To understand the processing of type 1 plasminogen activator inhibitor (PAI-1) into the storage granules of platelets, we utilized a eukaryotic expression vector (pRHC/CMV) to transfer the human cDNA for PAI-1 into AtT-20 cells, a mouse pituitary cell line known to sort proteins in a regulated fashion. Immunofluorescence staining of PAI-1-transfected AtT-20 clones revealed co-localization of PAI-1 with an endogenously produced and stored hormone (i.e. adrenocorticotropic hormone, ACTH). Stimulation of PAI-1-transfected AtT-20 cells with a secretagogue resulted in the release of both active PAI-1 and the latent form. In comparison, PAI-1-transfected Chinese hamster ovary cells (i.e. a non-packaging cell line) did not release PAI-1 in response to a secretagogue and exhibited immunoreactivity for PAI-1 primarily confined to the Golgi region. Percoll density gradient fractionation of AtT-20 cells revealed a codistribution of PAI-1 and ACTH in cellular compartments of the same density. The half-life of PAI-1 activity at 37°C was prolonged in intact granules (t½p 6 h) in comparison with its half-life in lysed granules (t½h 2 h). These studies demonstrate the presence of a new functional property associated with the PAI-1 molecule that directs this inhibitor into the storage secretory pathway.

Type 1 plasminogen activator inhibitor (PAI-1) is the primary physiological inhibitor of vascular tissue-type plasminogen activator (t-PA) (1, 2). The ability of PAI-1 to neutralize the activity of t-PA and other serine proteases (e.g. urokinase, factor Xa, activated protein C) has led to the classification of this inhibitor in the serine protease inhibitor (serpins) superfamily (1, 2). This inhibitor is produced as an M, 50,000 glycoprotein by a wide variety of cells and is present in blood either at low concentrations in plasma or in a large storage pool within platelets (3–11). Agonist-induced platelet activation is known to cause the release of active PAI-1 which has led to the concept that PAI-1 is stored in conjunction with other hemostatic proteins within platelet α-granules (3, 4, 7, 9–11). The presence of PAI-1 antigen in megakaryocytes (12, 13), the hemopoietic precursor of platelets, suggests that PAI-1 may be deposited into storage organelles (i.e. α-granules) during the maturation of these cells. However, no information exists on the ability of PAI-1 to be specifically targeted into storage granules or if the presence of PAI-1 in α-granules is a non-specific entrapment event that occurs during megakaryocytopoiesis.

Research from a number of laboratories indicates that PAI-1 is synthesized in an active form, but it is a relatively labile inhibitor that is rapidly converted into an inactive form at 37°C with a half-life of approximately 1 h (1). The conformation of PAI-1 resulting from inactivation at 37°C is commonly referred to as latent PAI-1, because inhibitory activity can be revealed by treatment with denaturants or negatively charged phospholipids (1). In light of the observation that platelets possess low biosynthetic capabilities (14), it is not unexpected that the majority of PAI-1 is present within platelets in a latent form. Although vitronectin is known to be capable of increasing by 2-fold the half-life of PAI-1 activity in solution (37°C) (15), preliminary data of Pressnier et al. (15) suggest that complexes between vitronectin and PAI-1 are not present in nonactivated platelets. Therefore, little information exists on the mechanisms that account for the presence of active PAI-1 stored within platelets that have a half-life span of 9–12 days in the circulation (14).

Two distinct pathways are known to be responsible in eukaryotic cells for the secretion of proteins from cells (17, 18). The "constitutive" pathway externalizes proteins rapidly using post-Golgi vesicles and does not require an external stimulus for release of a compound into the extracellular milieu (17, 18). In the "regulated" pathway, proteins are stored in secretory granules until the cells are stimulated to secrete in response to the appropriate stimuli (17, 18). A number of tumor-derived cell lines exhibit both a constitutive and a storage secretory pathway, and these cell lines have been used as in vitro model cell systems for analyzing the processing of proteins into these two pathways (17, 18). A classical system is the mouse pituitary tumor cell line, AtT-20, that has been shown to divert a majority of the endogenously synthesized adrenocorticotropic hormone (ACTH) into the regulated storage pathway (19, 20). Treatment of AtT-20 cells with the appropriate secretagogue (e.g. 8-BrcAMP) results in release of the contents of the secretory granule (19, 21). These cells have been shown to have the capacity, after transfection with the appropriate DNA, to package heterologous peptide hormones and enzymes into the regulated secretory pathway. For example, proinsulin (21), trypsinogen (20, 22), human growth hormone (23), and peptidylglycine α-amidating monoxygenase (24) are transported to the regulated pathway with a similar efficiency as the endogenous hormone ACTH. This cell line has also proven useful in studies investigating the sorting of two platelet α-granule proteins into the regulated secretory pathway, including P-selectin (25, 26) and von Willebrand Factor (27). In light of this information, this study was designed to investigate the applicability of utilizing the AtT-20 cell line as a model system for analyzing the processing of PAI-1 into storage granules.

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Plasminogen Activator Inhibitor in Storage Granules

MATERIALS AND METHODS

Plasminogen Construction and Transfection of Eukaryotic Cells—The cDNA encoding the full-length molecule of human PAI-1, including the signal peptide, was kindly provided by Dr. David Ginsburg (University of Michigan) (28). The entire coding region for PAI-1 was excised using the EcoRI site at the 5' end of the cDNA and NsiI site at nucleotide 1328 in the 3' nontranslated region and transferred into an EcoR/PSelI-digested pBlueScript II KS plasmid vector (Stratagene, La Jolla, CA). Two unique restriction sites in the multiple cloning region of pBluescript II KS (i.e. HindIII at the 5' end and XbaI at the 3' end) were then used to transfer the PAI-1 cDNA in an orientation-specific manner into a HindIII/XbaI-digested pRC/CMV plasmid vector (Invitrogen, San Diego, CA).

Growth and Transfection of Eukaryotic Cells—AtT-20 cells and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). AtT-20 cells were cultured at 37 °C in the presence of a 15% CO2 atmosphere, whereas a 5% CO2 atmosphere was used for CHO cells. AtT-20 and CHO cells were transfected with 2.5 μg of the DNA construct (pRC/CMV/PAI-1) by utilizing Lipofectin according to the manufacturer's instructions (Life Technologies, Inc.). After 48 h, stable cells were selected by treatment with the antibiotic Geneticin (G-418; Life Technologies, Inc.) at 0.5 mg/ml for AtT-20 cells and 2 mg/ml for CHO cells, and individual clones were isolated by ring cloning onto polylysine films. Colonies were subsequently screened for the expression of soluble human PAI-1 antigen as described below.

Immunofluorescence Analysis—AtT-20 and CHO cells were grown for 4 days on polylysine-coated glass coverslips (12-mm diameter; Fisher) in serum-containing media supplemented with 2 mM 8-Br-CAMP (Sigma) as described by Burguess et al. (20) to induce the expression of cellular granules in AtT-20 cells. Both cell lines were fixed with 4% paraformaldehyde for 20 min at 4 °C, washed with PBS, and incubated (22 °C, 20 min) with 0.2 μg/ml of fluorescein-conjugated goat anti-rabbit IgG (1:25 dilution, Sigma; monoclonal antibody 380 against PAI-1; Immune IgG, 10 mg/ml) as described previously (29-31) by using immobilized t-PA (22 °C, 20 min) with 0.2 μg/ml of trypsin (0.5 mg/ml) and 0.05 unit/ml of a-thrombin. The solutions were mixed and layered onto the dish film, the Triton X-100-washed SDS-gel was layered onto the film of the nitrocellulose with Blotto, washed, and the radioactivity associated with each dot determined in a γ-counter.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Reverse Fibrin Autoantody—SDS-PAGE and reverse fibrin autography were performed as described previously (28, 31). Briefly, samples were subjected to SDS-PAGE, and the electrophoresed gel was subsequently soaked for 1.5 h 2.5% Triton X-100. Fibrin-agar indicator films were prepared containing 1% agarose, 2.4 mg/ml fibrinogen, 25 μg/ml human plasminogen, 0.5 unit/ml α-thrombin, and 0.05 unit/ml urokinase (all final concentrations). The solutions were mixed and poured on prewarmed glass plates. After solidification of the fibrin-agar film, the Triton X-100-washed SDS-gel was layered onto the film and incubated in a humid chamber. PAI activity is revealed by the development of opaque lysis-resistant zones in the otherwise clear indicator film.

RESULTS

Production and Deposition of PAI-1 within PAI-1-transfected AtT-20 Cells—From three transfection experiments with the vector pRC/CMV/PAI-1, 10 colonies were isolated that were neomycin-resistant and produced human PAI-1 antigen. Based upon the production of PAI-1 antigen over a 24-h period, one high PAI-1 producer (i.e. clone AtT-20-pRC-CMV/PAI-1-3F, 201.5 μg/ml/105 cells) was selected for further studies. Because our initial screening utilized a murine monoclonal antibody capable of reacting against total PAI-1 (i.e free PAI-1, t-PA/PAI-1-complexes, etc.), we decided to employ reverse fibrin autography to gain an insight into the levels of free PAI-1 produced and associated with the AtT-20 cells (Fig. 1). Conditioned media and cell lysates of both nontransfected and PAI-1-transfected AtT-20 cells were fractionated by SDS-PAGE and placed onto a fibrin-agar film that was supplemented with both plasminogen and urokinase to initiate fibrin degradation. After 2 h at 37 °C, lysis-resistant zones were evident in both the cell lysates (Fig. 1, lanes 1 and 2) and conditioned media samples (lanes 3 and 4) that co-migrated with purified PAI-1. The size of the lysis-resistant zones were significantly larger in the samples from the PAI-1-transfected AtT-20 (lanes 2 and 4) than those from nontransfected cells (lanes 1 and 3). Immunoprecipitation experiments revealed that the majority of the inhibitor activity present in lanes 2 and 4 could be depleted utilizing Sepharose beads conjugated to a monoclonal antibody against human PAI-1, whereas no decrease in the lysis-resistant zones was observed utilizing this Sepharose-antibody complex to immunoprecipitate the samples of nontransfected cells (data not shown).
AtT-20 cells by reverse fibrin autography. Samples from nontransfected lanes 1 and 3 and PAI-1-transfected AtT-20 cells (clone 4F, lanes 2 and 4) were subjected to SDS-PAGE and analyzed for PAI-1 activity by reverse fibrin autography as described under “Materials and Methods.” Samples included cell lysates (lanes 1 and 2, $10^6$ cells/lane) and the corresponding media conditioned by $10^5$ cells ($\pm 100$ μl/lane, lanes 3 and 4). Lanes 5 contained nonconditioned medium (100 μl), and lane 6 contained 100 ng of purified human PAI-1. Position of $M_r$ markers are indicated.

To investigate if human PAI-1 produced in the transfected cells was contained within storage granules, experiments were directed at co-localizing this inhibitor with an endogenously produced and stored hormone (i.e. ACTH) (20). Double label immunofluorescence experiments were employed initially to ascertain the distribution of these two molecules. Fig. 2 indicates that the staining for PAI-1 (Fig. 2A) and for ACTH (Fig. 2B) could be co-localized in AtT-20 cell processes. No staining for ACTH (Fig. 2D) or PAI-1 (Fig. 2E) was observed if the primary antibody was replaced with rabbit IgG or nonimmune mouse. As a control in these experiments, CHO cells were transfected with the pRC/CMV/PAI-1 construct, and stable colonies were selected based upon their resistance to G-418 and production of human PAI-1. A similar analysis for human PAI-1 in CHO and ACTH revealed that PAI-1 was primarily confined to the Golgi region in CHO cells with little staining in the cell periphery (Fig. 2G). No staining for ACTH was observed in the PAI-1-transfected CHO cells (Fig. 2H).

Characterization of PAI-1 Released and Stored by PAI-1-Transfected AtT-20—It is well known that the secretory products contained with dense core secretory granules can be released following stimulation with certain secretagogues (e.g. 8-Br-cAMP) (19, 20, 22). Further evidence for the presence of PAI-1 within storage granules could be obtained if the release of this inhibitor was increased in the presence of an appropriate secretagogue. Therefore, PAI-1-transfected AtT-20 and CHO cells were incubated for three separate 1-h incubation periods in the presence or absence of the secretagogue 8-Br-cAMP and the conditioned media assayed for PAI-1 antigen. The first two successive 1-h incubation periods shown in Fig. 3 were employed to determine the constitutive expression of PAI-1 in both cell lines. Fig. 3A indicates that the presence of 8-Br-cAMP during the third incubation period with the PAI-1-transfected AtT-20 cells resulted in a 60-fold increase in the PAI-1 antigen content in the conditioned media, which is equivalent to 18% of the total cellular PAI-1. In contrast, no increase in the PAI-1 content of the media conditioned by CHO cells was observed upon their incubation with 8-Br-cAMP (Fig. 3B).

Although current information indicates that PAI-1 is synthesized in an active form (1), both the active and latent forms of PAI-1 have been detected stored in platelet α-granules (7, 11, 33, 34). To determine the applicability of this model cell system for understanding the PAI-1 molecule released from platelet storage organelles (i.e. α-granules), it was relevant to characterize the AtT-20 cell-associated and secretagogue-released forms of PAI-1. Fig. 4A indicates that both the active (specific activity: $4,158 \text{ units/mg}$) and latent form (specific activity following SDS treatment: $8,800 \text{ units/mg}$) of PAI-1 could be detected in the media conditioned by the AtT-20 cells incubated in the presence of the secretagogue 8-Br-cAMP. In comparison, neither form of PAI-1 could be detected in the media conditioned by these cells incubated in the absence of a secretagogue (Fig. 4A). Analysis of the cell lysates revealed that both the active (specific activity: $976 \text{ units/mg}$) and latent (specific activity following SDS treatment: $3,771 \text{ units/mg}$) forms were present in the PAI-1-transfected AtT-20 cells. Incubation of these cells with a secretagogue resulted in a decrease in both forms of PAI-1 (Fig. 4B). In contrast, only the active form could be detected in the samples of the CHO cells, which was not
Active PAI-1 is represented by the cellular membranes (20,25) and a high density, storage blotting. PAI-1 antigen (Fig. 3A) and clone CHO-pRClCMV/PAI-1-2C (B) were plated into 60-mm dishes (n = 8/cell) in complete media. The next day, the cells were washed twice with PBS and incubated in serum-free medium for three consecutive 1-h periods. The numbers 1, 2, and 3 refer to the consecutive incubation periods. During the final incubation period, the cells were maintained either in the presence (n = 4) or absence (n = 4) of 5 mM 8-Br-cAMP. The conditioned medium was harvested, centrifuged, and assayed for PAI-1 antigen as described under "Materials and Methods." Data are represented as mean ± standard deviation.

For PAI-1-transfected AtT-20 and CHO cells were washed twice with 20 mM EDTA, washed, resuspended in PBS, and counted. Conditioned media (A) and cell lysates (B) were assayed for PAI-1 activity using the functional immunosassays as described under "Materials and Methods." Data are represented as mean ± standard deviation.

The secretagogue-induced release of the active and latent form of PAI-1 (Fig. 4) would suggest that both forms of this inhibitor are stored within the transfected AtT-20 cells. Evidence for this concept would be obtained by the isolation and analysis of PAI-1-containing dense core secretory granules. For this reason, PAI-1-transfected AtT-20 cells were subfractionated on a Percoll density gradient and the isolated fractions analyzed for the presence of three secretory proteins by dot blotting. PAI-1 antigen (Fig. 5A) was recovered as two well separated bands: (i) a low density band that has been observed to correspond to rough endoplasmic reticulum, Golgi apparatus, cellular membranes (20,25) and (ii) a high density, storage granule-containing region (20,25). In agreement with previous published reports (20,25), Fig. 5B indicates that the endogenously synthesized and stored hormone, ACTH, was associated with both the low density fractions (i.e. endoplasmic reticulum, etc.) and the high density fractions. In contrast, laminin, a secreted extracellular matrix protein that is known to be transported exclusively via the constitutive secretory pathway (20), was detected only in the region with low density (Fig. 5C). Dense granules present within fractions 14 and 15 of the Percoll gradient were pooled and analyzed for PAI-1 activity. Fig. 6 (inset) demonstrates that both the active and latent form of this inhibitor were associated with these dense granules. To determine if the active form of PAI-1 is stabilized
within these storage organelles, the Percoll-isolated dense granules were incubated at 37 °C and the activity of PAI-1 monitored over a 48-h period. Fig. 6 indicates that the half-life of PAI-1 activity in the isolated granules was 5.5 h in comparison with purified active human PAI-1, which decayed with a half-life of 1 h. Lysis of the Percoll-isolated granules with Triton X-100 prior to their incubation at 37 °C reduced the half-life of PAI-1 activity to 2 h (Fig. 6).

**DISCUSSION**

Platelet PAI-1 has been established to play a key role in regulating the fibrinolytic system (35, 36). Although PAI-1 was first detected in platelets in 1984 (3, 7), little is known about its origin (e.g. uptake from the plasma or synthesis in megakaryocytes) or the mechanisms that stabilize this relatively labile inhibitor within platelets, which have a life span in the circulation of 9–12 days and are formed over a period of 3–5 days during megakaryopoiesis (14). This study provides morphological and biochemical evidence indicating that PAI-1 contains a functional region or domain that enables this inhibitor to be involved in both the targeting and stabilization of PAI-1 into a functional molecule (26). More specifically, chimeric constructs of the cytoplasmic region of P-selectin and the luminal domain from another plasma membrane protein (i.e. tissue factor) were observed to be routed into secretory granules, thus demonstrating that the 35-amino acid cytoplasmic domain of P-selectin contains the necessary information for its storage (26). The presence of this domain on P-selectin’s cytoplasmic region has been speculated to play a direct role in sorting by permitting its direct interaction with the underlying submembrane cytoskeleton (18), whereas prevailing theories (17, 37) would require that a soluble protein (e.g. PAI-1) contain a signal that interacts with a membrane-associated receptor dedicated to facilitate sorting. Because a highly conserved sequence of amino acids has not been identified within the diverse group of proteins that have not been sorted into the regulated secretory pathway, Kizer and Tropsha (38) have proposed a degenerate (40) motif occupying one side of an amphipathic α-helix may function as a targeting signal for this pathway. Analysis of the amino acid sequence of PAI-1 (28) reveals that the degenerate motif proposed by Kizer and Tropsha (38) is present in several regions of PAI-1 (i.e. Ser172-Leu175, Thr184-Leu192, Leu296-Leu298, Thr305-Leu313). As an alternative to a sorting signal, the ability of certain secretory products to form molecular aggregates with each other and condense into electron dense material within the Golgi has been proposed as a mechanism to initiate sorting by excluding nonaggregating molecules from the forming dense core secretory granule (18, 37). It is known that several factors appear to play a role in the aggregation or condensation of molecules within the trans Golgi, including a high calcium concentration and a low pH. Experiments with agents that disrupt the internal pH within the trans Golgi have provided biochemical evidence, indicating that mildly acidic pH plays a role in protein condensation and sorting of several molecules (17, 18, 39). In light of these data, it is relevant to note that PAI-1 also exhibits a low isoelectric point (i.e. 4.5–5) (1) and that the activity of this molecule is prolonged at mildly acidic pH (40).

Our study also provides data indicating that the activity status of PAI-1 stored in the transfected AtT-20 cells is similar to its activity status within porcine (34), canine (33), and human platelets (4, 6, 9, 33, 34). Present data indicate that platelet PAI-1 is composed of two distinct forms: the majority in a latent but denaturant activatable form and a second population comprising its active form (4, 6, 9, 33, 34). Thus, the active and latent denaturant-activatable form of PAI-1 are present in a 1:3 ratio both in platelets and the transfected AtT-20 cells, and these two forms of PAI-1 can be released from both platelets and the transfected AtT-20 cells following agonist-induced secretion (Fig. 4). In addition, stabilization experiments revealed that the activity of PAI-1 within the isolated dense core secretory granules exhibit a half-life ($t_{1/2}$) of 5 h at 37 °C in comparison with the relative lability of this inhibitor in solution ($t_{1/2} = 1$ h, Fig. 6). Preliminary data from our group indicate that activity of PAI-1 within isolated platelet α-granules also appears to be stabilized ($t_{1/2} = 5$ h). Therefore, the data in this report indicate that the secretion experiments with AtT-20 cells and PAI-1 are applicable for dissecting the mechanisms involved in both the targeting and stabilization of PAI-1 into storage granules.

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