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Recombinant Semliki Forest Virus Enhanced Plasminogen Activator Inhibitor 1 Expression and Storage in the Megakaryocytic Cell Line MEG-01

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Abstract Platelet plasminogen activator inhibitor I (PAI-1), a trace α-granule protein, is a key physiological regulator of fibrinolysis. Because information on the packaging of PAI-1 into α-granules during megakaryocytopoiesis may reveal novel approaches for controlling hemostasis, this study investigated basal, plasmid-mediated, and alphavirus-mediated PAI-1 packaging into α-granules-like structures in the megakaryocytic cell line MEG-01. Differentiation of MEG-01 cells with phorbol myristate acetate (PMA) was observed to result in a four-fold increase in both secreted and cell-associated PAI-1 antigen over a four day period. Subcellular fractionation of PMA-treated MEG-01 cells on 45% self-forming Percoll gradients was employed to separate low density membrane and Golgi-rich fractions from a high density granule-containing region. A subsequent 30–60% pre-formed Percoll gradient was employed to remove contaminating lysosomes from the PAI-1/glycoprotein IIbIIIa-containing granules. Electron microscopy showed that these MEG-01 granules share a similar size distribution (350–600 nm) and morphology to platelet α-granules. PAI-1 (40 ng/mg protein) in isolated MEG-01 storage granules was ~10% of the levels present in isolated platelet α-granules. To elevate PAI-1 production/storage, two expression systems were investigated. Experiments with plasmids encoding PAI-1 and β-galactosidase resulted in low transfection efficiency (0.001%). In contrast, Semliki Forest virus (SFV)-mediated gene transfer increased cellular PAI-1 by 31-fold (1,200 ng/10^6 cells at 10 MOI) in comparison to mock-infected cells. Pulse-chase experiments demonstrated that SFV/PAI-1 mediated gene expression could enhance PAI-1 storage 6–9-fold, reaching levels present within platelets. To document the ability of PAI-1 to be stored in a rapidly releasable form in MEG-01 cells, we isolated platelet-like particles from the media conditioned by the cells and examined secretagogue-induced release of PAI-1. Particles from SFV/PAI-1 infected cells display a 5-fold enhanced secretion of PAI-1 following treatment with ADP in comparison to particles incubated in the absence of secretagogue. These results suggest that SFV mediated gene expression in MEG-01 cells provides a useful framework for analyzing the production and storage of α-granule proteins. J. Cell. Biochem. 82: 277–289, 2001. © 2001 Wiley-Liss, Inc.

Key words: platelets; megakaryocytes; storage granules; gene transfer; Percoll fractionation; fibrinolysis

Plasminogen activator inhibitor 1 (PAI-1) is the primary physiological inhibitor of vascular tissue-type plasminogen activator (t-PA) with rate constants greater that 10^7/M/s for both single- and two-chain t-PA, as well as urokinase, which results in the formation of high molecular weight, inactive PA/PAI-1 complexes [reviews in Van Meijer and Pannekoek, 1995; Wiman, 1996; Eitzman and Ginsburg, 1997; Vaughan, 1998]. PAI-1 is produced as a Mr 50000 glycoprotein and is present in blood either at low concentrations in plasma (10–25 ng/ml) or in a large storage pool within platelets [reviews in Van Meijer and Pannekoek, 1995; Wiman, 1996; Eitzman and Ginsburg, 1997; Vaughan, 1998]. Platelet-rich thrombi are more resistant to thrombolysis than erythrocyte-rich thrombi and this resistance to lysis is mediated by the release of PAI-1 from platelets [reviews in

Abbreviations used: SFV, Semliki Forest virus; PAI-1, plasminogen activator inhibitor I; PMA, phorbol myristate acetate; ELISA, enzyme-linked immunosorbent assay; CM, conditioned media; PDI, protein disulfide isomerase; vWF, von Willebrand factor; ECL, enhanced chemiluminescence; gpIIbIIIa, glycoprotein IIbIIIa.

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Van Meijer and Pannekoek, 1995; Wiman, 1996; Eitzman and Ginsburg, 1997; Vaughan, 1998]. Immunoelectron microscopy confirms the localization of PAI-1 to the α-granules [Wang et al., 1994; Prendes et al., 1999]. Functional activity of PAI-1 in the α-granules appears to be maintained through its interactions with other proteins into a high molecular weight complex that is calcium-dependent [Lang and Schleef, 1996a]. Because platelets contain only remnants of endoplasmic reticulum (ER)/Golgi apparatus and carry out little de novo protein synthesis [Rabellino et al., 1981], it has been established that the majority of α-granule proteins are packaged during platelet formation in bone marrow megakaryocytes [review in Sims and Gewirtz, 1989].

Because megakaryocytes are terminally differentiated and comprise ~0.05–0.5% of nucleated cells in bone marrow [review in Sims and Gewirtz, 1989], studies on the packaging of α-granule proteins have utilized a series of model tissue culture lines [reviews in Saito, 1997; Arvan and Castle, 1998; Tooze, 1998]. For example, the mouse pituitary AtT-20 line has been used to demonstrate that α-granule protein PAI-1 [Gombau and Schleef, 1994], multimerin [Hayward et al., 1999], von Willebrand factor (vWF) [Wagner et al., 1991], and P-selectin [Disdier et al., 1992; Koedam et al., 1992] contain functional domains that direct their routing into storage granules. Although an AtT-20 cDNA library has been used to identify a PAI-1 binding protein in the regulated secretory pathway [Lang et al., 1996b], this cell line does not synthesize a complete panel of α-granule proteins and thus, shows limited value with respect to dissecting protein–protein interaction within α-granules. An alternative approach employs cell lines that exhibit features of the megakaryocytic lineage to understand packaging of platelet proteins [review in Saito, 1997]. These cell lines typically proliferate in suspension and can be induced to differentiate along the megakaryocytic lineage with several agents (e.g., phorbol myristate acetate, PMA). Moreover, α-granule-like structures have been documented in several of these megakaryocytic cell lines [Greenberg et al., 1988; Ogura et al., 1988; Fugman et al., 1990]. PAI-1 expression has been described in the megakaryocytic cell line MEG-01 [Alessi et al., 1994; Wohn et al., 1997], Dami [Hill et al., 1996], UT7 [Madoiwa et al., 1999], CHRF-288 [Konkle et al., 1993], and K562 [Alitalo et al., 1989; Schuster et al., 1993]. Information on ultrastructural localization of PAI-1 in these cells is limited to studies with the Dami cell line in which labeling for this molecule was detected within an extensive network of convoluted organelles that did not exhibit a dense matrix typical of platelet α-granules but rather resemble vacuoles [Hill et al., 1996]. To initiate studies on the packaging of PAI-1 into α-granules, the MEG-01 cell line appeared relevant based upon, (i) this line readily proliferates in tissue culture, (ii) differentiation results in the expression of cellular markers restricted to megakaryocytic lineage [Ogura et al., 1988], (iii) differentiated MEG-01 cells are able to form long beaded processes, which rupture to produce platelet-like particles with size and function similar to individual platelets [Takeuchi et al., 1998], and (iv) a microgranular distribution for PAI-1 has been detected in MEG-01 cells exhibiting a differentiated phenotype [Alessi et al., 1994]. In this study, we examined basal and gene transfer-enhanced PAI-1 production/packaging in MEG-01 cell line.

MATERIALS AND METHODS

Growth of Eukaryotic Cells

MEG-01 and BHK cells were obtained from the American Tissue Culture Collection (Rockville, MD) and propagated in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal bovine serum (GIBCOBRL) at 37°C in a 5% CO_2 humidified atmosphere.

Quantitation of PAI Antigen

PAI-1 antigen was measured utilizing a two-site enzyme-linked immunosorbent assay (ELISA) as described previously [Schleef et al., 1989]. Briefly, the assay employed an immobilized monoclonal antibody (Mab) (clone 2D2) specific for human PAI-1 as a catcher to bind PAI-1 in a sample. The bound PAI-1 was immunologically detected by incubation with affinity-purified rabbit anti-PAI-1 (10 μg/ml) followed by sequential incubation with alkaline phophatase conjugated goat anti-rabbit immunoglobulin (IgG) (1:2000, Zymed) and *p*-nitrophenolphosphate (Zymed).

PAI-2 antigen was assayed utilizing the IMUBIND PAI-2 ELISA kit as instructed by the manufacturer (American Diagnostica, Inc.).

Analysis of PAI-1 Production

MEG-01 cells (2×10^6 cells/35 mm diameter dish/condition) were incubated in the presence or absence of 10^{-8} M PMA for a period of 96 h. At 24 h intervals, the conditioned media (CM) were harvested. Cells were washed twice with PBS. The PBS washes and conditioned media were spun briefly to pellet loose cells, which were combined with washed cells and lysed in a solution containing 150 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP40, and 0.5% deoxycholate supplemented with Complete protease inhibitors (Roche). CM and cell lysates were assayed for PAI-1 antigen in the two-site ELISA.

Subcellular Fractionation of MEG-01 Cells Using Percoll Gradients

MEG-01 cells were fractionated on Percoll density gradients utilizing protocols previously employed for the isolation of platelet α-granules [Lang and Schleef, 1996a]. Briefly, 10^8 MEG-01 cells were collected and washed twice in 200 ml RCD buffer (180 mM NaCl, 38 mM KCl, 1.7 mM $NaHCO_3$, 21.2 mM Na-citrate, 27.8 mM D-glucose, 1.1 mM $MgCl_2$, 1 mM theophyllin). The cells were pelleted by low speed centrifugation (1,500*g*, 10 min, RT), resuspended in 20 ml of RCD buffer and the cells were disrupted by sonication (5×7 s pulse). The cell homogenates were pooled and subjected to centrifugation (12,000*g*, 15 min, 4°C) to pellet cell membranes and subcellular organelles. These pellets were resuspended in 13 ml of 45% Percoll (Pharmacia LKB Biotechnology, Inc.) in 0.15 M NaCl in a Beckman quick-seal centrifuge tube (16×76 mm), and centrifuged (20,000*g*, 30 min, 4°C; Beckman L7-65 ultracentrifuge, Vti-65 rotor). Fractions (800 μl) were collected manually from the top.

To separate storage granules away from lysosomes, fractions with a density 1.057–1.073 g/ml were collected and diluted 1:1 (by volume) with RCD buffer. The diluted sample was loaded on the top of a preformed 30–60% Percoll step gradient. This was generated by successive layering of 1 ml volumes of a series of decreasing concentration of Percoll (60, 55, 50, 45, 40, 35, and 30%; 1 ml/concentration) into Beckmann ultraclear centrifuge tube (9×19 mm). The gradient was centrifuged (14,000*g*, 30 min, 4°C) in a SW 41 swinging bucket rotor. Fractions (800 μl) were collected from the top of the gradient and stored at −80°C until assayed. The densities of Percoll gradients were calibrated utilizing density marker beads (Pharmacia) in conjunction with refractive indexes.

Assays for β-Glucuronidase, Glycoprotein IIbIIIa, Protein Disulfide Isomerease (PDI), and von Willebrand Factor (vWF)

β-glucuronidase activity in each fraction was determined utilizing diagnostic kit #325-A as instructed by the manufacturer (Sigma). The distributions of glycoprotein IIbIIIa, PDI, and vWF were determined by dot blotting essentially as previously described [Gombau and Schleef, 1994]. Briefly, 20 μl of each fraction was applied to Immobilon-P membrane (MILLIPORE) using a 96-well BRL dot blotting apparatus. The membrane was blocked with Blotto (5% no-fat dry milk in 10 mM Tris-HCl, pH 7.5) for 1 h and incubated (30 min, 22°C) with anti-CD61 (Pharmingen; diluted 1:300), which reacts specifically with β3 subunit of glycoprotein IIbIIIa, anti-PDI (Stressgen Biotechnologies Corp; diluted 1:500) or anti-vWF (DAKO; diluted 1:250) in Blotto. Following the incubation with either horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulins (Ig) (Zymed; diluted 1:4000) or HRP conjugated anti-rabbit Ig (Amersham; diluted 1:2000), bound antibodies were detected using the enhanced chemiluminescence system (ECL, Amersham). Relative quantitations were made by densitometry using Alphaimager 2000 Documentation and Analysis System (Alpha Innotech Coporation).

PAI-1 and β-Galactosidase Expression in Transiently Transfection of MEG-01 and BHK Cells

MEG-01 cells (10^6/35 mm dish/condition) were treated with PMA (10^{-8} M) for 1 day to induce attachment. Transient transfections of MEG-01 cells with 1 μg of pRC/CMV/PAI-1 or pCDNA3.1/His/LacZ control vector (Invitrogen) in combination with the following transfection reagents, Geneporter 2 (Gene Therapy Systems), Fugene 6 (Roche), Cytofectene (Bio Rad), Superfect (Qiagen), Lipofectamine (Life technologies), Dmire-C (Life Technologies), Effectene (Qiagen), Dotap (Roche), Lipofectine (Life Technologies), and calcium phosphate precipitates (Life Technologies) were performed as instructed by manufacturers. After 36 h of incubation, conditioned media and cell lysate were harvested and assayed for PAI-1 antigen

as described above. Transfection efficiency was determined using β-gal staining kit (Invitrogen) as instructed by manufacturer.

DNA Based SFV System and Construction of SFV Vectors

SFV Plasmids (pSCAhelper, pSCAβ, and pSCA1) were generous gifts of Dr. Bremner, University of Toronto, Canada. As a control, a SFV construct encoding PAI-2 cDNA fused to the signal peptide for PAI-1 was obtained (SFV/signal peptide-PAI-2, a generous gift from Dr. Ny of University of Umeå, Sweden [Mikus et al., 1993]). To expand the limited number of sites for cloning, we utilized restriction sites BamHI and XmaI for the ligation of a previously described synthetic polylinker [Meanger et al., 1997] into the multiple cloning region of pSCA1 to generate pSCA1m. Plasmid pSCA1/PAI-1 was constructed by ligating full-length human PAI-1 cDNA clone B4 [Ginsburg et al., 1986] into pSCA1m utilizing restriction sites ClaI and XmaI. Virus production was performed as previously described [DiCiommo and Bremner, 1998]. Briefly, BHK cells (10^7 cells) were co-transfected with a mixture of plasmids (9 μg pSCAhelper combined with either 5 μg of pSCAmPAI-1 or pSCAβgal) and 30 μl Superfect (Qiagen) supplemented with DMEM to a final volume of 5 ml. Cells were incubated at 37°C for 48 h. Media containing SFV particles were harvested. The virus stocks were activated as previously described [DiCiommo and Bremner, 1998]. The titers of virus stocks (SFV/PAI-1 or SFV/β-gal) were determined by counting the number of BHK cells expressing recombinant proteins following SFV infections. X-gal staining was employed for SFV/βgal titers while an immunofluorescence assay with anti-PAI-1 monoclonal antibody (American Diagnostic #380) and rhodamine conjugated rabbit anti-mouse IgG (Calbiochem) was utilized for SFV/PAI-1.

SFV Infection

All SFV infections of MEG-01 cells were performed at 37°C and 5% CO_2 for 24 h in serum-containing growth media in the presence or absence of PMA as indicated in the text. Infected cells were processed as described above except otherwise indicated. Cell viability was determined at the indicated time utilizing trypan blue stain as instructed by the manufacturer (Life Technologies).

Tran^{35}S-Metabolic Labeling and Immunoprecipitation

MEG-01 cells (2×10^6 cells/35 mm diameter dish) were incubated in the absence or presence of 10^{-8} M PMA for 72 h, and starved for 30 min in methionine-free media (Life Technologies). Cells were infected with SFV/PAI-1 or SFV/β-gal (5MOI, 24 h, 37°C) in methionine-free RPMI media containing 10% dialyzed FCS, 10^{-8} M PMA, and Tran ^{35}S-label (250 μCi/ml, ICN). CM and cells were collected separately. A duplicate series of cultures were chased with complete media for 6 h to allow for release of all constitutively secreted proteins. Post-chased cells were washed two times in PBS and cell lysates prepared by a 10 min incubation on ice in a buffer containing 150 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA, 1% NP40, and 1X Complete protease inhibitors (Roche). Cell lysates and media were spun (14,000*g*, 20 min, 4°C). Samples were precleared by incubation (2 h, 4°C) with 20 mg protein-A Sepharose (Pharmacia) on a rotating shaker. Protein-A Sepharose were removed by brief centrifugation and the supernatants were immunoprecipitated with either 3 μl of rabbit serum anti-PAI-1 [Schleef et al., 1989] or normal rabbit serum (Sigma) overnight on a shaker at 4°C. Protein-A sepharoses preblocked with unlabeled cell lysate were added to samples, and incubated with constant agitations (20 min, RT). The beads were spun down, and washed by repeating three 15 min incubations on ice in 1ml of radioimmuno-precipitation assay (RIPA) buffer (150 mM NaCl, 1% NP40, 0.3% deoxycholate, 50 mM Tris HCl pH 6.8, and 0.1% SDS). After the last wash, the beads were recovered, resuspended in 90 μl of SDS PAGE sample buffer, boiled for 4 min, and fractionated on 8% SDS-PAGE. Gels were fixed in 10% acetic acid and 30% methanol, treated with Amplify (30 min, Amersham), dried, and exposed to film at −70°C for autoradiography. PAI-1 bands were excised and counted using Beckman model LS3801 liquid scintillation system.

RESULTS

Characterization of Basal PAI-1 Production

Figure 1 details the basic characteristics of PAI-1 production in MEG-01 cells over a 4 day period either in the presence or absence of the differentiation-inducing agent, PMA. Non-trea-

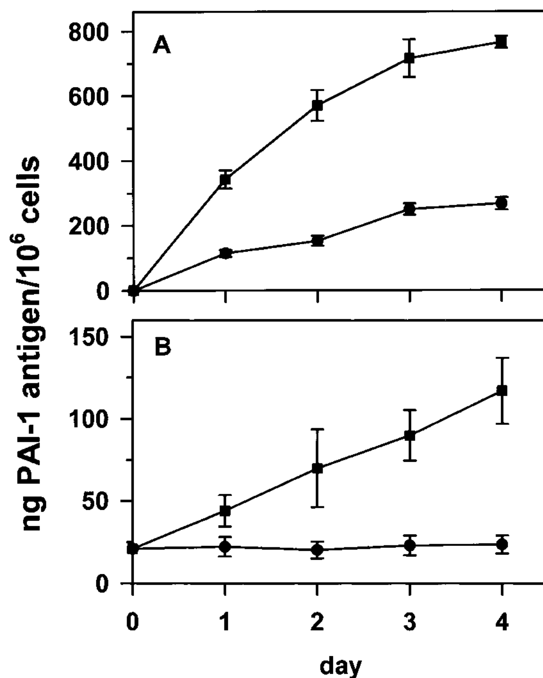

Fig. 1. Effects of PMA on PAI-1 production. MEG-01 cells (2×10^6 cells/35 mm dish, n = 4 dishes/condition) were incubated either in the presence (■) or absence (●) of 10^{-8}M PMA for 0–96h. At the indicated times, CM (panel **A**) and cell lysates (panel **B**) were harvested and assayed for PAI-1 antigen in the two-site ELISA.

ted MEG-01 cells release 110 ng ± 15 ng of PAI-1/10^6 cells over the first 24 h period and ~55 ng PAI-1/10^6 cells/24 h over the following 72 h (Fig. 1 panel A). Intracellular PAI-1 levels of non-treated cells remained constant at ~25 ng/10^6 cells (Fig. 1B). PMA-treatment of MEG-01 cells increases the rate of PAI-1 secretion into the CM by 3.6-fold over the first 48 h, where upon the rate decreases to 50 ng PAI-1/10^6 cells over the last 24 h (Fig. 1A). In comparison, the cell-associated PAI-1 levels continuously increase over the entire 96 h period to levels that were 4.6-fold over the levels associated with non-stimulated cells (Fig. 1B).

To determine if the increases in cell-associated PAI-1 observed following PMA treatment over a 4 day period results in a corresponding increase in its storage within intracellular granules, we attempted to isolate α-granule-like organelles from PMA-treated MEG-01 cells. Our laboratory and other investigators have observed that fractionation of AtT-20 cells [Koedam et al., 1992; Gombau and Schleef, 1994] and platelet [Lang and Schleef, 1996a] homogenates on self-forming Percoll gradients results in the rough ER, constitutive secretory vesicles, Golgi apparatus, and cell membranes migrating to the low density region, whereas storage granules migrate to the high density region of the gradient. Figure 2A indicates that fractionation of MEG-01 cells treated 1 day with PMA results in the distribution of PAI-1 antigen into both the low and high density region of the gradient. PAI-1 levels in the high density fractions increased daily as cells are cultured following PMA treatment, reaching a peak of 860 ng PAI-1 antigen/ml at a density 1.067 g/ml at the end of fourth day. To further characterize the organelles in the high density region, the distribution of a classical α-granule protein (i.e., glycoprotein IIbIIIa) was also examined. Figure 2B indicates that glycoprotein IIbIIIa co-distributed in the gradient with PAI-1 in 4 day PMA-treated MEG-01 cells. In contrast, the ER protein marker, protein disulfide isomerase (PDI), was found only in the low density region of the gradient. Analysis of a lysosomal marker, β-glucuronidase, reveals that these organelles distribute at a slightly lighter density (peak activity at a density 1.063 g/ml) than storage granules; however the migration of lysosomes partially overlaps that of granule markers in the high density region (Fig. 2B).

To separate PAI-1/glycoprotein IIbIIIa-containing granules apart from lysosomes, fractions (density 1.063–1.073 g/ml) were further centrifuged on a preformed 30–60% Percoll step gradient. Figure 2C indicates that β-glucuronidase was restricted to the the first four fractions (Zone I, density 1.030–1.041), whereas PAI-1 and glycoprotein IIbIIIa were distributed not only into first four fractions but also in the fractions with higher density (Zone II with density 1.042–1.056 g/ml and Zone III with density 1.057–1.138 g/ml). The latter two zones were essentially free of lysosomes, and contain peak PAI-1 level of 58 and 34 ng/mg protein, respectively. To compare the distribution profile of MEG-01 storage granules with platelet α-granules, platelet homogenates were fractionated on 45% Percoll and 30–60% step gradients as described for MEG-01 cells. Figure 2D reveals that PAI-1-containing platelet granules also migrated into three zones, but the levels of PAI-1 antigen in Zone II and III were more than ten-fold higher than the respective levels in MEG-01 storage granules (Zone II: 800 ng/mg platelet protein vs. 60 ng/mg MEG-01 protein;

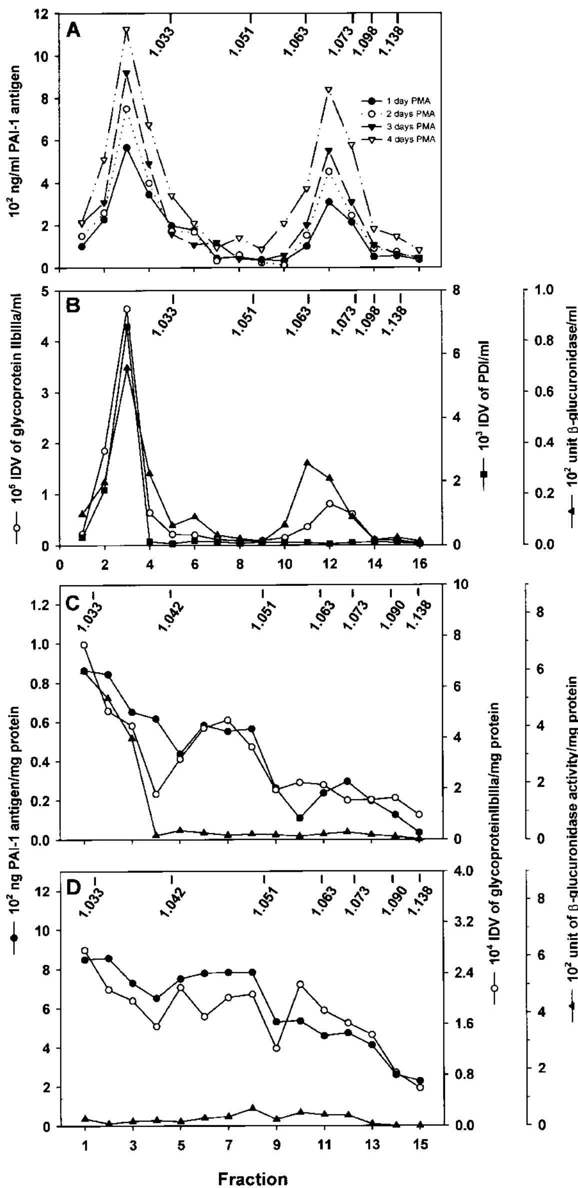

Fig. 2. Fractionation of MEG-01 cell homogenates on Percoll. Panel **A:** MEG-01 cells were treated with PMA for 1–4 days. Cells (10^8/day) were harvested at the indicated time and sonicated, and the cellular organelles centrifuged on a self-generating 45% Percoll density gradient. Fractions were collected from the top of gradient and assayed for PAI-1. Panel **B:** Percoll density gradient fractions derived from 4 days PMA-treated MEG-01 cells were also assayed for β-glucuronidase, glycoprotein IIbIIIa, and PDI. Panel **C:** Dense granule-containing fractions (density 1.063–1.73 g/ml) from 4 day PMA-treated cells were isolated as described above and further fractionated on a preformed 30–60% Percoll gradient. Fractions were collected from the top of gradient and assayed for PAI-1, glycoprotein IIbIIIa, and β-glucuronidase. Panel **D:** Platelet homogenates were fractionated on a 45% Percoll gradient. Fractions with a density 1.063–1.73 g/ml were further centrifuged on a 30–60% Percoll gradient. Fractions were collected and assayed for PAI-1, glycoprotein IIbIIIa, and β-glucuronidase. Plots shown are derived from one experiment, which are representative of three separate experiments.

Zone III: 500 ng/mg platelet protein vs. 30 ng/mg MEG-01 protein). Electron microscopy (Fig. 3) revealed that MEG-01 dense granules of Zone II (panel A) and III (panel B) share a similar size distribution (350–600nm) and are morphologically similar to platelet α granules as described in the literature [review in White, 1998]. However, the contents of granules in Zone III appear more compacted than those in Zone II in agreement with their respective density.

Plasmid Mediated-Transient Transfection Analysis

Because PAI-1 (~46 ng/mg protein) in isolated MEG-01 storage granules was ~10% of the levels present in isolated platelet α-granules, we investigated the possibility of increasing the amount of PAI-1 in MEG-01 α-granule-like organelles by elevating PAI-1 expression by gene transfer. MEG-01 cells were PMA treated for 1 day to induced cell attachment before transfection with plasmid encoding PAI-1 (pRC/CMV/PAI-1) or β-galactosidase (pCDNA3.1/His/LacZ Control vector). Figure 4A shows the low efficiency of transient plasmid transfection of MEG-01 cells that were mediated by ten standard transfection reagents. For example, Superfect-mediated plasmid transfer only yielded 25 ng PAI-1 antigen above basal levels (Fig. 4A) and a transfection efficiency of 0.001% (Fig. 4C). In contrast, Superfect-mediated plasmid transfection of BHK cells resulted in high PAI-1 expression (1,310 ng in CM and 225 ng /10^6 cells, Fig. 4B) and transfection efficiency (55%, Fig. 4D). Optimizing plasmid concentration for Superfect-mediated transfection did not significantly increase the levels of cell associated PAI-1 (Fig. 4A insert, ○) or CM PAI-1 (Fig. 4A inserts, ●), or transfection effciency (Fig. 4C insert). We observed that transient transfections with MEG-01 cells in suspension also gave low transfection efficiency and PAI-1 expression (data not shown).

SFV-Mediated Gene Transfer

Viral vectors are another useful tool to express heterologous proteins in non-proliferating cells [reviews in Schlesinger and Dubensky, 1999; Stone et al., 2000]. In an attempt to elevate PAI-1 expression in MEG-01 cells, we selected an alphavirus system (i.e., DNA-based SFV vector) because in comparison to other viral vectors, this type of approach has been reported to result in the highest recombinant

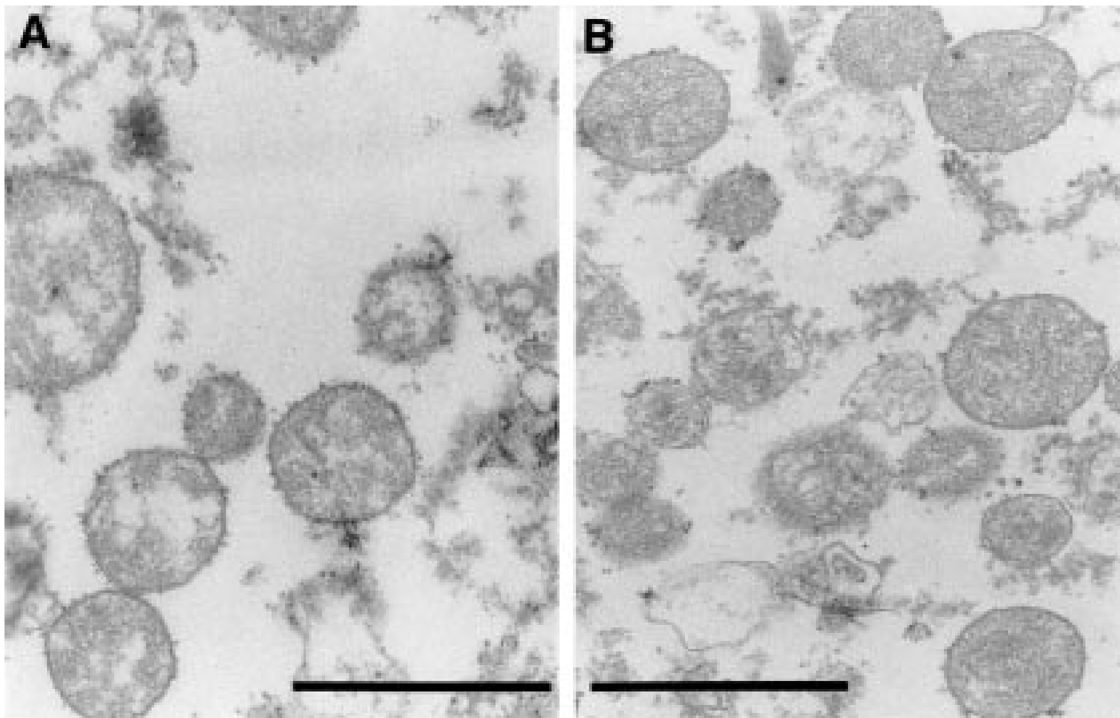

Fig. 3. Electron microscopy images of α-granule-like storage granules of MEG-01. Storage granules from 4 day PMA-treated MEG-01 cells were isolated by utilizing two successive Percoll gradients as described in the legend to Figure 2C. Zone II (density 1.042–1.056 g/ml, panel **A**) and Zone III (density 1.057–1.138 g/ml, panel **B**) were collected, fixed, and analyzed by electron microscopy. Scale bar indicates 1 μm.

protein yields in a broad range of mammalian cells [DiCiommo and Bremner, 1998]. SFV/PAI-1 virions were produced and used to infect MEG-01 cells in the presence or absence of PMA. Figure 5A indicates that a 24 h infection of MEG-01 cells with increasing virions results in elevated PAI-1 in both CM and cells (e.g., ~8,000 ng and ~1,200 ng/10^6 cells, respectively, at 50 MOI or greater). At 24 h postinfection, viabilities of SFV/PAI-1 and mock-infected cells were similar (Fig. 5B, □, ■); however, longer infection periods led to a decrease in cell viability (data not shown). The production of another α-granule protein, vWF, was not affected at less than 10 MOI (Fig. 5B). MEG-01 cells infected with a control virus SFV/β-gal revealed that the production of PAI-1 (Fig. 5A inset) and vWF (Fig. 5B) was not affected at less than 10 MOI. These results prompted us to utilize SFV/PAI-1 at $\leq$5 MOI and an infection period of 24 h in subsequent SFV infections.

Because continued incubation of MEG-01 cells with PMA enhances basal PAI-1 deposition into α-granule-like storage organelles (Fig. 2A), we investigated the possibility of elevating intracellular PAI-1 by increasing the period of PMA-treatment prior to SFV/PAI-1 infection. Consistent with the results in Figure 5A, SFV/PAI-1 infection (5 MOI) of MEG-01 cells in the presence of PMA for 24 h results in a 7.6-fold increase in cell associated PAI-1 in comparison to cells incubated in the absence of virus (Fig. 6A, 460 and 60 ng/10^6 cells, respectively). Pretreatment with PMA for a 24, 48, or 72 h prior to SFV/PAI-1 infection results in a respective 15, 78, and 87% increase in cell associated PAI-1 over non-stimulated cells. Panel B indicates that increasing PMA-pretreatment period results in a continuous rise in cellular levels for the control α-granule protein vWF (~2-fold increase in 72 h PMA pretreated cells relative to non-stimulated cells). These levels were not affected by SFV infection (Fig. 6B) and thus, suggest that this SFV/PAI-1 infection protocol has no deleterious effect on cellular protein production.

To investigate if the observed increase in cellular PAI-1 leads to a concomitant elevation in its storage, we performed a series of pulse-chase experiments. Table I shows a representative experiment utilizing ^{35}S-biosynthetic labeling to monitor PAI-1 production. To define the

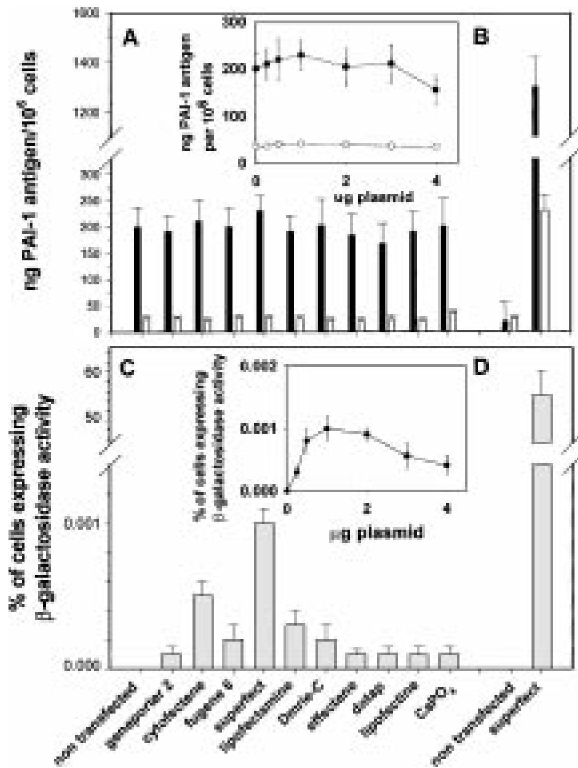

Fig. 4. PAI-1 and β-galactosidase expression in transiently transfected MEG-01 cells. The indicated transfection reagents (n = 3/transfection condition) were utilized to mediate transfer of plasmids pRC/CMV/PAI-1 (panel **A-B**) and pCDNA3.1/His/LacZ control vector (panel **C-D**) into MEG-01 (panel A and C) and BHK cells (panel B and D) as described in Materials and Methods. After a 36 h incubation period, conditioned media (solid bars) and cell lysates (open bars) were harvested and assayed for PAI-1 antigen (panel A and B). β-galactosidase expression was measured by staining with X-gal (panel C and D). Transfection efficiency indicates number of positively stained cells as a percentage of total in dish. Inserts: PAI-1 production (top panel: CM, solid symbols; cell lysates, open symbols) and transfection efficiency (bottom panel: closed symbols) were obtained at the indicated plasmid concentration utilizing a constant ratio of Superfect to DNA (4:1).

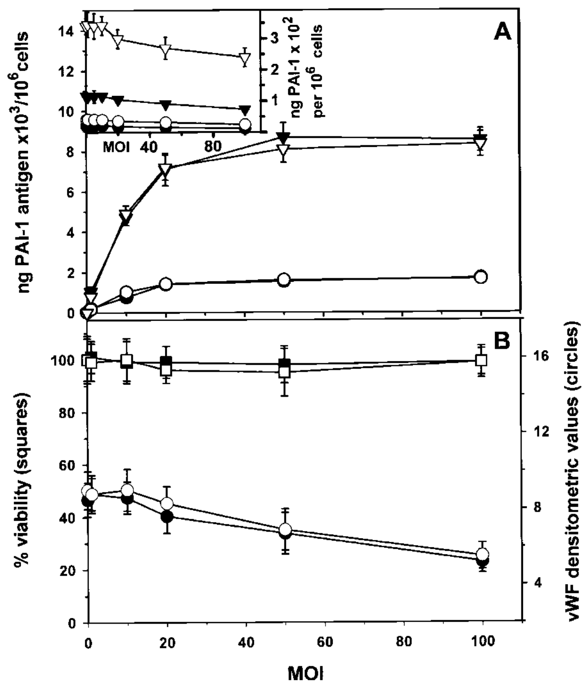

Fig. 5. SFV transduction of MEG-01 cells over 24 h period. Panel **A:** MEG-01 cells (10^6 cells/35 mm diameter dish/condition) were either infected with SFV/PAI-1 or SFV/β-gal (see inset) at the indicated MOI either in the presence (open symbols) or absence (closed symbols) of PMA (n = 4 dishes/condition). At 24 h postinfection, CM (triangles) were collected and cell lysates (circles) prepared. PAI-1 antigen was assayed in the two-site ELISA. Inset: PAI-1 antigen of samples (CM, triangles; cell lysates, circles) derived from cells incubated in the presence (open symbols) or absence (closed symbols) of PMA and the indicated MOI of SFV/β-gal for 24 h. Panel **B:** MEG-01 cells were either mock-, SFV/PAI-1- or SFV/β-gal-infected at the indicated MOI in the presence of PMA (n = 4 dishes/condition) for 24 h. Lysates from SFV/PAI-1- (○) or SFV/β-gal- (●) infected cells were prepared and vWF production was determined as described in the Materials and Methods. Duplicate cultures of SFV/PAI-1 (□) and mock (■) infection were assayed for cell viability.

basal level of PAI-1 production, cells were infected with a control virus SFV/β-gal in the presence of PMA. Immunoprecipitation of total biosynthetically labeled PAI-1 produced over a 24 h period yielded $3.2 \pm 0.4 \times 10^4$ cpm/10^6 cells. A 6 h chase period is typically employed to determine the amount of labeled protein deposition into storage granules [review in Chavez et al., 1994] and this protocol yielded $1.5 \pm 0.2 \times 10^3$ cpm/10^6 cells of ^{35}S-labeled PAI-1. Mock-infected cells exhibit PAI-1 production and storage that is similar to SFV/β-gal infected cells. SFV/PAI-1 infected cells show an increase of 7.5-fold in production and 5.3-fold in storage of ^{35}S-labeled PAI-1. Differentiation of MEG-01 cells by a 72 h PMA treatment period followed by infection with control SFV/β-gal results in 66–87% of basal PAI-1 production/storage. These production levels confirm the data in Figure 1 that demonstrate PAI-1 antigen accumulation during the 72–96 h of PMA treatment is less than the first 24 h. Differentiation of cells with PMA for 72 h followed by infection with SFV/PAI-1 yielded a respective 10- and 8-fold increase in PAI-1 production and storage in comparison to basal values. Isolation of storage granules from cells treated by this optimized PMA-SFV-PAI-1 regiment indicates that PAI-1 storage (300–600

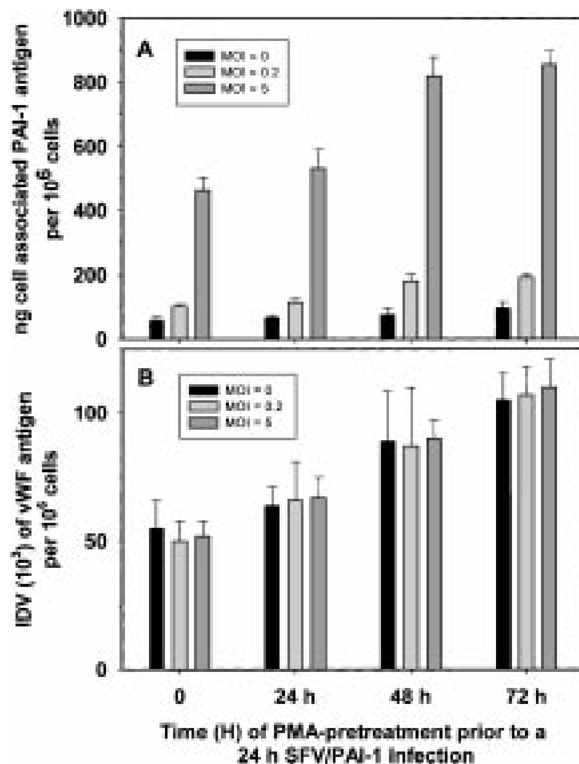

Fig. 6. Length of PMA pretreatment on SFV transduction of PAI-1. MEG-01 cells (10^6 cells/dish, n = 4 dishes/condition) were incubated either in the absence or presence of PMA for 1 to 3 days. At the indicated times, cells were treated with fresh PMA for 24 h in the absence or presence of SFV/PAI-1. Cell lysates were collected and assayed for PAI-1 antigen (panel **A**) and vWF antigen (panel **B**) as described in the Materials and Methods.

ng PAI-1/mg MEG-01 protein) was comparable to platelet α-granules (data not shown).

Because MEG-01 cells produce platelet-like particles that exhibit structures similar to α-granules in human platelets [Takeuchi et al., 1998], we further examined the packaging of PAI-1 in this system by isolating platelet-like particles derived from the SFV- and non-infected MEG-01 cells. Figure 7A indicates that particles derived from the SFV/PAI-1-infected cells contain 540 ± 57 ng PAI-1 antigen/mg protein (closed bar, treatment 2) or approximately 135 ± 14 ng/10^8 particles as calculated by direct particle counting. This value is analogous to the levels of PAI-1 reported for platelets (~60 ng PAI-1 antigen/10^8 platelets) [review in Booth, 2000] and represent 11-fold greater PAI-1 antigen in particles derived from SFV/PAI-1-infected cells in comparison to particles derived from non- or SFV/β-gal-infected cells. We subsequently build upon the observation that MEG-01 platelet-like particles rapidly increase surface expression (18–29 %) of an α-granule protein, P-selectin, following stimulation with thrombin [Takeuchi et al., 1998] and investigated if the PAI-1 stored within these platelet-like particles is packaged in a rapidly releasable form. Treatment of platelet-like particles derived from SFV/PAI-1 infected cells with 5 μM ADP released 33 ± 3 ng PAI-1/mg particles, which is a 5-fold stimulation of PAI-1 release relative to particles incubated in the absence of secretagogue (Fig. 7B, treatment 2). Furthermore, the agonist-induced release of PAI-1 from platelet-like particles derived from SFV/PAI-1 infected cells was 16-fold higher than agonist-induced release of PAI-1 from particles derived from SFV/β-gal- or non-infected cells. To demonstrate the specificity of SFV-mediated PAI-1 packaging, we obtained and employed the construct SFV/signal peptide-PAI-2 (SFV/SP-PAI-2) that routes this protein to the ER-dependent secretory pathway [Mikus et al., 1993]. In agreement with the

TABLE I. PAI-1 Production and Storage in PMA-and SFV-Treated MEG-01 Cells

Time (hour) of PMA pretreatment	24 h infection in presence of PMA	Total PAI-1 (cpm) produced over 24 h in the presence of PMA	Amount (cpm) retained after 6 h chase	Amount retained as % of total produced
0	Mock	$3.1 \pm 0.4 \times 10^4$	$1.5 \pm 0.3 \times 10^3$	4.7 ± 0.9
	SFV/β-gal	$3.2 \pm 0.4 \times 10^4$	$1.5 \pm 0.2 \times 10^3$	4.5 ± 0.9
	SFV/PAI-1	$2.4 \pm 2.5 \times 10^5$	$8.0 \pm 1.1 \times 10^3$	3.0 ± 0.8
	Mock	$2.2 \pm 0.2 \times 10^4$	$1.3 \pm 0.2 \times 10^3$	6.0 ± 0.7
72	SFV/β-gal	$2.1 \pm 0.3 \times 10^4$	$1.3 \pm 0.1 \times 10^3$	6.1 ± 0.8
	SFV/PAI-I	$3.2 \pm 2.2 \times 10^5$	$1.2 \pm 2.1 \times 10^4$	4.5 ± 1.2

MEG-01 cells were incubated in the absence or presence of PMA for 72 h. Non-stimulated or PMA-differentiated cells (2×10^6 cells/35 mm dish, n = 4 dishes/condition) were mock-, control SFV/β-gal-, or SFV/PAI-1- (5 MOI; 37°C) infected in methionine-free media supplemented with ^{35}S-[trans] labels and PMA for 24 h. A duplicate series of cultures were washed and chased with complete media for 6 h. Post-chased cell lysates were immunoprecipitated to determine the level of PAI-1 retained within cells.

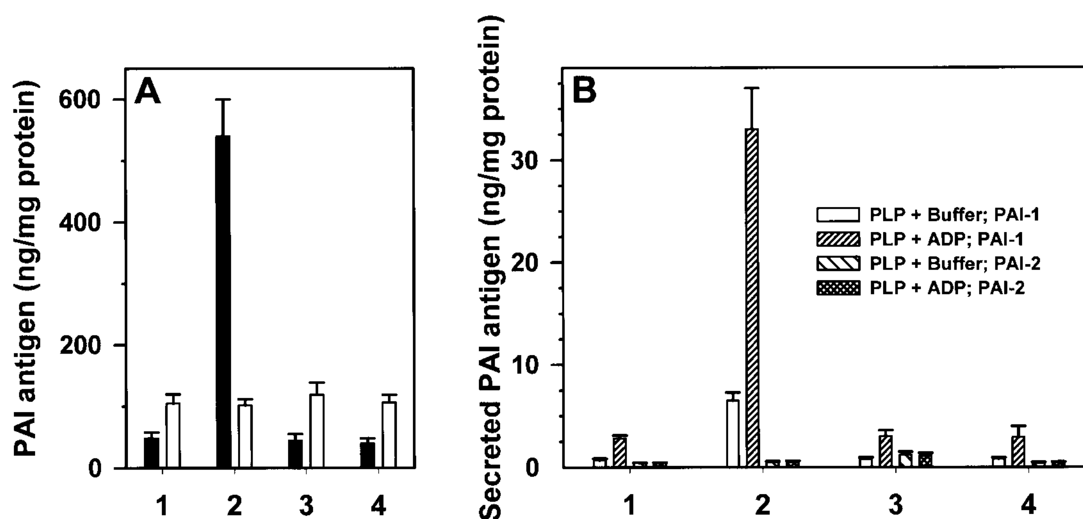

Fig. 7. Effects of secretagogue on the release of PAIs from MEG-01 platelet-like particles (PLP). Panel **A:** Platelet-like particles were isolated from 3-day PMA-treated MEG-01 cells (2 x 10^6, n = 3/treatment) that were either mock- (treatment 1), SFV/ PAI-1- (treatment 2), SFV/ SP-PAI-2- (treatment 3) or SFV/ β-gal-infected (treatment 4) for 24 h and assayed for PAI-1 (closed bars) and PAI-2 (open bars) antigens in the respective two-site ELISAs. Panel **B:** Platelet-like particles (n = 3/condition) derived from either mock- (treatment 1), SFV/PAI-1- (treatment 2), SFV/SP-PAI-2- (treatment 3), or SFV/β-gal-infected (treatment 4) cells were washed twice and incubated for 15 min in the absence or presence of 5 μM ADP. The releasates were harvested by centrifugation and assayed for PAI-1 or PAI-2 antigens.

observations of Booth and co-workers [Alford et al., 1993], PMA-treated MEG-01 cells contain low levels of PAI-2 (Fig. 7A). Although the levels of PAI-2 in media conditioned by SFV/SP-PAI-2 infected cells were ~14-fold higher than basal levels observed for non-, SFV/PAI-1, or SFV/β-gal infected cells (data not shown), SFV/SP-PAI-2 infection did not significantly increase the level of PAI-2 contained within platelet-like particles (Fig. 7A, open bars). Moreover, the treatment of these particles with 5 μM ADP did not stimulate the release of PAI-2 in comparison to particles incubated in the absence of secretagogue (Fig. 7B, treatment 3, bar 3 vs. 4). Our data demonstrate that SFV infection following PMA induced differentiation of MEG-01 cells provides a useful approach for studies of α-granule protein production and storage within platelets.

DISCUSSION

This report provides a detailed analysis of basal and SFV-mediated PAI-1 production in MEG-01 cells. Previous studies on PAI-1 production in MEG-01 cells have been restricted to semi-quantitative analyses utilizing either immunohistochemistry [Alessi et al., 1994], in situ hybridization [Alessi et al., 1994], or reverse transcription-polymerase chain reaction (RT-PCR) [Wohn et al., 1997]. For example, Wohn et al. [1997] reported a biphasic profile for PAI-1 mRNA expression following PMA treatment with the highest level detected 6 h post-treatment followed by a second but smaller peak at the 3 day time point. Our observations extend these studies by demonstrating that, (i) differentiation of MEG-01 cells was found to increase the basal production of PAI-1 by 3–4 fold over a 4 day period of PMA treatment and (ii) PAI-1 antigen continues to be produced and stored for several days following cell-treatment with PMA. Although published studies on other megakaryocytic cell lines [Alitalo et al., 1989; Konkle et al., 1993; Schuster et al., 1993; Hill et al., 1996; Madoiwa et al., 1999] have utilized a variety of techniques to investigate PAI-1 expression, quantitative information on the production of this protein is limited to the studies of the K562 myeloid cell line [Schuster et al., 1993] in which a limited time course (i.e., ≤24h) is described following cell treatment with PMA (e.g., 150 ng PAI-1 antigen/10^6 cells/24 h). In addition to information on PAI-1 production, we demonstrate that a portion (5%) of PAI-1 is stored in MEG-01 cells (Table I). Although the storage efficiency in MEG-01 cells is low in relation to AtT-20 cell line [Moore and Kelly, 1985], this megakaryocytic cell line permits the studies of PAI-1 interaction with other α-

granule proteins. Moreover, we designed a protocol for the isolation of α-granule-like structures from MEG-01 cells. Fractionation on Percoll gradients has proven useful for the isolation of storage granules from several model cell lines and a Percoll-based protocol has been utilized previously by our group for the isolation of platelet α-granules [Lang and Schleef, 1996a]. In our present study, PAI-1/gpIIbIIIa-containing granules isolated from MEG-01 exhibited a density (i.e., 1.04–1.13 g/ml) comparable to published values for platelet α-granules [Gogstad, 1981; Lang and Schleef, 1996a]. Previous studies have shown that a minor level of lysosomes co-purifies with vWF-containing storage granules in one-step Percoll density gradient [Vischer and Wagner, 1993]. We observed a similar level of lysosome contamination in PAI-1/gpIIbIIIa-containing granules derived from fractionation of MEG-01 homogenates on a 45% Percoll gradient; therefore, a second Percoll gradient was employed to reduce the level of contaminating lysosomes. Isolated MEG-01 storage granules display a spherical form with dense core matrix and size that characterize platelet α-granules.

Another important feature of this study is the use of gene transfer to increase PAI-1 expression in differentiated MEG-01 cells. Based upon reports employing liposome-mediated gene transfer as a means to generate stable-transfected megakaryocytic cell lines [Wang et al., 1999; Clemetson et al., 2000], we investigated the utility of several commercially-available liposomal reagents to mediate PAI-1 cDNA transfer into MEG-01 cells; however, data in Figure 4 indicates that these reagents in transient transfection experiments resulted in poor expression. Electroporation has also been used for the transfer of plasmids into solution-phase (non-differentiated) megakaryocytic cell lines [Minami et al., 1998; Wang et al., 1999], but it is difficult to adopt these procedures to large quantities of differentiated cells that are adherent to culture dishes. Although retroviral transduction of non-differentiated megakaryocytic cell lines has been described [Abe et al., 1993; Kiffmeyer et al., 1994], this approach also has limited applicability for PMA-differentiated cells, which are non-proliferating and resistant to retrovirus-mediated gene transfer [Miller et al., 1990]. While both adenovirus and SFV are potential vectors for gene transfer into PMA-differentiated MEG-01 cells, we selected a DNA-based SFV system because of several positive attributes. Firstly, unlike adenovirus, SFV will infect almost any mammalian cell [Berglund et al., 1996]. Secondly, viral RNA amplification by SFV replicase is extremely proficient and results in the highest level of target protein production (i.e., up to 50% of total cellular proteins) [Berglund et al., 1996; DiCiommo and Bremner, 1998]. Thirdly, in contrast to adenovirus that is directly infectious in humans, biohazard is minimized in the SFV system by a point mutation in the viral glycoprotein gene p62, which prevents infection unless the virus is cleaved with α-chymotyrpsin [DiCiommo and Bremner, 1998]. Fourthly, the current SFV system produces no detectable contaminating wild-type virus (e.g., $</8.7 \times 10^7$ infectious particles) [DiCiommo and Bremner, 1998] and involves only a co-transfection of two plasmids into mammalian cells for 2 days to directly generate virus recombinants. In contrast, adenovirus systems typically require an in vivo homologous recombination between plasmids and linear viral DNA to generate adenovirus recombinants. This process also results in a high background of contaminating parental wild-type virus [Graham and Prevec, 1991], which necessitates repeated rounds of plaque purification (~2–4 months). Our data utilizing SFV system indicates that PAI-1 production was elevated with no deleterious effects on its storage efficiency (Table I). SFV-mediate gene transfer into differentiated MEG-01 cells is efficient and increases the amount of PAI-1 in storage granules to the level present in platelet α-granules.

Our study also employs MEG-01 platelet-like particles to demonstrate the relevance of SFV-infected cells as a model for megakaryocytic α-granule protein expression and packaging. Previous studies [Takeuchi et al., 1991, 1998; Yamazaki et al., 1999] have shown that these platelet-like particles and freshly isolated human platelets share similar functions and structures. For example, thrombin treatment of MEG-01 platelet-like particles results in increased surface expression of membrane protein P-selectin and these particles transform from a spherical shape to irregular forms with filopods that mediate their aggregation [Takeuchi et al., 1998]. Our observations extend these studies by documenting that MEG-01 platelet-like particles exhibited a regulated secretion of the α-granule protein PAI-1 (Fig. 7B). More-

over, our data using the construct SFV/SP-PAI-2 revealed that not all proteins are routed into the regulated secretory pathway of megakaryocytic cells (Fig. 7). The specificity for the targeting of protein into MEG-01 storage granules suggests that this approach provides a rapid and valuable tool for studies of packaging of PAI-1 and other α-granule proteins.

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