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 immunological 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 pTrecHis 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-Sephadex, 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) 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, 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 hematopoietic 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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* This work was supported by National Institutes of Health Grants HL49584 and HL49563 (to R. R. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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¶ 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-morpholinepropane-sulfonic 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-BK-cyclic AMP) results in release of the contents of the secretory granule (24, 26). These cells have been shown to have the capacity, after transfaction 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 monoxygenase (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, thus permitting the rapid enrichment based upon its interaction with PAI-1 to identify a bacteriophage’s genetic material, thus permitting the rapid isolation of the surface-expressed protein/peptide is contained within the phage (34–38). In this system, the cDNA encoding the vector pCMB3H, a variant of the phagemid pComb3 (43). The final library consisted of 1.5 × 106 clones.

**Purification of Storage Granule Protein-23**

**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 NaHCO3, 21.2 mM sodium citrate, 27.8 mM d-glucose, 1.1 mM MgCl2, 1 mM theophyllin, pH 6.5) to a final concentration of 107/ml and sonicated using an Astranon Ultrasonic processor XL (Heat Systems Inc., Farmingdale, NY) five times (4 °C, 5 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 isonicotic 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 1.086–1.101 g/ml. Rabbit antibodies directed against α-granules were prepared by immunizing a New Zealand White rabbit with 0.5 ml of isolated α-granules (106 mg of protein/injection) by subcutaneous injection in Freunds adjuvant at 8-week intervals for a period of 6 months utilizing standard techniques. The IgG fraction of the antisera 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).

**cDNA Isolation and Reverse Transcription—**AtT-20 and Dami cells (1.5 × 106 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 pCMB3H—**AtT-20 cell cDNA was amplified in a Perkin-Elmer 9600 thermal cycler utilizing the forward primer (5'-CAGTCGTCGAGGNNNTG-3') that contained an internal XhoI site (underlined) and, in separate reactions, reverse primers (i.e. 5'-TGGGCAACTGTGTTANNNNNN-3', 5'-TGGGCAACTGTCANNNNNN-3') that contained an internal Spel 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 bp was electroeluted from this gel. This material was digested with an excess amount of restriction enzymes Spel and XhoI (50 and 200 units/μg DNA, respectively) and ligated into the vector pCMB3H, a variant of the phagemid pComb3 (43). The final library consisted of 1.5 × 106 clones.

**Phage Production and Enrichment—**Phages 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 A600 of 0.8, infected with VCSM13 helper phage (4 × 1010 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 3900) 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 1013 to 1012 colony-forming units) or 15 times (third to sixth round of panning) or 10 times (second round of panning), 5 times (second round of panning), or 10 times (first 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 5 μl of 2 N Tris base. The initial phage input was determined by titering on selective plates. The final
Purification of Storage Granule Protein-23

Preparation and Purification of His-A-61 Construct—The cDNA insert encoding clone A-61 was removed from paranHA vector using the Xho I site on the 5’-end of the insert and a BstBI site following the cDNA sequence encoding the decapeptide tag. The A-61-decapeptide cDNA sequence was isolated by agarose gel electrophoresis and ligated into the expression vector pAraHA (43). The ligation mixture was transformed into E. coli DH12S cells and grown overnight in SB containing 30 \( \mu \)g/ml chloramphenicol. Single colonies of A-61-decapeptide 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 by TBS containing 4 \( \mu \)l phenylmethysulfonyl 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 0.1\% affinity-puriﬁed antibodies against the A-61 fusion protein. The SDS-gel-purified A-61 fusion protein 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 probe was purified using Sephadex G-50 minispin columns (Worthington) resulting in a speciﬁc activity of 10 \( \times \)10^6 cpm/µg. Hybridization of the labeled probe to the nylon membrane was performed in 5 \( \times \) SSPE, 5 \( \times \) Denhardt's solution, 0.5\% SDS, and 50 \( \mu \)g/ml fresh denatured salmon sperm DNA (Life Technologies, Inc.) for 15 h at 65 °C followed by washing in 0.1 \( \times \) SSC, 0.1\% SDS at 60 °C. Hybridization to a \( ^{32}P \)-labeled 2.0-kilobase pair human \( \beta \)-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 with staining as described by the manufacturer. Single colonies of A-61-decapeptide cDNA-pAraHA were grown for 8 h, induced by incubation (30 °C, 16 h) with \( 1% \) arabinose, harvested, lysed by TBS containing 4 \( \mu \)l phenylmethysulfonyl fluoride (final concentration; Sigma) by four freeze/thawing cycles, and centrifuged, the cell-free supernatants were incubated (1.5 h, 37 °C) in microtiter wells coated with 0.1\% affinity-puriﬁed antibodies against the A-61 fusion protein. The SDS-gel-purified A-61 fusion protein 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 probe was purified using Sephadex G-50 minispin columns (Worthington) resulting in a speciﬁc activity of 10 \( \times \)10^6 cpm/µg. Hybridization of the labeled probe to the nylon membrane was performed in 5 \( \times \) SSPE, 5 \( \times \) Denhardt's solution, 0.5\% SDS, and 50 \( \mu \)g/ml fresh denatured salmon sperm DNA (Life Technologies, Inc.) for 15 h at 65 °C followed by washing in 0.1 \( \times \) SSC, 0.1\% SDS at 60 °C. Hybridization to a \( ^{32}P \)-labeled 2.0-kilobase pair human \( \beta \)-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.

Northern Analysis—RNA (10 \( \mu \)g/ml) from AT-T20 and DAMI cells was separated by denaturing electrophoresis in formaldehyde-contain-
chromatographed on a Sephacryl S-100 column (100 × 1 cm, Pharmacia; 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. Column was eluted with 15 column volumes of Polybuffer 74 (Pharmacia; diluted 1:8 with distilled water) (4). 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) (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 to 5 parts whole blood) via cardiac puncture of 12-week-old unanesthetized 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, 10 mM Tris, pH 7.4) either in the absence or presence of calcium ionophore (1 μM A23187). The resulting pellets were resuspended in 5% fetal calf serum. To 5% fetal calf serum RPMI 1640 medium, 1 ml of resuspended cells were added to round-bottomed 24-well plates (Corning). Platelet-derived PAI-1 was affinity-purified from serum-free supernatants of platelet-derived conditioned media utilizing affinity-purified rabbit antibodies against PAI-1. Antisera directed against PAI-1 were affinity-purified from serum-free supernatants of platelet-derived conditioned media utilizing affinity-purified rabbit antibodies against PAI-1. The presence of purified PAI-1 was confirmed by immunoblotting utilizing anti-A-61 as the primary antibody and anti-β-glycoprotein I as the secondary antibody. The results indicated that PAI-1 was present in the platelet-derived conditioned media.

**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<sub>405</sub>, 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 CAMTES12; EMBL accession number X85865), whereas nucleotides 154–342 of clone A-61 are 83% identical with a longer human cDNA clone (78G05; EMBL accession number P00897). Northern blotting analysis utilizing a 32P-labeled A-61 probe revealed a single transcript in AtT-20 (Fig. 2A, lane 1) as well as in DAMI cells (Fig. 2A, lane 2), a human megakaryocytic cell line known to produce small amounts of storage granules (38). To determine the characteristics of the molecule corresponding to clone A-61, the cDNA insert was subcloned into the prokaryotic expression

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**Table I**

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

| Clone   | Solid-phase PAI-1 | Soluble PAI-1/ | Solid-phase rabbit anti-α-granules |
|---------|-------------------|----------------|-----------------------------------|
|         |                   | solid-phase anti-PAI-1 |                                  |
| A-61    | 0.54              | 2.35            | 0.74                              |
| A-104   | 5.17              | 1.32            | 0.62                              |
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. 2B, lane 1) that reacted positively utilizing antibodies to the decapeptide tag (Fig. 2B, lane 3) in comparison with the eluant of cells transformed with vector alone (Fig. 2B, 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. 2C 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-A-61 (lane 1 versus lane 3), whereas the purified A-61 fusion construct was recognized in the presence or absence of reducing agents (lane 2 versus lane 4). These observations are in accord with the presence 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 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.
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. 3B 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, 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. 4A 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. 4B 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 a-granule constituent, raises the possibility that SGP-23 might associate with other a-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 a-granule proteins. Fig. 5A

![Image](88x490 to 258x729)

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.

![Image](332x440 to 530x729)

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 (107/ml) were incubated either in the absence or presence of calcium ionophore followed by pelleting of the platelets by centrifugation. The samples (106 platelets (lanes 1 and 4); supernatant of 106 nonactivated platelets (lanes 2 and 5); releaseate of 106 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.
Fig. 6. Purification of SGP-23 from AtT-20-conditioned media. A, AtT-20-conditioned media were dialyzed against column buffer (1 mM CaCl₂, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4) and the dialysate was passed sequentially through a concanavalin A-Sepharose column (30 x 1 cm), a heparin-Sepharose column (30 x 1 cm), and a DEAE-Sephacel column (30 x 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. A₂₈₀ (●) 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 x 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 A₂₈₀ (●) 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 x 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 A₂₈₀ (●), 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
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-Sephalac with an elution of SGP-23 between 0.04 and 0.08 M NaCl (Fig. 6A). Molecular sieve chromatography on Sephacryl S-100 was employed as a second step to select for proteins in the 15–30-kDa region (Fig. 6B). Subsequent purification steps employed chromatofocusing on Polybuffer exchanger 94 with SGP-23 eluting at an acidic pH of 4.6–4.2 (Fig. 6C) 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. 6C, 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. 6C, 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 18-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 10⁶ 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

| Purification step            | Total volume | Protein concentration | Total protein | SGP-23 concentration | Total SGP-23 |
|-----------------------------|--------------|-----------------------|---------------|----------------------|-------------|
|                             | ml           | µg/ml                 | µg            | µg/µl                | 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         |
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 Storage Granule Protein-23 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. 2C 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 Aspergillus 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 (25, 64). Fractionation 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, fibrinogen, matrix protein, 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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