. (1987) J. Mol. Biol. 195, 621–636
32. Sharma, S., Rodgers, L., Brandsma, J., Gething, M.-J., and Sambrook, J. (1985) EMBO J. 4, 1479–1489
33. Wirth, D. F., Lodish, H. F., and Robbins, P. W. (1979) J. Cell Biol. 81, 154–162
34. Doyle, C., Roth, M. G., Sambrook, J., and Gething, M.-J. (1985) J. Cell Biol. 100, 704–714
35. Coughlin, P., Nicholl, J., Sun, J., Salem, H., Bird, P., and Sutherland, G. (1995)
36. Ooms, L., Nicholl, J., Bird, P., and Sutherland, G. R. (1995) Chromosome Res. 3, 447
37. Evans, E., Cooley, J., and Remold-O'Donnell, E. (1995) Genomics 28, 235–240

Intracellular Location of PI-6