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Purification of Storage Granule Protein-23

A NOVEL PROTEIN IDENTIFIED BY PHAGE DISPLAY TECHNOLOGY AND INTERACTION WITH TYPE I PLASMINOGEN ACTIVATOR INHIBITOR*

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Type 1 plasminogen activator inhibitor (PAI-1) is a key regulator of the fibrinolytic cascade that is stored in a rapidly releasable form within platelet α-granules. To identify proteins that may participate in the targeting or storage of this potent inhibitor, this report investigates the applicability of utilizing filamentous bacteriophages to display proteins expressed by cells containing a regulated secretory pathway and their enrichment based upon an interaction with PAI-1. For this purpose, RNA was extracted from AtT-20 cells (*i.e.* a classical model cell system for intracellular protein sorting), reverse transcribed, amplified using polymerase chain reaction primers containing internal restriction sites, and cloned into the phagemid pCOMB3H for expression as fusion constructs with the bacteriophage gene III protein. *Escherichia coli* was transformed with the phagemids and infected with VCSM13 helper phage, and the resulting AtT-20 cDNA-bacteriophage library was enriched by panning against solid- and solution-phase PAI-1. The enriched cDNA library was subcloned into a prokaryotic expression vector system that replaces the gene III protein with a decapeptide tag for immunologic quantitation. One novel cDNA clone (*i.e.* A-61), which preferentially recognized solution-phase PAI-1 and reacted positively with antibodies derived from a rabbit immunized with α-granules, was subcloned into the prokaryotic expression vector pTrcHis to create a construct containing an N-terminal six-histidine purification tag. This construct was expressed in *E. coli*, purified by nickel-chelate chromatography followed by preparative SDS-polyacrylamide gel electrophoresis, and utilized for the generation of polyclonal antibodies. Immunoblotting analysis employing antibodies against the purified A-61 construct revealed a 23-kDa protein present in the regulated secretory pathway of AtT-20 cells. The 23-kDa molecule was purified from media conditioned by AtT-20 cells by ion exchange chromatography on DEAE-Sephacel, molecular sieve chromatography on Sephacryl S-100, chromatofocusing on Polybuffer exchanger 94, and affinity chromatography on PAI-1-Sepharose. N-terminal amino acid sequencing of a 16-kDa Lys-C proteolytic fragment of the 23-kDa storage granule protein was employed to confirm its identity with the cDNA sequence of clone A-61. These data indicate that phage display of cDNA libraries fused to the C-terminal region of the gene III protein and their enrichment via an interaction with a target molecule can be utilized to define other proteins present within a particular cellular pathway.

Type-1 plasminogen activator inhibitor (PAI-1)[1] is the primary physiological inhibitor of vascular tissue-type plasminogen activator (for reviews, see Refs. 1 and 2). The role of PAI-1 as a key physiological regulator of the fibrinolytic system is supported by the correlation of bleeding disorders in a number of patients that have a deficiency in blood PAI-1 activity (3–6). Sequence analysis of the cDNA encoding PAI-1 has led to the classification of this inhibitor in the serpin superfamily (1, 2). This inhibitor is produced as a M_r 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 (7–15). The presence of PAI-1 mRNA and antigen in megakaryocytes (16–18), 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.

Current information indicates that PAI-1 is synthesized in an active form, but it is rapidly converted into an inactive form at 37 °C with a half-life of approximately 1 h (for a review, see Ref. 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 detected following treatment with denaturants or negatively charged phospholipids (for reviews, see Refs. 1 and 2). In light of the observation that platelets possess low biosynthetic capabilities (19), 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) (for a review, see Ref. 20), recent data from our group (21) indicate that complexes between vitronectin and PAI-1 are not present in nonactivated platelets. Therefore, little information exists on the proteins that interact and stabilize PAI-1 stored within platelets that have a mean life span of 9–12 days in the circulation (19).

Two distinct pathways are known to be responsible for the secretion of proteins from eukaryotic cells (for reviews, see Refs. 22 and 23). The "constitutive" pathway externalizes proteins rapidly using post-Golgi vesicles and does not require an

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U64446.

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[1] The abbreviations used are: PAI-1, type 1 plasminogen activator inhibitor; serpin, serine protease inhibitor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SGP-23, 23-kDa storage granule protein; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ACTH, adrenocorticotropic hormone; Mops, 4-morpholinepropanesulfonic acid.

external stimulus for release of a compound into the extracellular milieu (22, 23). In the "regulated" pathway, proteins are stored in secretory granules until the cells are stimulated to secrete in response to the appropriate stimuli (22, 23). A number of tumor-derived cell lines exhibit both a constitutive and a regulated 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 (22, 23). 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 (24, 25). Treatment of AtT-20 cells with the appropriate secretagogue (*e.g.* 8-Br-cyclic AMP) results in release of the contents of the secretory granule (24, 26). 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 (26), trypsinogen (25, 27), human growth hormone (28), and peptidylglycine α-amidating monooxygenase (29) are transported to the regulated pathway with a similar efficiency as the endogenous hormone, ACTH. This cell line has also proven useful for investigating the packaging of two proteins stored in both endothelial cells and platelets (*i.e.* P-selectin (30, 31) and von Willebrand's factor (32)). Furthermore, transfection experiments with full-length PAI-1 cDNA demonstrated that this inhibitor is also packaged into AtT-20 dense core storage granules (33). Analysis of PAI-1 within the isolated storage granules has revealed (i) the presence of both active and latent PAI-1 in a ratio comparable with the situation in human platelets and (ii) that PAI-1 activity is stabilized within the secretory granules (33). Taken together, this cell line appears to be a useful model system for identification of proteins within the regulated secretory pathway that interact with PAI-1 and potentially participate in its targeting or stabilization.

The ability of filamentous bacteriophages to display proteins on their surface has been used for the generation of libraries of recombinant antibody fragments (for reviews, see Refs. 34–36) and peptide libraries (for reviews, see Refs. 37 and 38). Antibody fragments or peptides are expressed as fusion proteins with the bacteriophage's gene III or gene VIII surface protein, and the characteristics of the surface-expressed molecule (*e.g.* affinity or interaction with a ligand) can be used as a means to enrich the phage (34–38). In this system, the cDNA encoding the surface-expressed protein/peptide is contained within the bacteriophage's genetic material, thus permitting the rapid identification and cloning of a molecule. This study was initiated to investigate the applicability of this system to identify proteins that potentially interact with PAI-1 within the regulated secretory pathway. In this report, we describe the construction of an AtT-20 cDNA-bacteriophage library and its enrichment based upon its interaction with PAI-1 to identify a novel 23-kDa protein that is present within the regulated secretory pathway.

MATERIALS AND METHODS

Growth of Cultured Cells—AtT-20 cells, DAMI cells (a cell line established from an individual with megakaryoblastic leukemia (39)), and a transformed human fibroblast cell line (SV40 WI38 VA13 2RA) were obtained from the American Tissue Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) in the presence of either a 5% $C0_2$ (DAMI cells and transformed fibroblasts) or 15% CO_2 atmosphere (AtT-20 cells (33)).

Purification of PAI-1 and the Preparation of Antibodies to PAI-1—Native PAI-1 was purified from the media conditioned by transformed human lung fibroblasts as described previously (33, 40). Antibodies to PAI-1 were raised in New Zealand rabbits and affinity-purified on Sepharose-PAI-1 columns as described previously (33).

Preparation of Antibodies to Platelet α-Granules—Human platelets were isolated and utilized for the preparation of α-granules as described previously (21). Briefly, platelets were diluted in homogenization buffer (108 mM NaCl, 38 mM KCl, 1.7 mM $NaHCO_3$, 21.2 mM sodium citrate, 27.8 mM D-glucose, 1.1 mM $MgCl_2$, 1 mM theophyllin, pH 6.5) to a final concentration of 10^9/ml and sonicated using an Astrason Ultrasonic processor XL (Heat Systems Inc., Farmingdale, NY) five times (4 °C, 3 s of sonication on setting 2 followed by a 15-s pause between each sonication). Samples were centrifuged (15 min, 2,000 × *g*); the membrane/organelle/cytosol-containing supernatants were pooled and mixed 1:1 with 40% metrizamide solution (Accurate Chemical and Scientific Co., Westbury, NY); and this mixture was layered on top of a two-step gradient consisting of 1 ml of 35% metrizamide underlayered with 0.5 ml of 38% metrizamide. Following centrifugation (1 h, 4 °C, 100,000 × *g*), the α-granules were harvested from the 20–35% metrizamide interface. This preparation was mixed with an equal volume of 90% stock isotonic Percoll (1 ml of 1.5 M NaCl with 9 ml of Percoll; Pharmacia Biotech, Inc.) and ultracentrifuged (4 °C, 30 min, 20,000 × *g*). The α-granules were recovered as an opaque band at a density of 1.06–1.1 g/liter. Rabbit antibodies directed against α-granules were prepared by immunizing a New Zealand White rabbit with 0.5 ml of isolated α-granules (20 mg of protein/injection) dissolved in Freund's adjuvant over a period of 6 months utilizing standard techniques. The IgG fraction of the antiserum was isolated by ammonium sulfate precipitation and affinity-purified utilizing CNBr-activated Sepharose beads that were coupled to α-granule proteins according to the manufacturer's instructions (Pharmacia).

RNA Isolation and Reverse Transcription—AtT-20 and DAMI cells (1.5×10^8 cells, 10^6 cells/ml) were harvested separately into guanidine thiocyanate followed by extraction of total RNA according to the procedures described by Chomczynski and Sacchi (41) and as detailed previously (42). AtT-20 RNA (15 μg) was reverse transcribed utilizing the commercially available First Strand cDNA synthesis kit (Boehringer Mannheim) and oligo$(dt)_{15}$ primers.

Construction of cDNA Library in pCOMB3H—AtT-20 cell cDNA was amplified in a Perkin-Elmer 9600 thermal cycler utilizing the forward primer (5′-CAGTCGCTCGAGRNNATG-3′) that contained an internal *Xho*I site (underlined) and, in separate reactions, reverse primers (*i.e.* 5′-TGGGCAACTAGTGTANNNNNN-3′; 5′-TGGGCAACTAGTC-CANNNNNN-3′) that contained an internal *Spe*I site (underlined). The following protocol, known as "touchdown" PCR, was utilized to derive specific PCR products: 5 min at 94 °C, 20 cycles consisting of 30 s at 94 °C, 45 s at 53 °C (lowering temperature 0.5 °C each cycle), 3 min at 70 °C, followed by 5 cycles utilizing 30 s at 94 °C, 45 s at 43 °C, and 3 min at 70 °C. PCR products from each library were pooled and subjected to agarose gel electrophoresis, and the region between 3000 and 200 base pairs was electrophoretically eluted from the agarose gel. This material was digested with an excess amount of restriction enzymes *Spe*I and *Xho*I (50 and 200 units/μg DNA, respectively) and ligated into the vector pCOMB3H, a variant of the phagemid pComb3 (43). The final library consisted of 1.5×10^6 clones.

Phage Production and Enrichment—Phagemids were transformed into *Escherichia coli* XL1-Blue cells and grown in super broth medium (SB; 30 g/liter tryptone, 20 g/liter yeast extract, and 10 g/liter Mops, pH 7) at 37 °C supplemented with tetracyclin (10 μg/ml) and carbenicillin (50 μg/ml). Cultures were grown to an A_{600} of 0.8, infected with VCSM13 helper phage (4×10^{11} plaque-forming units/ml), and grown 2 additional h. Kanamycin was added (70 μg/ml), and the culture was incubated overnight. Phage were isolated from liquid culture by polyethylene glycol 8000 and NaCl precipitation (43). Phage pellets were suspended in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl), 1% bovine serum albumin (BSA). Enrichment of phages was performed on microtiter plates (Costar 3690) under the following conditions. Wells were precoated with 1 μg of PAI-1 or 2 μg of affinity-purified rabbit antibodies to PAI-1. Wells were washed twice with water and blocked for 1 h with 3% (w/v) BSA in TBS. Wells coated with PAI-1 were directly incubated (2 h, 37 °C) with 50 μl of mixed phage (typically 10^{11} to 10^{12} colony-forming units). Alternatively, 50 μl of phage ($>10^8$ phage) were incubated (30 min, 37 °C) with 20 ng of PAI-1 (final concentration) followed by incubation (2 h, 37 °C) on polyclonal anti-PAI-1-coated wells. Following incubation, the wells were washed once (first round of panning), 5 times (second round of panning), or 10 times (third to sixth round of panning) with TBS, 0.05% Tween solution. After a final rinse in distilled water, the adherent phage were eluted by incubation (10 min, 22 °C) with 50 μl of elution buffer (0.1 M HCl, adjusted to pH 2.2 with glycine) containing 1 mg/ml BSA. The eluant was removed and neutralized with 3 μl of 2 M Tris base. The initial phage input was determined by titering on selective plates. The final

phage output was determined by infecting 1 ml of logarithmic phase XLI-Blue cells with the neutralized eluant for 15 min at room temperature and plating equal aliquots on carbenicillin plates.

Preparation and Analysis of Soluble Recombinant AtT-20 Proteins That Bind to PAI-1—Phagemid DNA from the panned library was isolated, digested with *Sfi*I, and ligated into the arabinose-inducible expression vector pAraHA (43, 44). The ligation mixture was transformed into *E. coli* DH12S cells and grown overnight in SB containing 30 μg/ml chloramphenicol. Single colonies of AtT-20 cDNA/pAraHA in *E. coli* were picked, grown for 8 h, and induced by incubation (30 °C, 16 h) with 1% arabinose. The bacteria were harvested, lysed into TBS containing 4 μM phenylmethylsulfonyl fluoride (final concentration; Sigma) by four freeze/thawing cycles, and centrifuged, and the cell-free supernatants were incubated (1.5 h, 37 °C) in microtiter wells coated with PAI-1 (1 μg/well) or affinity-purified rabbit antibodies against α-granules (1 μg/well). Alternatively, the bacterial lysates were incubated (0.5 h, 37 °C) with 20 ng of soluble PAI-1 followed by incubation (1 h, 37 °C) of the mixture in wells coated with affinity-purified rabbit antibodies against PAI-1. Controls included wells coated with 1 μg/ml of BSA or normal rabbit IgG. After washing, bound protein was detected by incubating the washed wells with alkaline phosphatase-labeled antidecapeptide (mouse monoclonal anti-YPYDVPDYAS (45), 1:500 dilution, 50 μl/well, 2 h, 37 °C) followed by kinetic measurement of the resulting color change at 405 nm after the addition of the substrate *para*-nitrophenylphosphate. The cDNA inserts were sequenced from both strands using the dideoxytermination method (46) coupled with primers synthesized according to the sequences that are either 5′ or 3′ of the *Sfi*I site in the vector pAraHA (43).

Northern Analysis—RNA (10 μg/lane) from AtT-20 and DAMI cells was separated by denaturing electrophoresis in formaldehyde-containing 1% agarose gels and transferred to Hybond-N nylon membranes (Amersham Corp.) as described previously (42). The cDNA sequence encoding clone A-61 was labeled with [^{32}P]dCTP by random priming using the DECAprime II DNA labeling kit (Ambion Inc., Austin, TX) as described by the manufacturer. The labeled probe was purified using Sephadex G-50 minispin columns (Worthington) resulting in a specific activity of 10^9 cpm/μg. Hybridization of the labeled probe to the nylon membrane was performed in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 50 μg/ml fresh denatured salmon sperm DNA (Life Technologies, Inc.) for 15 h at 65 °C followed by washing in 0.1 × SSC, 0.1% SDS at 60 °C. Hybridization to a ^{32}P-labeled 2.0-kilobase pair human β-actin probe (Clontech) was used to confirm approximate equal loading in all lanes. The blot was exposed for 3 days to Kodak XAR autoradiographic film.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed according to the procedures described by Laemmli (47). Gels were processed either by silver staining as described previously (48), or the electrophoresed proteins were transferred to nitrocellulose as described previously (42, 49). The nitrocellulose was blocked by incubation (1 h, 22 °C) with 5% nonfat milk in PBS, 0.1% Tween 20. The washed blots were incubated (1 h, 22 °C) with a primary antibody (*i.e.* monoclonal anti-decapeptide, 1 μg/ml (45); affinity-purified rabbit anti-A-61, 1 μg/ml, described below) diluted in phosphate-buffered saline (PBS; 50 mM phosphate, pH 7.4, 150 mM NaCl) supplemented with 0.1% Tween 20 followed by the appropriate horseradish peroxidase-labeled secondary antibody (Amersham). For detection of the bound antibodies by enhanced chemiluminescence, the washed blots were soaked (0.125 ml/cm^2) for 1 min with a 1:1 mixture of detection reagent 1 and reagent 2 of the ECL Western blotting detection kit (Amersham), the excess reagent was drained, and the blots were exposed to Kodak XAR film. Quantitation of a 23-kDa protein in various samples (*e.g.* column fractions) was accomplished by subjecting serial dilutions of these samples to SDS-PAGE in parallel with a standard dose response of purified A-61 fusion protein. Following the transfer of the electrophoresed proteins to nitrocellulose, the intensities of the immunoreactive bands corresponding to the 23-kDa protein and the A-61 purified protein were determined by laser densitometry and ImageQuant software (both from Molecular Dynamics).

Preparation and Purification of His$_6$-A-61 Construct—The cDNA insert encoding clone A-61 was removed from pAraHA vector using the *Xho*I site on the 5′-end of the insert and a *Bst*BI site following the cDNA sequence encoding the decapeptide tag. The A-61-decapeptide cDNA sequence was isolated by agarose gel electrophoresis and ligated in-frame into a *Xho*I/*Bst*BI-cleaved pTrcHisA (Invitrogen Corp., San Diego, CA). *E. coli* XL1-Blue was transformed separately with pTrcHis/A-61 and pTrcHisA (vector control), and single colonies were picked and confirmed by cDNA sequencing. pTrcHis/Å-61-transformed and pTrcHis-transformed *E. coli* (1 liter of culture/preparation) were induced with isopropyl-β-D-thiogalactopyranoside for 4 h, and the bacteria were pelleted and resuspended in nickel-chelate column equilibration buffer (300 mM NaCl, 50 mM phosphate buffer, pH 8). The bacteria were lysed by incubation (20 min, 30 °C) with 100 μg/ml lysozyme (Sigma) followed by three freeze/thawing cycles. The lysates were clarified by centrifugation and passed separately over nickel-chelate columns (5 × 1 cm, Invitrogen) previously equilibrated with column buffer. The column was washed with column buffer supplemented with 0.1% Triton X-100, 10% glycerol and eluted with an imidazole gradient (0–0.5 M) in column buffer, and the eluted material was analyzed by SDS-PAGE followed by silver staining or immunoblotting using the anti-decapeptide as the primary antibody. The A-61 fusion protein was observed to elute between 0.25 and 0.4 M imidazole. These fractions were pooled, concentrated 10-fold with Centriprep-10 spin columns (Amicon), and subjected to preparative SDS-PAGE on 3-mm-thick gels employing a 12% separating gel and a 4% stacking gel. Following electrophoresis (16 h, 22 °C, 50 V constant voltage), the separating gel was sectioned into 2-mm slices, and the slices were agitated (16 h, 4 °C) in PBS containing 0.1% Triton X-100 (2 ml/slice). Analytical SDS-PAGE followed by immunoblotting with the anti-decapeptide was used to identify fractions containing the A-61 fusion protein. The SDS-gel-purified A-61 fusion protein was injected into a New Zealand White rabbit (100 μg/injection, eight injections over a 4-month period) in the presence of Freund's adjuvant utilizing standard protocols. The purified A-61 fusion protein was also coupled to CNBr-activated Sepharose (500 μg of A-61 construct/ml of packed beads; Pharmacia) according to the manufacturer's instructions. The IgG fraction of the antisera directed against the A-61 fusion protein was isolated utilizing previously described protocols (40), and 100 mg of IgG was circulated (16 h, 4 °C) through the A-61-Sepharose column. The column was washed with PBS and eluted with 0.2 M glycine-HCl, pH 2.5, and the eluted antibodies were immediately neutralized with 1 M Tris-HCl, pH 8.1.

Analysis of AtT-20 Cells with Antibodies Directed against the Purified A-61 Fusion Protein—Cell lysates were prepared by detaching washed cells with PBS supplemented with 20 mM EDTA, followed by centrifugation (1000 × g, 10 min), resuspension of the cell pellet in PBS, and dilution to 10^6 cells/ml in PBS containing 0.5% Triton X-100.

Isolation of dense core storage granules from AtT-20 cells was performed as described previously (33). Briefly, AtT-20 cells (five 75-cm^2 tissue culture flasks) were washed twice with PBS and detached by scraping into 10 ml of homogenization buffer (0.2 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.2). The cells were centrifuged, washed with PBS, and resuspended in 2 ml of homogenization buffer. The suspension was subjected to 20 strokes in a Dounce homogenizer (size 19; Kontes Glass Co., Vineland, NJ) at 4 °C. Nuclei and cellular debris were removed by centrifugation (600 × g, 10 min), and the supernatant was centrifuged (10,000 × g, 5 min) to collect cell membranes and granules. The pellet was resuspended in 200 μl of homogenization buffer, layered on 13 ml of 20% Percoll, 0.25 M sucrose, 20 mM Tris-HCl, pH 7.2, in a Beckman quick-seal centrifuge tube (16 × 76 mm), and centrifuged (20,000 × g, 30 min) in a Beckman L7–65 ultracentrifuge using a Vti65 rotor. Fractions (800 μl) were collected sequentially from the top.

To stimulate cells with secretagogues, AtT-20 (10^6 cells/dish) were plated in 60-mm diameter tissue culture dishes, and the cells were allowed to equilibrate by incubation (37 °C, 24 h) in growth media. The cells were washed four times over a 2-h period with serum-free media and incubated in 3 ml of serum-free media in the absence or presence of a secretagogue (*i.e.* 5 mM 8-Br-cyclic AMP or 56 mM KCl (25, 30)). Conditioned media were collected after 1 h at 37 °C.

Purification of a 23-kDa Protein from AtT-20 Cells—AtT-20 cells were grown to confluence in 162-cm^2 tissue culture flasks, and the cells were washed 3 times with PBS and incubated in 25 ml of serum-free media. The conditioned media were harvested after 24 h, and the process was repeated. The conditioned media were dialyzed (4 °C) against 30 volumes of 10 mM Tris-HCl, pH 7.4 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and the dialysis buffer was changed 4 times over a 3-day period. Based upon experiments utilizing a series of different Pharmacia column resins (described under "Results"), the dialyzed AtT-20-conditioned media (5 liters/preparation) were passed sequentially through a concanavalin A-Sepharose column (30 × 1 cm), a heparin-Sepharose column (30 × 1 cm), and a DEAE-Sephacel column (30 × 1 cm) previously equilibrated in 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4. The columns were washed, and the DEAE-Sepharose column was disconnected and eluted separately, employing a 0–0.15 M NaCl linear gradient (500-ml gradient, 5 ml/fraction) followed by 0.5 M NaCl in 10 mM Tris-HCl, pH 7.4. Fractions containing the 23-kDa A-61 immunologically related protein were pooled, concentrated to 1 ml, and

chromatographed on a Sephacryl S-100 column (100 × 1 cm, Pharmacia; 20 ml/h, 2 ml/fraction) equilibrated in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4. Fractions containing the 23-kDa A-61 immunologically related protein were pooled, dialyzed against 0.025 M imidazole-HCl, pH 7, and subjected to chromatofocusing by application to a Polybuffer exchanger 94 column (20 × 0.5 cm, Pharmacia; 10 ml/h) previously equilibrated with 0.25 M imidazole-HCl, pH 7. The column was eluted with 15 column volumes of Polybuffer 74 (Pharmacia; diluted 1:8 with distilled water, pH 4, with HCl). Fractions containing the 23-kDa A-61 immunologically related protein were pooled, applied to a PAI-1-Sepharose column (500 μg of PAI-1 coupled to 1 ml of packed CNBr-Sepharose according to instructions provided by Pharmacia), and eluted with 0.5 M NaCl in 10 mM Tris-HCl, pH 7.4. Proteolytic digestion of the purified 23-kDa protein was performed by incubation (37 °C, 0.5–4 h) with Endoproteinase Lys-C (sequencing grade, 1:50 ratio; Promega). The mixtures were subjected to electrophoresis in duplicate, and the electrophoresed peptides were transferred to Immobilon PSQ (Millipore). Immunoblotting utilizing anti-A-61 as the primary antibody revealed a 16-kDa fragment utilizing a 1-h period of incubation. The region corresponding to this fragment was excised from a nonblocked Immobilon sheet and subjected to Edman degradation utilizing a Procise model 492 N-terminal amino acid sequencing apparatus (Applied Biosystems, Foster City, CA).

Detection of Storage Granule Protein-23 (SGP-23) Associated with Platelets and Its Specificity for PAI-1 in Comparison with Other Proteins—Murine blood was collected into acid citrate dextrose (0.025 M citric acid, 0.85 M sodium citrate, 2% dextrose; 1 part acid citrate dextrose, 5 parts whole blood) via cardiac puncture of halothane-anesthetized mice. Platelet-rich plasma was prepared by centrifugation of anticoagulated whole blood (160 × *g*, 15 min). The platelet-rich plasma was aspirated and centrifuged (680 × *g*, 20 min). The platelet pellet was washed twice by centrifugation with Tris-buffered saline (0.15 M NaCl, 0.2 M Tris-HCl). Murine platelets (10^9/ml) were incubated (20 min, 37 °C) either in the absence or presence of calcium ionophore (1 μM, Sigma), centrifuged (10,000 × *g*, 10 min, 4 °C), and subjected to SDS-PAGE followed by immunoblotting using either affinity-purified antibodies to A-61 or normal rabbit IgG. To investigate the ability of SGP-23 to interact with PAI-1 in comparison with a number of other proteins, CNBr-activated Sepharose (25 μl) was incubated overnight in the presence of 2 μg of either PAI-1 or the following purified proteins: ovalbumin, BSA, IgG, hemoglobin, aprotinin, tissue-type plasminogen activator, fibrinogen, vitronectin, fibronectin, or ACTH; Sigma). The beads were washed, blocked with 0.1 M Tris-HCl, pH 7.4, and incubated with 0.5 ml of dialyzed AtT-20 cell-conditioned media. The beads were washed and boiled in the presence of SDS-sample buffer under reducing conditions, and the eluted material was analyzed by SDS-PAGE followed by immunoblotting with anti-A-61.

RESULTS

Construction of an AtT-20 cDNA Library on the Surface of Filamentous Bacteriophages and the Selective Enrichment of cDNA Clones with PAI-1—AtT-20 RNA was reverse transcribed and amplified utilizing *Xho*I- and *Spe*I-containing primers, and the resulting PCR products were ligated into the phagemid pCOMB3H. The AtT-20 cDNA-bacteriophage library was enriched or "panned" by utilizing PAI-1 bound either directly to microtiter wells or immunoabsorbed to antibody-coated microtiter wells. The cDNA inserts present within the panned library were subcloned into a prokaryotic expression vector system (*i.e.* pAraHA) in order to replace the C-terminal region encoding the bacteriophage gene III protein with a sequence encoding a decapeptide tag for immunologic analysis. *E. coli* was transformed, 120 clones were induced with arabinose, and the resulting lysates were analyzed for their ability to bind to PAI-1 by an enzyme-linked immunoassay. A series of 30 clones that interacted with either solid-phase PAI-1 and/or solution-phase PAI-1 were sequenced, and following the elimination of duplicates, two distinct clones were identified (Table I). Analysis of these clones on microtiter wells coated with antibodies directed against α-granule proteins revealed that the proteins expressed by these two clones reacted positively with these antibodies (*i.e.* >0.5 net change in A_{405}; Table I).

Detection and Subcellular Localization of an AtT-20 Cell Protein Immunologically Related to Clone A-61—Because the potential existed that PAI-1 may have associated with the plastic microtiter wells in a manner that would mask a particular site on the molecule, we selected for further analysis clone A-61, which reacts preferentially against the solution-phase form of PAI-1. Data base searches (plus/plus orientation) of the sequence encoding clone A-61 (Fig. 1) indicated that the 3′-end of clone A-61 (*i.e.* nucleotides 288–342) is 91% identical with nucleotides 1–57 of a cDNA clone for an expressed sequence tag derived from human mRNA (clone CAM tEST1E2; EMBL accession number X85685), whereas nucleotides 154–342 of clone A-61 are 83% identical with a longer human cDNA clone (78G05; EMBL accession number F00897). Northern blotting analysis utilizing a ^{32}P-labeled A-61 probe revealed a single transcript in AtT-20 (Fig. 2*A*, *lane 1*) as well as in DAMI cells (Fig. 2*A*, *lane 2*), a human megakaryocytic cell line known to produce small amounts of storage granules (39). To determine the characteristics of the molecule corresponding to clone A-61, the cDNA insert was subcloned into the prokaryotic expression

TABLE I

Enzyme-linked immunoassay of AtT-20 clones for their affinity for PAI-1 and their reactivity toward antibodies directed against α-granules

Single colonies of AtT-20 cDNA/pAraHA in *E. coli* were picked, grown for 8 h, and induced by incubation (30 °C, 16 h) with 1% arabinose. The bacteria were harvested and lysed, and the cell-free supernatants were incubated (1.5 h, 37 °C) in microtiter wells coated with PAI-1 (1 μg/well) or affinity-purified rabbit antibodies against α-granules (1 μg/well). Alternatively, the bacterial lysates were incubated (0.5 h, 37 °C) with 20 ng of soluble PAI-1 followed by incubation (1 h, 37 °C) of the mixture in wells coated with affinity-purified rabbit antibodies against PAI-1. After washing, bound protein was detected by incubation with alkaline phosphatase-labeled anti-decapeptide followed by measurement of the change in A_{405} during incubation with *para*-nitrophenylphosphate. Background consisted of wells coated with either BSA or normal rabbit IgG.

Clone	Assay		
	Solid-phase PAI-1	Soluble PAI-1/ solid-phase rabbit anti-PAI-1	Solid-phase rabbit anti-α-granules
	$\Delta A_{405} - \Delta A_{405\ (background)}$		
A-61	0.54	2.35	0.74
A-104	5.17	1.32	0.62

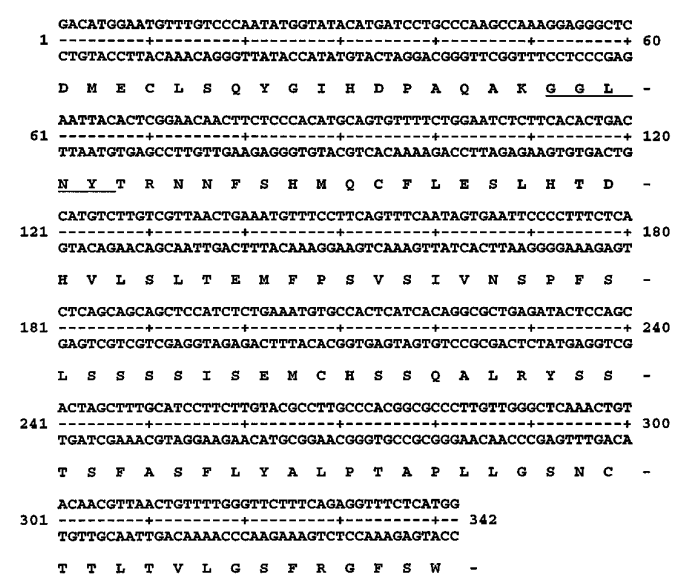

FIG. 1. **cDNA sequence of the insert encoding clone A-61.** The insert encoding clone A-61 was sequenced in pAraHA as described under "Materials and Methods," and the sequence shown immediately follows the *Xho*I site, whereas the 3′-region immediately precedes the *Spe*I site.

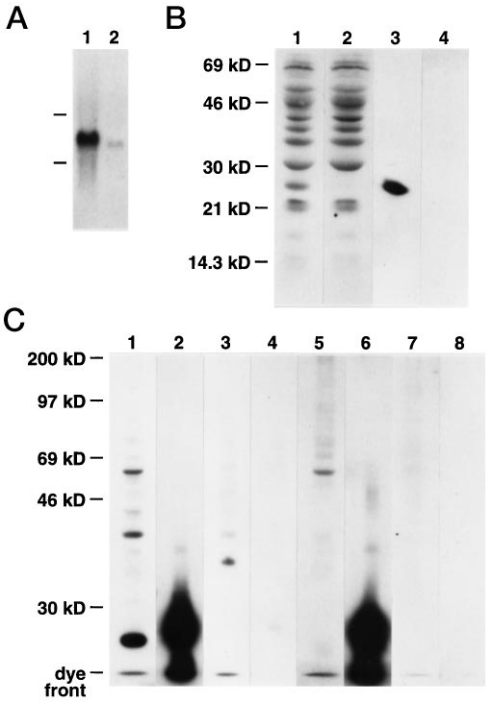

FIG. 2. **Application of clone A-61 for the analysis of AtT-20 RNA and protein.** *A*, Northern blotting analysis of AtT-20 and DAMI cell mRNA with clone A-61. Total RNA was isolated from AtT-20 (*lane 1*) and DAMI (*lane 2*) cells, subjected to agarose electrophoresis (10 μg/lane), transferred to nitrocellulose, and probed with a ^{32}P-labeled A-61 probe. The positions of 28 and 18 S ribosomal RNA are indicated. *B*, prokaryotic expression of pTrcHis/A-61. pTrcHis/A-61-transformed (*lanes 1* and *3*) or pTrcHis-transformed (*lanes 2* and *4*) *E. coli* (1 liter of culture/preparation) were induced with isopropyl-β-D-thiogalactopyranoside for 4 h, and the bacteria preparations were lysed and fractionated on nickel-chelate columns as described under "Materials and Methods." The material eluting between 0.25 and 0.4 M imidazole was pooled, subjected to SDS-PAGE (50 μg/lane), and analyzed by silver staining (*lanes 1* and *2*) or by immunoblotting employing anti-decapeptide as the primary antibody (*lanes 3* and *4*). *C*, identification of an immunologically related protein in AtT-20 cells with anti-A-61. AtT-20 cells (*lanes 1*, *3*, *5*, and *7*; 10^5 cells/lane) and purified A-61 (*lanes 2*, *4*, *6*, and *8*; 500 ng/lane) were subjected to SDS-PAGE under reducing (*lanes 1–4*) or nonreducing (*lanes 5–8*) conditions, transferred to nitrocellulose, and analyzed by immunoblotting with antibodies directed against A-61 (*lanes 1*, *2*, *5*, and *6*) or normal IgG (*lanes 3*, *4*, *7*, and *8*).

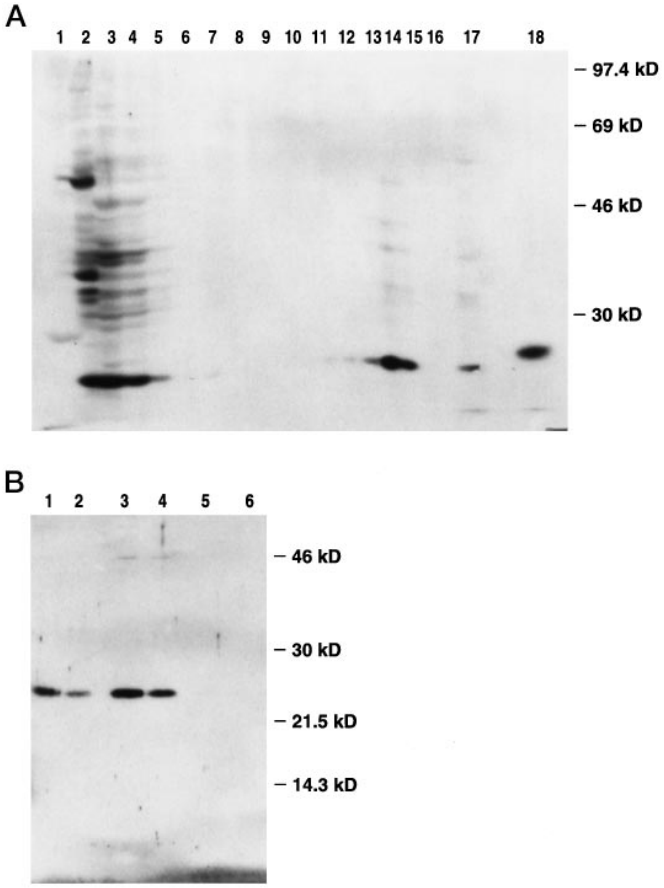

FIG. 3. **Detection of a 23-kDa protein immunologically related to clone A-61 in the regulated secretory pathway of AtT-20 cells.** *A*, immunoblotting analysis of Percoll fractionated AtT-20 cells with anti-A-61. AtT-20 cells were homogenized, and the 10,000 $\times$ *g* pellet was layered on 20% Percoll. After centrifugation at 20,000 $\times$ *g* for 30 min, 800-μl fractions were collected from the top (*lanes 1–16*) and analyzed by SDS-PAGE and immunoblotting using antibodies against A-61. *Lane 17* contains 100 μl of cell homogenate, and *lane 18* contains 100 ng of the purified A-61 fusion protein. *B*, agonist-induced release of 23-kDa protein from AtT-20 cells. AtT-20 cells were washed three times with serum-free media over a 2-h period and then incubated for 1 additional h in serum-free media supplemented with KCl (56 mM; *lanes 1* and *2*) or 8-bromo-cyclic AMP (5 mM; *lanes 3* and *4*) or in serum-free media alone (*lanes 5* and *6*). The conditioned media were harvested and analyzed as described above.

vector pTrcHisA, which links a His_6 tag onto the N-terminal end of the molecule. The construct was expressed in *E. coli*, and the fusion protein was enriched on nickel-resin columns. One additional protein (24.5 kDa) was enriched in the eluant obtained from the pTrcHis/A-61-transformed cells (Fig. 2*B*, *lane 1*) that reacted positively, utilizing antibodies to the decapeptide tag (Fig. 2*B*, *lane 3*) in comparison with the eluant of cells transformed with vector alone (Fig. 2*B*, *lanes 2* and *4*, respectively). The M_r of this additional protein is in accord with a molecule composed of the His_6 tag (39 amino acids), A-61 (115 amino acids), and the C-terminal fusion region containing a decapeptide tag (17 amino acids). Preparative SDS-PAGE was used as a final purification step, and the purified protein was used to raise antibodies in rabbits. The antiserum was affinity-purified on columns composed of the purified A-61 fusion protein conjugated to Sepharose 6B beads, and the affinity-purified antibodies were employed to probe the AtT-20 lysates for proteins immunologically related to A-61. Fig. 2*C* indicates that the affinity-purified anti-A-61 reacted with a limited series of proteins in the AtT-20 cell lysates (*lane 1*) that were not present in the blots probed with normal rabbit IgG (*lane 3*). For example, a 23-kDa protein in the AtT-20 lysates was only detected following reducing SDS-PAGE conditions by affinity-purified anti-61 (*lane 1 versus lane 5*), whereas the purified A-61 fusion construct was recognized in the presence or absence of reducing agents (*lane 2 versus lane 6*). These observations are in accord with the absence of a signal peptide in the pTrcHisA expression vector, resulting in the cytoplasmic localization of the A-61 fusion protein and hence the generation of antibodies to a molecule that lacked proper disulfide bonds.

Two important characteristics of a protein present in the regulated secretory pathway in AtT-20 cells are (i) its presence in dense core secretory granules and (ii) an ability to be released following treatment of the cells with known secretagogues (22, 23). To determine if any of the A-61-related proteins are contained within storage granules of AtT-20 cells, these cells were subfractionated on a Percoll density gradient, and the isolated fractions were analyzed by SDS-PAGE and immunoblotting using antibodies to A-61. Although several immunoreactive proteins were detected in the low density fractions (Fig. 3*A*, *lanes 2–4*), which have been reported to contain cellular membranes, endoplasmic reticulum, Golgi apparatus, etc. (25, 30), a 23-kDa protein was prominently detected in the high density fractions (*lanes 14–15*) known to contain the dense core

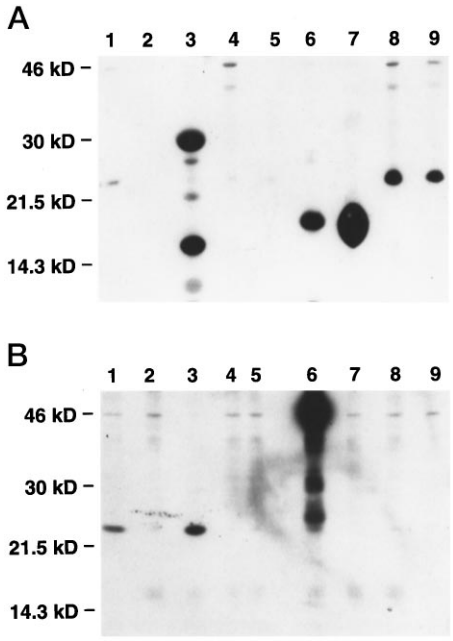

FIG. 4. **Affinity of the 23-kDa A-61-related protein for various chromatographic matrices.** *A*, conditioned media of AtT-20 cells (0.5 ml) were incubated with 25 μl of the following Pharmacia column matrices: Sepharose 6B (*lane 2*), concanavalin A-Sepharose (*lane 3*), heparin-Sepharose (*lane 4*), blue Sepharose (*lane 5*), wheat germ lectin-Sepharose (*lane 6*), lentil lectin-Sepharose (*lane 7*), DEAE-Sepharose (*lane 8*), and lysine-Sepharose (*lane 9*). The beads were washed three times and boiled in the presence of 100 μl of SDS-sample buffer under reducing conditions. The eluted soluble proteins were subjected to SDS-PAGE and immunoblotting analysis using antibodies against the A-61 fusion protein. *Lane 1* contains 100 μl of AtT-20 cell-conditioned media. *B*, CNBr-activated Sepharose (25 μl) was incubated overnight alone (*lane 2*) and in the presence of 2 μg of PAI-1 (*lane 3*), ovalbumin (*lane 4*), BSA (*lane 5*), IgG (*lane 6*), hemoglobin (*lane 7*), aprotinin (*lane 8*), or tissue-type plasminogen activator (*lane 9*). The beads were washed, blocked with 0.1 M Tris-HCl, pH 7.4, and incubated with 0.5 ml of AtT-20 cell-conditioned media. The beads were washed and then boiled in the presence of SDS-sample buffer under reducing conditions, and the eluted material was analyzed as described above. *Lane 1* contains 100 μl of AtT-20-conditioned media.

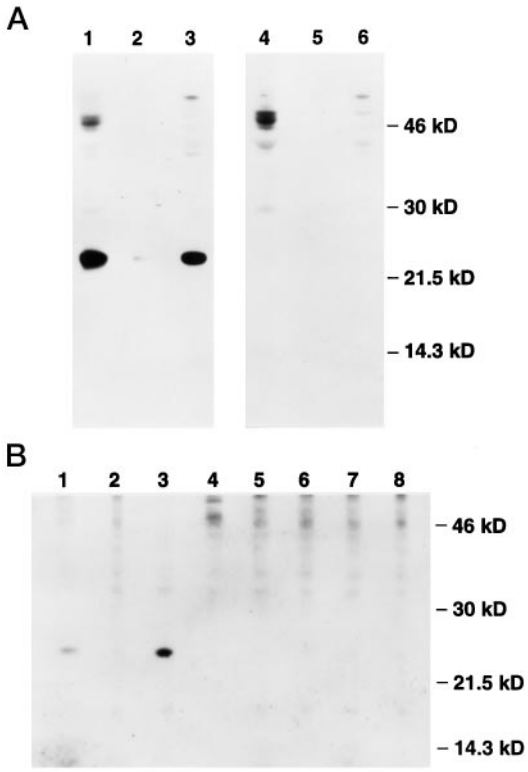

FIG. 5. **Detection of SGP-23 associated with platelets and its specificity for PAI-1 in comparison with other constituents of storage granules.** *A*, murine platelets (10^9/ml) were incubated either in the absence or presence of calcium ionophore followed by pelleting of the platelets by centrifugation. The samples (10^8 platelets (*lanes 1* and *4*); supernatant of 10^8 nonactivated platelets (*lanes 2* and *5*); releasate of 10^8 A23187-activated platelets (*lanes 3* and *6*)) were subjected to SDS-PAGE and immunoblotting using either affinity-purified antibodies to A-61 (*lanes 1–3*) or normal rabbit IgG (*lanes 4–6*). *B*, CNBr-activated Sepharose (25 μl) was incubated overnight alone (*lane 2*) and in the presence of 2 μg of PAI-1 (*lane 3*), fibrinogen (*lane 4*), vitronectin (*lane 5*), fibronectin (*lane 6*), ACTH (*lane 7*), or BSA (*lane 8*). The beads were washed, blocked with 0.1 M Tris-HCl pH 7.4, and incubated with 0.5 ml of AtT-20 cell-conditioned media. The beads were washed and then boiled in the presence of SDS-sample buffer under reducing conditions, and the eluted material was analyzed as described above. *Lane 1* contains 100 μl of AtT-20-conditioned media.

secretory granules (25, 30). Control dot blotting experiments performed as described previously (33) confirmed that this high density region contained the endogenously synthesized and stored hormone ACTH (data not shown). Further evidence for the presence of this 23-kDa protein in the regulated secretory pathway would be obtained if the release of this molecule could be demonstrated in the presence of an appropriate secretagogue. Therefore, AtT-20 cells were grown overnight in six-well microplates, washed repeatedly, and treated either in the absence or in the presence of a secretagogue. Fig. 3*B* indicates a representative experiment in which treatment of the AtT-20 cells with secretagogues (*i.e.* KCl (*lanes 1* and *2*); 8-bromo cAMP (*lanes 3* and *4*)) resulted in an increase in the levels of the 23-kDa protein in the conditioned media in comparison with the levels present in the conditioned media in the absence of a secretagogue (*lanes 5* and *6*). These data indicate that the 23-kDa protein immunologically related to clone A-61 is present in the regulated secretory pathway, and this molecule is referred to as SGP-23 based upon its subcellular distribution and M_r under denaturing/reducing conditions.

Interaction of SGP-23 with Chromatographic Resins and Proteins and Its Release from Platelets—Studies utilizing the AtT-20 cell line have revealed that proteins deposited into secretory granules are also released slowly from these cells into the culture media (25, 28). Therefore, a series of experiments were performed with the media conditioned by AtT-20 cells to investigate the ability to utilize this material as a source of SGP-23 and establish the affinity of SGP-23 for various chromatographic resins. Fig. 4*A* indicates that SGP-23 released from AtT-20 cells (*lane 1*) bound to DEAE-Sepharose (*lane 8*) and lysine-Sepharose (*lane 9*), but this protein did not associate with Sepharose beads alone, concanavalin A Sepharose, heparin-Sepharose, blue-Sepharose, wheat germ lectin-Sepharose, or lentil lectin-Sepharose (*lanes 2–7*, respectively). Bands in *lanes 3*, *6*, and *7* correspond to lectins that are dissociated from the beads under boiling and SDS-reducing conditions. Fig. 4*B* indicates that SGP-23 bound to PAI-1 coupled to Sepharose beads (*lane 3*) but did not associate with beads coated with ovalbumin, BSA, goat IgG, hemoglobin, aprotinin, or tissue-type plasminogen activator (*lanes 4–9*, respectively). Immunoreactive bands in *lane 6* correspond to the heavy and light chain of IgG dissociated from the beads under boiling and SDS-reducing conditions.

The ability of SGP-23 to exhibit a specificity for Sepharose beads coated with PAI-1, an α-granule constituent, raises the possibility that SGP-23 might associate with other α-granule proteins. To extend our analysis to this group of proteins, it was first necessary to establish that SGP-23 is a platelet protein and that it is released from platelets by agonists known to induce the secretion of soluble α-granule proteins. Fig. 5*A*

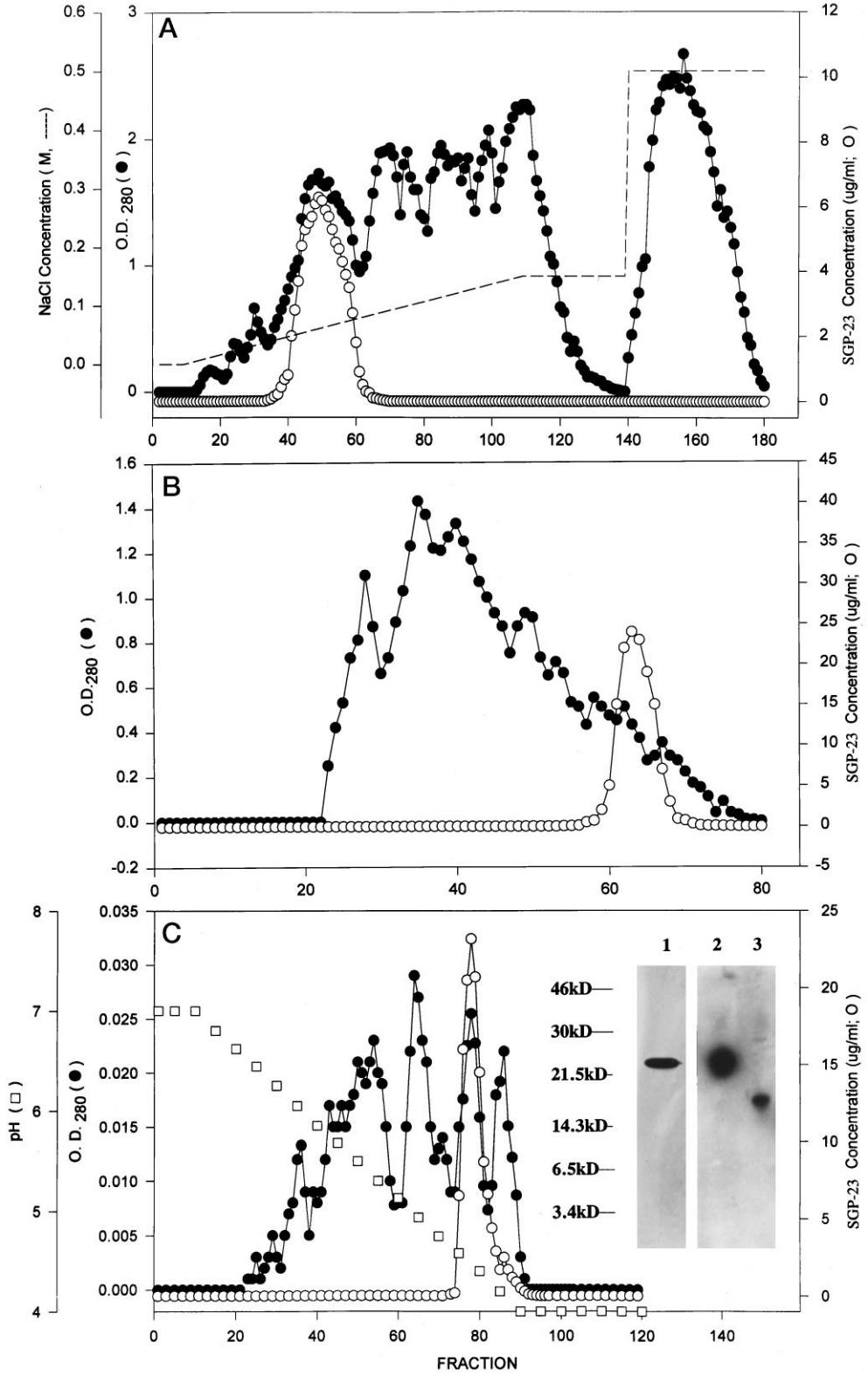

FIG. 6. **Purification of SGP-23 from AtT-20-conditioned media.** *A*, AtT-20-conditioned media were dialyzed against column buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and the dialysate was passed sequentially through a concanavalin A-Sepharose column (30 × 1 cm), a heparin-Sepharose column (30 × 1 cm), and a DEAE-Sephacel column (30 × 1 cm) previously equilibrated in column buffer. The columns were washed, and the DEAE-Sephacel column was disconnected and eluted separately, employing a 0–0.15 M NaCl linear gradient (500-ml gradient, 5 ml/fraction) followed by 0.5 M NaCl in 10 mM Tris-HCl, pH 7.4. *Dashed line*, NaCl concentration. A_{280} (●) and SGP-23 (○) were measured as described under "Materials and Methods." *B*, fractions containing SGP-23 from the DEAE-Sephacel column were pooled, concentrated to 1 ml, and chromatographed on a Sephacryl S-100 column (100 × 1 cm, Pharmacia; 20 ml/h, 2 ml/fraction) equilibrated in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4. Fractions were analyzed for A_{280} (●) and SGP-23 (○). *C*, Sephacryl S-100 fractions containing SGP-23 were pooled, dialyzed against 0.025 M imidazole-HCl, pH 7, and applied to a Polybuffer exchanger 94 column (20 × 0.5 cm, Pharmacia; 10 ml/h) previously equilibrated with 0.25 M imidazole-HCl, pH 7. The column was eluted with 15 column volumes of Polybuffer 74 (Pharmacia; diluted 1:8 with distilled water, pH 4, with HCl). Values for A_{280} (●), pH (□), and SGP-23 concentration (○) are shown. *Inset*, ampholines were removed from the SGP-23/Polybuffer-containing fractions by affinity chromatography on PAI-1-Sepharose as described under "Materials and Methods." The SGP-23 preparation (1 μg) was

TABLE II
Summary of purification of SGP-23

Purification step	Total volume	Protein concentration	Total protein	SGP-23 concentration	Total SGP-23
	ml	*μg/ml*	*μg*	*μg/ml*	*μg*
Starting material	5000	2454	12,270.00	0.18	900
DEAE-Sepharose	100	1125	112.50	4.77	477
Sephacryl S-100	15	375	5.62	18.91	284
Polybuffer exchanger 74	10	23	0.23	15.81	158
PAI-1-Sepharose	6	21	0.13	20.35	122

indicates that a 23-kDa protein can be detected associated with mouse platelets using the affinity-purified antibodies directed against clone A-61 (*lane 1*), which is not detected in the platelet lysate sample probed with normal rabbit IgG (*lane 4*). Furthermore, this 23-kDa protein is also present in the releasate of platelets stimulated with calcium ionophore A23187 and analyzed by SDS-PAGE/immunoblotting with anti-A-61 (*lane 3*) but not in the supernatant of nonactivated platelets analyzed similarly (*lane 2*). Based upon this information, a series of known α-granule proteins, as well as the classical marker protein for AtT-20 storage granules (ACTH), were subsequently coupled to CNBr-Sepharose, and the affinity matrices were incubated with AtT-20-conditioned media. Analysis of the bound proteins by SDS-PAGE followed by immunoblotting using anti-A-61 revealed that SGP-23 bound to PAI-1-coated Sepharose beads (*lane 3*) but did not bind to Sepharose beads coated with fibrinogen (*lane 4*), vitronectin (*lane 5*), fibronectin (*lane 6*), ACTH (*lane 7*), or the negative control protein (BSA; *lane 8*). Taken together, these data suggest that SGP-23 preferentially associates with PAI-1.

Purification of SGP-23 from AtT-20 Cells—Based upon the aforementioned data, a purification scheme was optimized for the isolation of native SGP-23 (Fig. 6, Table II). This protocol utilized chromatography of AtT-20-conditioned media on DEAE-Sephacel with an elution of SGP-23 between 0.04 and 0.08 M NaCl (Fig. 6*A*). Molecular sieve chromatography on Sephacryl S-100 was employed as a second step to select for proteins in the 15–30-kDa region (Fig. 6*B*). Subsequent purification steps employed chromatofocusing on Polybuffer exchanger 94 with SGP-23 eluting at an acidic pH of 4.6–4.2 (Fig. 6*C*) and affinity purification on PAI-1-Sepharose. To further define the region within SGP-23 that cross-reacts with anti-A-61, the purified protein (Fig. 6*C*, *inset*, *lane 1*) was digested with endoproteinase Lys-C, which cleaves on the carboxylic side of lysine, resulting in the appearance of a 16-kDa immunoreactive product using anti-A-61 (Fig. 6*C*, *inset*, *lane 3*). Continued incubation with Lys-C resulted in the generation of smaller peptides with the concomitant loss of immunoreactivity to anti-A-61 (data not shown). N-terminal amino acid sequencing of the 16-kDa Lys-C fragment of SGP-23 revealed the sequence Gly-Gly-Leu-Asn-Tyr, which is identical to the amino acid sequence following the first lysine residue encoded by the A-61 cDNA (Fig. 1, *underlined*).

DISCUSSION

This report describes the identification and purification of a novel protein (SGP-23) present in the regulatory secretory pathway of AtT-20 cells. This protein was identified by the construction and subcloning of an AtT-20 cDNA library into the heavy chain site of phagemid pCOMB3H and by the expression and enrichment of phage by an interaction with PAI-1. The advantage of filamentous phage display cloning over conventional cloning is derived from the physical linkage of the cloned protein to the bacteriophage's coat protein and hence to the genetic material that encodes it. Fusion constructs with the gene III coat protein have been formed between not only antibodies and peptides but also a number of proteins (for a review, see Ref. 50). With regard to PAI-1, this system has been employed (i) for the generation of monoclonal antibody binding fragments (Fab) specific for PAI-1 (44), (ii) to identify structural epitopes on the PAI-1 molecule (51), and (iii) for the expression and display of functionally active PAI-1 (52). These latter studies (52) documented the feasibility of the system for the preparation of a large library of predominately single, random PAI-1 mutants applicable for the analysis of structure/function interactions. Our success with the expression of antibody domains and PAI-1 fused to the gene III protein suggested that a modified cloning strategy would permit the preparation and expression of cDNA libraries fused to the gene III coat protein. Because the presence of translational stop codons at the 3′-end of eukaryotic mRNA prevents the direct construction of fusion proteins N-terminal to the gene III protein, we investigated the applicability of utilizing 3′-PCR primers that contained the triplets GTA or CCA. Although annealing of these primers could occur within the encoding region and result in the expression of a truncated protein, annealing of these triplets under the conditions of touchdown PCR would convert a stop codon to either a tyrosine or a tryptophan. Furthermore, to promote the production of full-length protein, we designed 5′-PCR primers that contained a favorable translation initiation site (RNNATG). Utilizing this strategy, PCR products were obtained and subcloned between 200 and 3000 base pairs and resulted in a cDNA library of greater than 10^6 clones. Although our final screening was also based upon a positive reaction with immunologic reagents directed against proteins extracted from α-granules, these reagents may have contained antibodies raised against cytosolic proteins tightly attached to these organelles; thus, the possibility existed that the identified clones may have encoded α-granule-associated cytosolic proteins with an affinity for PAI-1. Therefore, we directed our efforts at documenting that one of these clones (*i.e.* clone A-61) encodes a protein present within and released from storage organelles. Our ability to detect the agonist-inducible release of a 23-kDa protein from both AtT-20 cells and platelets, as well as to subsequently develop a purification protocol for this protein, using antibodies raised against clone A-61 provides positive support for this strategy. Because clone A-61 was only 342 base pairs, our present data suggests that alignment of our PCR primers employed in the construction of the pCOMB3H library occurred within the region encoding for SGP-23 and thus resulted in the expression of a truncated molecule on the phage surface that was capable of interacting with PAI-1. Furthermore, the reactivity of proteins in AtT-20 cells with molecular

analyzed by SDS-PAGE (15% separating gel) and silver staining (*lane 1*). Alternatively, SGP-23 was incubated (1 h, 37 °C) either in the absence (*lane 2*) or presence (*lane 3*) of endoproteinase Lys-C (1:50 ratio) and subjected to SDS-PAGE (15% separating gel) followed by immunoblotting, utilizing antibodies against the purified A-61 fusion protein.

weight greater than SGP-23, coupled with the detection of an approximately 3-kilobase pair transcript for SGP-23 in AtT-20 and DAMI cells, is consistent with current information indicating that proteins (*e.g.* ACTH (22), insulin (22), platelet-derived growth factor (53, 54), etc.) that are targeted into storage granules are often synthesized as larger precursors and proteolytically processed in the secretory pathway. For example, platelet-derived growth factor is stored in α-granules as a dimer composed of two chains: (i) an A chain with a molecular mass of 16 kDa derived from three alternatively spliced transcripts of 1.9, 2.3, and 2.8 kilobase pairs (55) and (ii) a B chain with a molecular mass of 14 kDa derived from a 3.7-kilobase pair transcript (56, 57). Both chains are proteolytically processed at N-terminal and C-terminal ends following dimerization (58, 59). Thus, it is possible that the higher molecular weight proteins immunologically related to clone A-61 that were detected in Fig. 2*C* may represent precursors of SGP-23.

An alternative strategy to circumvent the stop codons present at the 3′-end of mRNA species has been described by Crameri and Suter (60). These investigators developed a phagemid (pJuFo) in which the cDNA library is subcloned C-terminal of the cDNA encoding the Fos leucine zipper flanked by cysteine residues. To capture the expressed cDNA library, the gene III product was modified by fusing the cDNA encoding the Jun leucine zipper flanked by cysteine residues N-terminal to the gene III protein. The Jun-gene III fusion protein is incorporated into phage particles during phage morphogenesis followed by Jun-Fos heterodimerization and disulfide bond formation, thus providing a covalent link to the phage surface for recombinant cDNA products expressed on the C terminus of the Fos leucine zipper. This system has been shown to be applicable for the analysis of proteins expressed by *Asperigillus fumigatus* that bind to human serum IgE (61), to demonstrate that the large subunit of human immunodeficiency virus-1 reverse transcriptase interacts with β-actin (62), and to identify additional proteins that might interact with Jun (63). Because the construction of a fusion product between the N-terminal region of a protein with the Fos leucine zipper may interfere with its biological activity/function, our protocol utilizing fusion between the C-terminal region of a protein and the gene III protein offers a complementary approach to the system described by Crameri and Suter (60).

In addition to providing evidence for the applicability of cDNA libraries fused to the C terminus of the gene III protein, our data also provide an insight into the process that may be involved in the storage of PAI-1. It is known that several factors appear to play a role in the aggregation or condensation of molecules within the trans-Golgi, including an elevated calcium concentration and a low ionic strength/pH (23, 64). Fractionation analysis of isolated/lysed platelet α-granules in a buffer that mimics the conditions known to be present within storage granules has recently revealed that PAI-1 interacts with a series of other α-granule proteins forming a ~25-nm structural unit (21). Our observation that SGP-23 preferentially interacts with PAI-1-Sepharose, in comparison with a number of other secreted proteins coupled to Sepharose, suggests that PAI-1 may contain a region or domain recognized by SGP-23, thus raising the possibility that a human homolog of SGP-23 may be one of the proteins that comprise these ~25-nm units of the platelet α-granule microenvironment. Because platelets are anucleated cell fragments with little biosynthetic capability and thus already contain proteins packaged in a secretagogue-releasable form, studies on the production and packaging of platelet α-granule proteins have been facilitated by employing model cell systems (*e.g.* AtT-20, DAMI, human erythroleukemic cells, etc.) that are able to biosynthetically produce and package either native or recombinantly expressed proteins into storage granules. Preliminary experiments in which a human erythroleukemic cDNA library was expressed on filamentous bacteriophages and enriched utilizing PAI-1 has revealed a cDNA insert highly homologous to clone A-61.[2] Our observations obtained from the interaction of SGP-23 with PAI-1-Sepharose suggests that the PAI-1/SGP-23 interaction is not strong and can be dissociated by elevating the ionic strength. The ability of proteins (*e.g.* SGP-23 and PAI-1) to specifically interact under the conditions present in the regulated secretory pathway would facilitate their co-deposition into storage granules, whereas their affinity would be reduced following release into the extracellular environment. In this manner, PAI-1 is not restricted to interacting only with molecules co-packaged within the secretory granule; thus, active PAI-1 released from platelet would be subsequently able to bind to a number of other biologically important molecules (*e.g.* fibrin, heparin, matrix proteins, etc.) (20). Recent information from our group indicates that human glioma U-251 cells also contain PAI-1 in a rapidly releasable form (65), which raises the possibility that the proteins and mechanisms involved in the packaging of PAI-1 into storage granules may not be solely limited to megakaryocytes/platelets and the hemostatic system.

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