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Research Article

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A Platelet Alpha Granule Membrane Protein That Is Associated with the Plasma Membrane After Activation

Characterization and Subcellular Localization of Platelet Activation-dependent Granule-External Membrane Protein

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Abstract

We have identified and purified a platelet integral membrane protein (140,000 mol wt), using the KC4 monoclonal antibody specific for activated platelets, that is internal in resting platelets but exposed on activated platelets (Hsu-Lin S.-C., C. L. Berman, B. C. Furie, D. August, and B. Furie, 1984, *J. Biol. Chem.* 259: 9121–9126.). The expression of the protein on the platelet surface is secretion-dependent. This protein has been named platelet activation-dependent granule-external membrane (PADGEM) protein. PADGEM protein is distinct from the surface glycoproteins of resting platelets, but identical to the S12 antigen, GMP-140. Using immunofluorescent staining, resting platelets failed to stain for PADGEM protein with the KC4 antibody, but after permeabilization showed a punctate staining of the cell interior. Thrombin-stimulated intact platelets stained with a peripheral rim pattern thus demonstrating the translocation of PADGEM protein from an internal location to the cell surface. PADGEM protein expression on the platelet surface at varying thrombin concentrations correlated with alpha granule release, as measured by the secretion of platelet factor 4. Further evidence for an alpha granule localization of PADGEM protein was provided by nitrogen cavitation of resting platelets followed by metrizamide density gradient centrifugation; PADGEM protein codistributed with platelet factor 4. Using immunoelectron microscopy, the protein was localized to the alpha granule in frozen ultrathin sections of resting platelets labeled using rabbit anti-PADGEM protein antibodies, whereas in thrombin-activated platelets, the plasma membrane was labeled. These studies indicate that PADGEM protein is a component of the alpha granule membrane of resting platelets and is incorporated into the plasma membrane upon activation and secretion.

Introduction

Platelets are anucleate blood cells that play a fundamental role in blood coagulation and hemostasis. These cells circulate in a resting state but upon activation undergo biochemical and morphological changes that include degranulation and the secretion of granule contents. The activated platelet membrane expresses receptors for fibrinogen (1–3), Factor Xa (4), Factor XIIIa (5), and thrombospondin (6), as well as procoagulant activity (7). The molecular basis for some of these changes remains obscure.

Using KC4, a monoclonal antibody specific to activated platelets, we have identified a protein on the surface of thrombin-activated platelets that is not expressed on resting platelets (8). This protein, termed KC4 antigen, is composed of a single polypeptide chain with an apparent molecular weight of 140,000. Approximately 13,000 molecules are expressed on the thrombin-activated platelet surface; activation with ADP, epinephrine, or collagen also leads to antigen exposure. At a high thrombin concentration, the extent of [^{14}C]serotonin release from serotonin-loaded platelets during activation was directly proportional to the amount of KC4 antibody that bound to the platelet surface. These previous results indicate that the expression of this antigen is secretion-dependent, but aggregation- and agonist-independent (8).

Using a similar approach with an activated platelet-specific monoclonal antibody S12, McEver and Martin have purified a protein antigen with essentially identical characteristics to the KC4 antigen (9). The antigen recognized by S12 was thought to reside on platelet glycoprotein (GP)[1] IIa (9, 10) located on the alpha granule (10). More recently, they have proposed that the S12 antigen is a newly identified protein, GMP-140 (11). In these studies, it is shown that the KC4 antigen and the S12 antigen are located on the same protein.

Our studies of the KC4 antigen indicated that it is an internal, integral membrane protein that becomes available on the platelet surface during activation and secretion (8). In the current study, we have determined that the KC4 antigen is an alpha granule membrane protein and confirm its equivalence to the protein GMP 140 recognized by the S12 monoclonal antibody (11). Since this protein becomes part of the plasma membrane after granule secretion, we have designated this protein platelet activation-dependent granule-external membrane (PADGEM) protein to give emphasis to its unique properties. Preliminary reports on PADGEM protein have been previously published (12, 13).

Methods

Platelet preparation. Platelet-rich plasma from normal human donors was prepared as previously described (8). Platelets were further purified by differential centrifugation or gel filtration, as indicated. For surface labeling with iodine, Western blot analysis, membrane preparation, subcellular fractionation and PADGEM protein purification, platelets were washed twice in Tris-buffered saline (TBS), 2.5 mM EDTA by centrifugation at 350 *g* for 20 min. For use in the solution-phase radioimmu-

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1. *Abbreviations used in this paper:* GP, glycoprotein; PADGEM, platelet activation-dependent granule external membrane; PAS, periodic acid Schiff; TBS, tris-buffered saline.

noassay, platelets were gel-filtered on a Sepharose 2B column equilibrated with Hepes buffer (14).

Proteins. The preparation of the KC4 monoclonal antibody specific for activated platelets and the KC4 antigen (PADGEM protein) by immunoaffinity chromatography have been previously described (8). Polyclonal anti-PADGEM protein antiserum was produced by immunization of a New Zealand white rabbit with 50 μg of purified PADGEM protein at monthly intervals. Antibodies specific for the PADGEM protein were affinity purified on a PADGEM protein-Sepharose 4B column. S12 monoclonal antibody (9) was the generous gift of Dr. R. McEver (University of Texas, San Antonio). KC4 antibody, S12 antibody and PADGEM protein were radiolabeled with ^{125}I-Na using the chloramine-T method (15). The platelets were surface-labeled with ^{125}I-Na or ^{131}I-Na using the lactoperoxidase method described by Phillips and Agin (16).

Immunofluorescence. Immunofluorescent staining of platelets was performed as previously described (17). Briefly, thrombin- or buffer-treated platelets were fixed and allowed to settle on poly-L-lysine-coated glass coverslips. For some experiments, the platelets were made permeable with 0.1% Triton X-100 for 3 min prior to staining. The cells were then incubated for 20 min with the monoclonal KC4 antibody, rinsed with TBS, 0.1% bovine serum albumin (BSA) and counterstained for 20 min with a fluorescein-labeled goat F(ab')$_2$ anti-mouse IgG. The platelets were viewed with a Zeiss Universal microscope (Carl Zeiss, Inc., Thornwood, NY) as described (17) and photographed with Tri-X Panchromatic film.

Differential release of granules and the expression of PADGEM protein. Release of the contents of alpha granules, dense granules and lysosomes, as well as the expression of PADGEM protein on the platelet surface, was measured as a function of the thrombin concentration used to activate the platelets. Gel-filtered platelets (3×10^8/ml) were evaluated for the expression of PADGEM protein with a direct solution phase radioimmunoassay (RIA). Platelets (400 μl) in Hepes buffer were mixed with 100 μl of ^{125}I-labeled KC4 antibody or 100 μl of TBS. After the addition of 10 μl of thrombin at various concentrations or TBS, the platelet mixture was incubated at 22°C for 15 min. Free and bound antibody were separated by density centrifugation in oil (18) and the pellet was assayed for ^{125}I.

In parallel experiments to determine the extent of [^{14}C]serotonin release, platelets were preloaded with [^{14}C]serotonin (19), reuptake of [^{14}C]serotonin was inhibited with imipramine (20) and secretion was arrested with the addition of 12 μl of 37% formaldehyde. The platelets were sedimented by centrifugation for 3 min in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA). A 200-μl aliquot of the supernatant was assayed and compared to the total [^{14}C]serotonin in the original platelet suspension. Immunoassays for platelet factor 4 (Abbott Laboratories, North Chicago, IL) and colorimetric assays for *N*-acetyl-β-glucosaminidase (21) were performed on the platelet supernatant in the absence of formaldehyde.

Isolation of platelet membranes. Washed fresh platelets, both resting and thrombin activated, were subjected to three freeze-thaw cycles. The membrane fractions, which were sedimented by centrifugation in a Beckman Microfuge for 2 min, were washed two times in TBS, 2.5 mM EDTA. Each membrane preparation was divided into two equal aliquots. One membrane aliquot was washed sequentially with TBS containing 10 mM EDTA, then 1 M NaCl, then 0.15 M NaCl (pH 10), and finally 0.15 M NaCl (pH 4). The control preparation was washed an equal number of times with TBS (pH 7.4). The membrane pellet was resuspended in TBS (pH 7.4) containing 0.1% Triton X-100, and then sonicated using a Heat Systems-Ultrasonics (Plainview, NY) sonicator model W-220. To determine whether PADGEM remained associated with the membranes, the four preparations of washed membranes were assayed for PADGEM protein using a competition RIA, described below. For determination of conservation of membrane mass, a parallel experiment was performed using ^{125}I-labeled membranes. Membranes were prepared from ^{125}I-surface labeled platelets and washed as above to remove peripheral membrane proteins. The labeled membranes (10^4 cpm) were added to the membrane samples and the radioactivity remaining after the different washing procedures was determined.

Subcellular fractionation and density gradient centrifugation. Platelets preloaded with [^{14}C]serotonin and washed in the presence of 2.5 mM EDTA, were resuspended in 0.27 M sucrose, 2 mM EDTA, pH 6.5, at a final concentration of 5×10^9/ml and disrupted by nitrogen cavitation using a modification of the method of Gogstad (22). After an initial pressurization at 1,200 psi for 15 min, granules and platelets were sedimented at 2,000 *g* for 20 min and subjected to a second cavitation at 1,200 psi for 10 min. Remaining whole platelets were removed by centrifugation at 350 *g* for 20 min and the supernatant used as the cell homogenate. The membranes of ^{131}I-surface labeled resting platelets were prepared separately to serve as a marker for the plasma membrane. Washed surface-radiolabeled platelets were disrupted by a freeze-thaw cycle and the membranes, collected by centrifugation at 12,000 *g* for 2 min, were washed three times in TBS prior to addition to the cell homogenate. After the cell homogenate was adjusted to 11.5% metrizamide, 2.5 ml was layered onto a 10-ml continuous metrizamide gradient (17.5–27.5% metrizamide containing 2 mM EDTA, pH 6.5) and centrifuged at 50,000 g_{av} for 2 h in a Beckman SW41 rotor. Fractions, 0.8 ml each, were collected from the top of the gradient, subjected to three freeze-thaw cycles and assayed for refractive index, protein content, subcellular organelle markers, and PADGEM protein. Protein content was determined by the method of Bradford (23). The lysosomal enzyme marker, *N*-acetyl β-glucosaminidase, was analyzed as described by Sellinger et al. (21). Platelet factor 4 was measured by RIA (Abbott). [^{14}C]Serotonin was quantitated in a Beckman LS1800 β-scintillation spectrometer and ^{131}I-labeled plasma membranes were assayed using a Beckman Gamma 8000 spectrometer. Lactate dehydrogenase was assayed by measuring the oxidation of NADH (24). The mitochondrial enzyme, succinic dehydrogenase, was assayed using iodonitrotetrazolium violet as a substrate (25). To quantitate PADGEM protein in gradient fractions, a competition solution-phase RIA was employed. Aliquots of gradient fractions (100 μl) were mixed with ^{125}I-labeled PADGEM protein in the presence of 0.1 mg of normal rabbit immunoglobulin and incubated for 2 h with rabbit anti-PADGEM protein antiserum. All dilutions for the RIA were made in TBS, 0.1% BSA. The resultant antibody-antigen complexes were precipitated by 3% (vol/vol) goat anti-rabbit IgG antiserum in TBS, 2.5% polyethylene glycol (PEG)-6000, 0.02% NaN_3. After centrifugation at 1,000 *g* for 20 min, the supernatant was decanted and the pellet assayed for ^{125}I.

Electrophoretic analyses of PADGEM protein. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed using 6% gels (26). Periodic acid-Schiff stain for carbohydrate was performed according to Fairbanks et al. (27). Western blots of platelet membrane proteins were performed as previously described (8) using standard methods (28). In studies involving the use of rabbit anti-PADGEM protein antibodies, blots were incubated for 1 h with TBS, 3% BSA; 0.1 volume of rabbit antiserum was added and the blots incubated for an additional 2 h. Excess primary antibody was removed with six washes with TBS. The blots were then exposed to ^{125}I-labeled goat F(ab')$_2$ anti-rabbit IgG in TBS, 3% BSA before being washed, dried, and autoradiographed.

Immunoelectron microscopy. Immunocytochemistry was performed as previously described (29). In brief, platelets were fixed and embedded in low gelling temperature agarose. Silver-gold ultrathin frozen sections were prepared using a Sorvall MT 2-B ultramicrotome (Sorvall Instruments, Newtown, CT) with an LCT-2 cryokit attachment, and sections were immunostained on electron micrograph (EM) grids. Bound rabbit anti-PADGEM protein antibody was detected using affinity purified biotinyl goat anti-rabbit Ig followed by 5 nm colloidal gold-avidin conjugate. Sections were negatively stained with 0.2% phosphotungstic acid and viewed on a Hitachi 12-A transmission electron microscope (Hitachi America, Inc., Allendale, NJ) with an accelerating voltage of 75 KV.

Materials. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide, iodonitrotetrazolium violet, NADH, and sodium pyruvate were obtained from Sigma Chemical Co. (St. Louis, MO). Triton X-100, ^{125}I-Na, ^{131}I-Na, [^{14}C]serotonin, and ^{125}I-labeled goat F(ab')$_2$ fragments to rabbit IgG were obtained from New England Nuclear (Boston, MA). Platelet factor 4 assay kits were purchased from Abbott and bovine thrombin from Parke-Davis (Detroit, MI). Fluorescein-conjugated goat anti-mouse IgG was

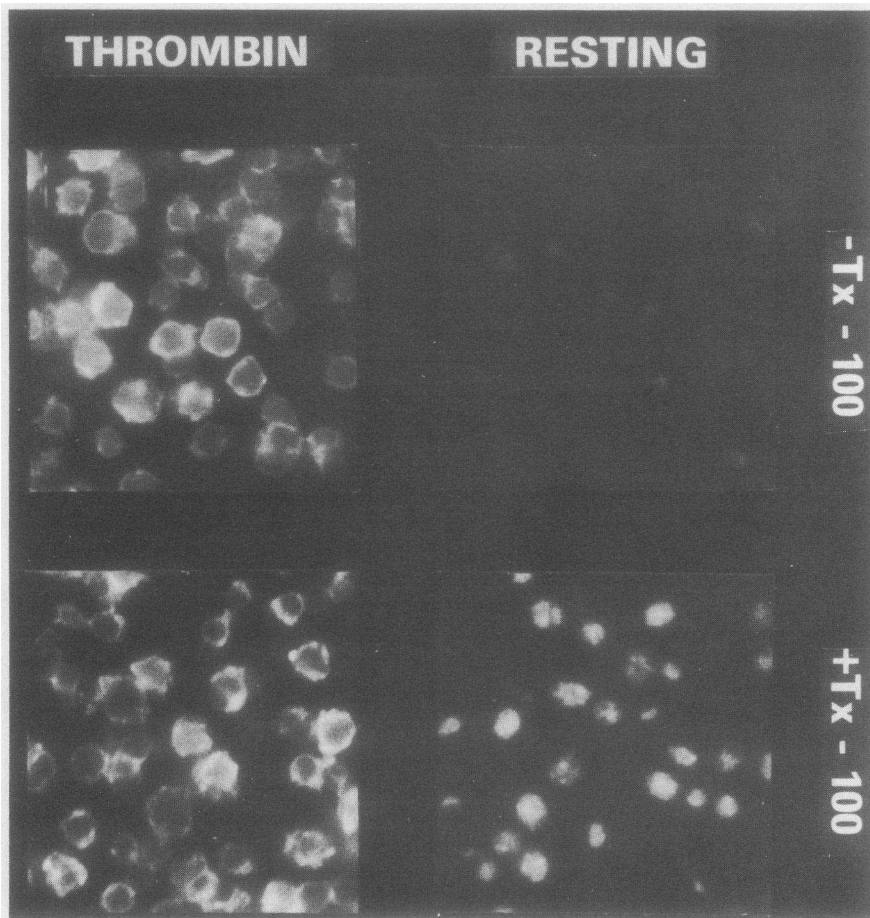

Figure 1. Immunofluorescent localization of the PADGEM protein in whole platelets. Intact or detergent permeabilized resting or thrombin stimulated platelets were stained with the monoclonal KC4 antibody, directed against PADGEM protein, and counterstained with fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG. Intact resting cells failed to demonstrate surface staining. However, resting cells permeabilized with Triton X-100 revealed extensive immunofluorescent staining occupying the cell center. Following thrombin stimulation, intact and permeabilized cells demonstrated a rim pattern of fluorescence consistent with a surface localization. × 1,300 magnification. Cells were treated with (+) or without (–) Triton X-100 (Tx-100).

obtained from Cappel Laboratories (Cochranville, PA); biotinyl goat anti-rabbit IgG and gold-avidin conjugate were purchased from Hyclone (Logan, UT). Metrizamide (centrifugation grade) was the gift of Nyegaard (Oslo, Norway). All other chemicals were of reagent grade.

Results

Immunofluorescent localization of PADGEM protein. When intact unstimulated, resting platelets were fixed and stained for PADGEM protein using the KC4 antibody, virtually no surface staining was seen (Fig. 1). However, intact thrombin-activated platelets demonstrated a rim staining pattern similar to that observed for the integral membrane proteins, GPIb and GPIIb/IIIa (30). This is in contrast to the punctate labeling pattern observed for alpha granule matrix proteins, such as thrombospondin and fibronectin (17). The surface-expressed PADGEM protein was derived from an internal pool, since unstimulated platelets that were permeabilized with Triton X-100 showed a central punctate fluorescence when stained for PADGEM protein. This central pool of fluorescence was greatly reduced in permeabilized thrombin-activated cells in comparison with resting cells. Similar results were obtained using polyclonal rabbit anti-PADGEM protein antibodies for fluorescent staining. In sum, these results indicate that PADGEM protein is internal in resting, unstimulated platelets; upon thrombin activation, PADGEM protein is translocated to the external plasma membrane and becomes diffusely distributed on this membrane surface.

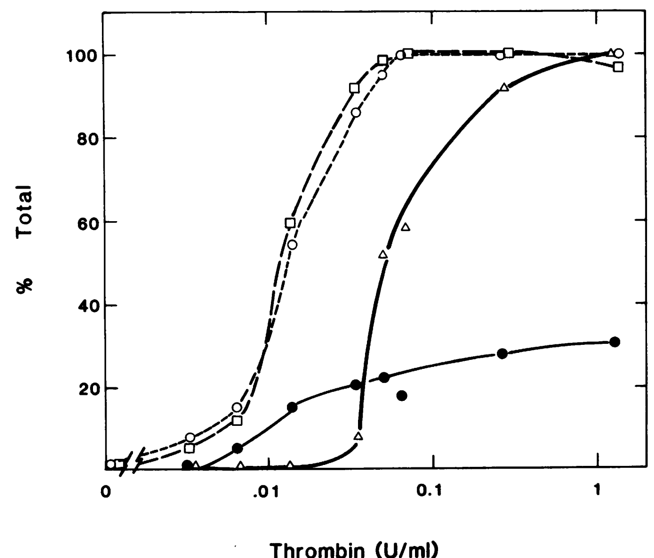

Figure 2. Differential release of granules and the expression of PADGEM protein. Platelets were stimulated with varying concentrations of thrombin (as indicated) and evaluated for the release of markers of the alpha granules, dense granules, and lysosomes. Platelets were evaluated for PADGEM protein (□) using the solution phase RIA. After centrifugation, the platelet supernatant was analyzed for [^{14}C]serotonin (△), platelet factor 4 (○) and *N*-acetyl β-glucosaminidase (●). The data are expressed as the percentage of the total amount released from a thrice freeze-thawed sample of gel-filtered platelets.

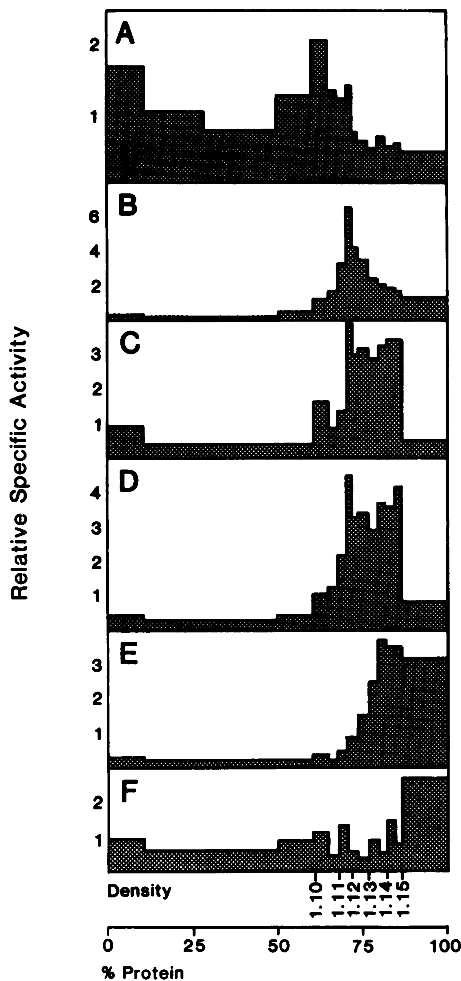

Figure 3. Density gradient analysis of subcellular fractions of resting platelets. Resting platelets were disrupted twice by nitrogen cavitation and subjected to metrizamide density gradient centrifugation. Gradient fractions were assayed for (*A*) ^{125}I-labeled plasma membranes; (*B*) *N*-acetyl β-glucosaminidase (lysosomes); (*C*) PADGEM protein; (*D*) platelet factor 4 (alpha granules); (*E*) succinic dehydrogenase (mitochondria); and (*F*) [^{14}C]serotonin (dense granules). Metrizamide density was calculated from the measured refractive index. The data are presented as relative specific activity (percent total marker/percent total protein) versus protein in each fraction.

Comparison of PADGEM protein expression and differential granule release. We compared the concentrations of thrombin required for PADGEM protein expression to those required for the secretion of markers from alpha granules (platelet factor 4), dense granules ([^{14}C]serotonin), and lysosomes (β-glucosaminidase). As previously described (31), platelet factor 4 release occurs at lower thrombin concentrations than those required for the release of the constituents of other storage granules (Fig. 2). The concentration dependence of PADGEM protein expression, as measured in a direct solution phase RIA, correlated with that for alpha granule release; in contrast, release of dense granule serotonin required much greater thrombin concentrations as did β-glucosaminidase release from lysosomes. These data indicate that the internal pool of PADGEM protein, which becomes expressed on the cell surface as a consequence of thrombin stimulation, resides with alpha granules.

PADGEM protein localization by subcellular fractionation. To further investigate the subcellular localization of PADGEM protein, we fractionated disrupted platelets on metrizamide density gradients. Disruption of the platelets was achieved by nitrogen cavitation such that the final cell homogenate contained <15% of the initial cytosolic lactate dehydrogenase while, on average, >60% of the granule contents were maintained. Each fraction was assayed for subcellular markers and PADGEM protein; the specific activity of each marker versus density is presented in Fig. 3. The plasma membrane, labeled with ^{131}I, migrated to a density of 1.10 (panel *A*), whereas lysosomes assayed by β-glucosaminidase had a density of 1.115 (panel *B*). Platelet factor 4, the marker of the alpha granules, was found at densities of 1.115–1.145 (panel *D*). Succinic dehydrogenase (panel *E*), a marker of mitochondria, and [^{14}C]serotonin (panel *F*), a marker of dense granules, migrated to densities of 1.14 and 1.15, respectively. These organelle densities are in agreement with those previously described (21). The migration of PADGEM protein (panel *C*) was compared to that of the subcellular markers and the distribution of PADGEM protein on the metrizamide gradient was identical to that of platelet factor 4. The similarity of the density distribution pattern of PADGEM protein to the alpha granules further indicates that PADGEM is a component of alpha granules.

Immunocytochemical localization of PADGEM protein by electron microscopy. The PADGEM protein was localized in frozen ultrathin sections of unstimulated platelets using immunoelectron microscopy. Polyclonal anti-PADGEM protein antiserum was prepared in rabbits and affinity purified using PADGEM protein covalently linked to agarose. The monospecificity of the anti-PADGEM protein antiserum was established by the Western blot technique (Fig. 4). The anti-PADGEM protein antiserum reacted with only a single protein in SDS-treated platelets and with purified PADGEM protein.

When ultrathin sections from resting platelets were stained with the affinity purified polyclonal anti-PADGEM protein antibodies, the gold particles were distributed at highest density about the alpha granule membrane (Fig. 5 *A*). Although some isolated particles were observed within the alpha granules, the density of particles about the membrane and within 200 Å of

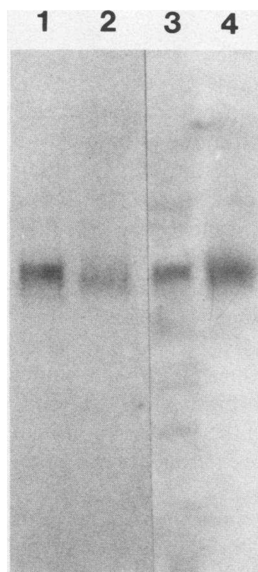

Figure 4. Antigenic specificity of anti-PADGEM protein antiserum. The specificity of the anti-PADGEM protein antiserum was detected by Western blot analysis. Proteins were subjected to electrophoresis on a polyacrylamide gel and transferred to nitrocellulose. The blot was treated either with ^{125}I-labeled KC4 antibody or with rabbit anti-PADGEM protein antiserum and then developed with ^{125}I-labeled goat F(ab')$_2$ anti-rabbit IgG. Lanes *1* and *3*, unlabeled resting platelets (10^8); lanes *2* and *4*, 5 μg purified PADGEM protein; lanes *1* and *2*, treated with ^{125}I-labeled KC4 antibody; lanes *3* and *4*, treated with rabbit anti-PADGEM protein antiserum and ^{125}I-labeled goat anti-rabbit IgG.

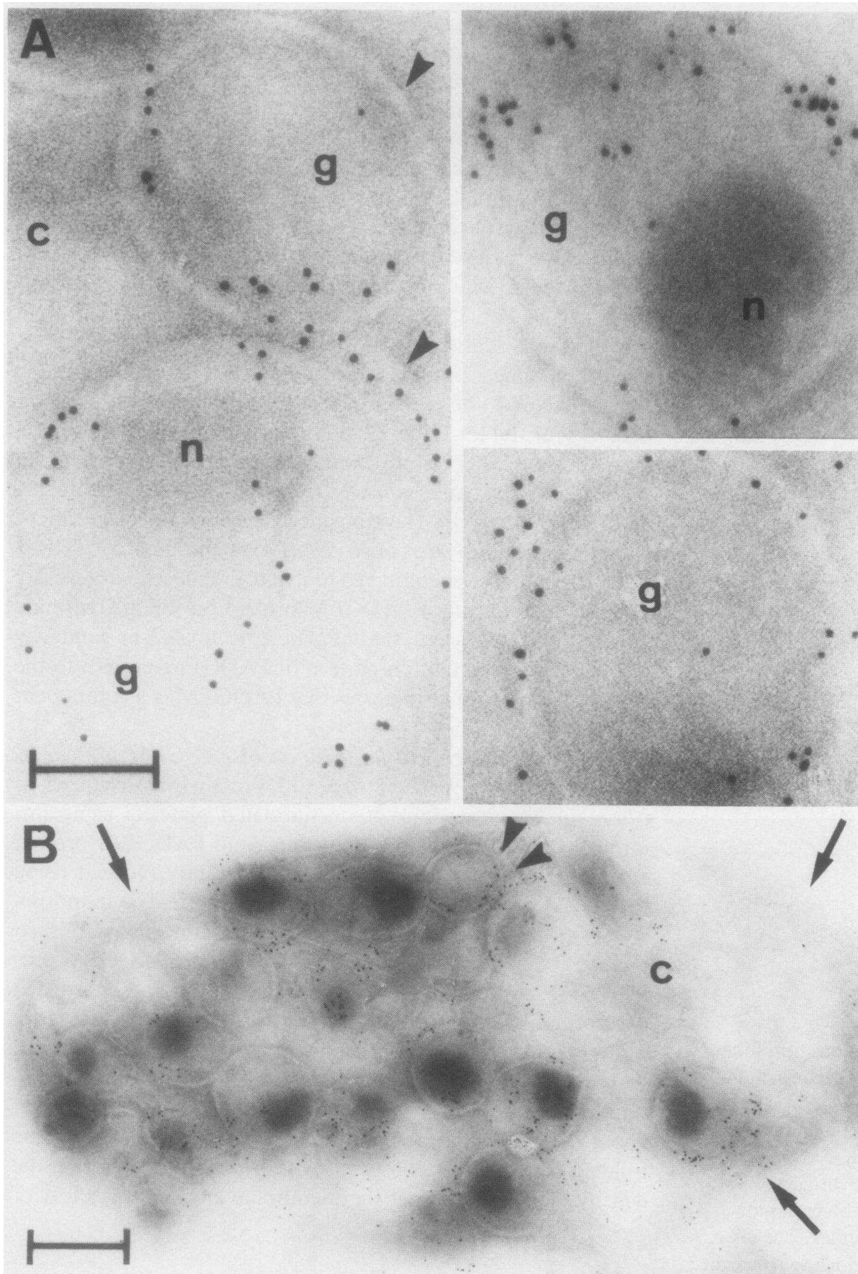

Figure 5. Indirect immunolabeling of the PADGEM protein in cryostat sections of human platelets. Ultrathin frozen sections were immunolabeled with affinity purified rabbit anti-PADGEM protein antibody as the primary antibody and biotinyl goat anti-rabbit IgG as the second antibody. Antibody-antigen complexes were visualized by means of a 5-nm colloidal gold-avidin conjugate. (*A*) represents a magnification of the alpha granules (g) shown in the whole cell in (*B*) (arrowheads). Cytosolic matrix (c) and alpha granule nuclei (n) are also indicated. Labeling patterns are suggestive of an alpha granule membrane localization. In the intact cell, (*B*), arrows indicate obliquely sectioned plasma membrane that is not labeled. Magnification, (*A*) × 154,000, (*B*) × 39,000; bars, (*A*) 0.1 μm, (*B*) 0.3 μm.

the bilayer (the upper limits of the distance from gold-labeled second antibody bound to the primary antibody bound to a membrane antigen) was greater than that of the antibody within the matrix of the alpha granule or the cytosol. No specific labeling of the plasma membrane was observed in the resting cells (Fig. 5 *B*). Similar experiments were performed with thrombin-activated platelets that had undergone degranulation and pseudopod formation. The external plasma membrane was heavily stained with anti-PADGEM protein antibody (Fig. 6 *A*). In contrast, only minimal labeling was observed when preimmune rabbit serum was used as the primary antibody (Fig. 6 *B*). These results are compatible with the translocation of the PADGEM protein from the alpha granule membrane to the external plasma membrane.

PADGEM protein as an integral membrane protein. Previous studies had shown that PADGEM protein is an integral membrane protein of the activated platelet plasma membrane (8). To determine whether PADGEM protein is an integral membrane protein of the alpha granule in resting platelets, platelet membranes (including surface and intracellular membranes) were analyzed for PADGEM protein. Membranes, prepared from resting and from thrombin-activated platelets, were washed either with TBS or sequentially with TBS/10 mM EDTA, 1 M NaCl, 0.15 M NaCl (pH 10.0), and 0.15 M NaCl (pH 4.0) to remove peripheral membrane proteins. Using a competition radioimmunoassay in which the membrane proteins were assayed by their ability to inhibit the binding of rabbit anti-PADGEM protein antibodies and ^{125}I-labeled PADGEM protein, equivalent quantities of PADGEM protein were detected in each membrane sample (Fig. 7). A parallel experiment, in which washed ^{125}I-labeled membranes were added to the membrane samples, showed equivalent retention of membrane following the two different washing procedures. These results indicate that PADGEM is an integral membrane protein in resting platelets and

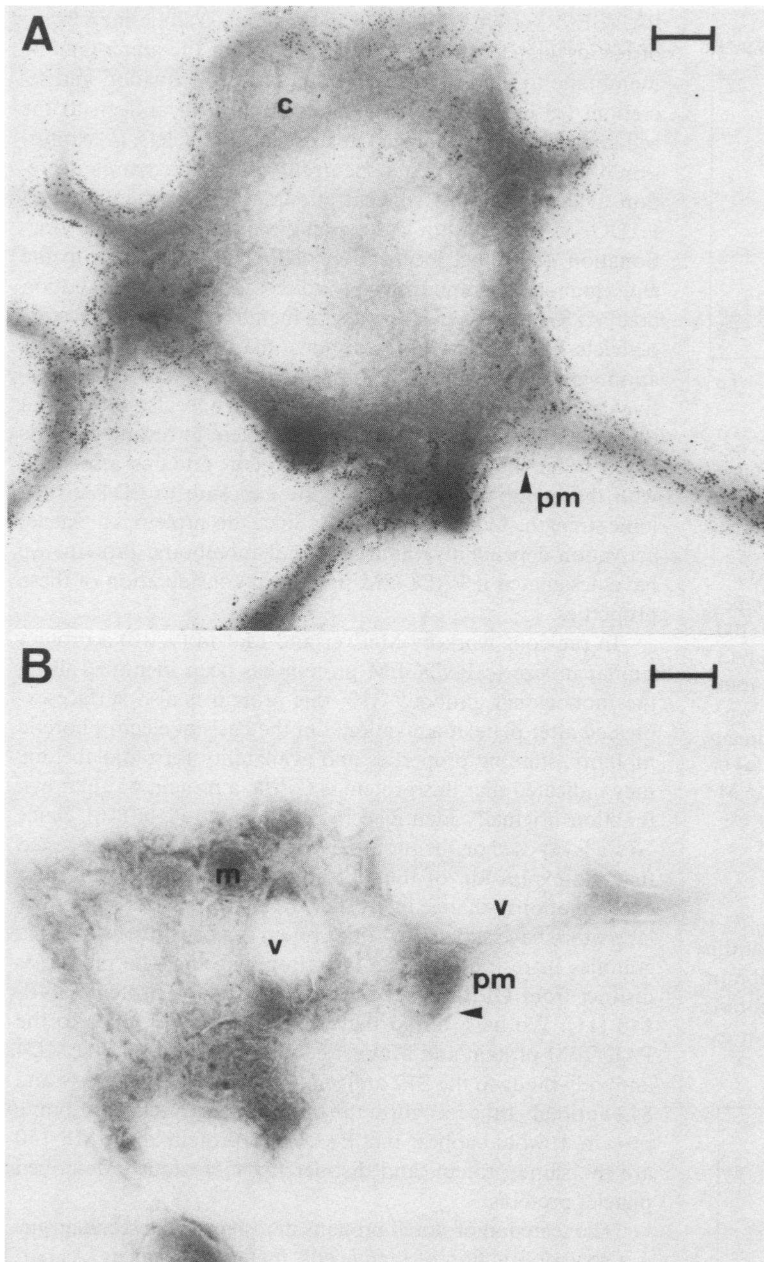

Figure 6. Indirect immunolabeling of the PADGEM protein in cryostat section of thrombin-stimulated human platelets. Platelets were activated with thrombin (1 U/10^8 platelets) for 1 min at room temperature. Frozen sections were stained as in Fig. 5, using rabbit anti-PADGEM as the primary antibody. (*A*) demonstrates heavy labeling of the extracellular face of the plasma membrane (pm). Alpha granules have been cleared from the cell interior. Minimal labeling of platelet cytosolic matrix (c) was observed. (*B*) represents a control section stained with preimmune serum as the primary antibody; mitochondria (m), vacuoles (v), and plasma membrane (pm) are indicated. Only occasional random colloidal gold particles were observed. Magnification, (*A*) and (*B*) × 54,000; bars = 0.2 μm.

is consistent with the previous finding (8) that PADGEM protein is an integral membrane protein in activated platelets.

Comparison of the PADGEM protein with other platelet glycoproteins. The KC4 monoclonal antibody (8) and the S12 monoclonal antibody (9) both bind to protein antigens on activated platelets with molecular weights of ~140,000. McEver and Martin (9) had reported that the S12 antibody binds to a glycoprotein that they believed to be GPIIa. More recently, they have designated this S12 antigen as GMP-140 (11). We had originally identified the KC4 antigen as a heretofore undescribed platelet membrane protein (8). This protein, now designated PADGEM protein, stains with PAS indicating that it is a glycoprotein (results not shown). To determine the relationship between the S12 antigen and the KC4 antigen, S12 antibody was labeled with ^{125}I for use in Western blot analysis. Nitrocellulose blots of platelet membrane proteins and purified PADGEM protein were incubated with ^{125}I-labeled S12. This reagent bound to purified PADGEM protein (data not shown) indicating that KC4 and S12 antibodies are directed against the same protein. Similar conclusions have been reached by McEver using ^{125}I-labeled KC4 antibody and crossed immunoelectrophoretic analysis (11).

The relationship of PADGEM protein and other known platelet glycoproteins was reevaluated. ^{125}I-labeled PADGEM protein was added to a solubilized ^{131}I-surface labeled platelet preparation and subjected to SDS polyacrylamide gel electrophoresis under nonreducing conditions. Analysis of the gel slices for ^{125}I and ^{131}I show that the PADGEM protein migrates between GPIIb and GPIIa and is not coincident with any labeled surface glycoproteins (Fig. 8).

Discussion

We have previously identified a new platelet protein with a molecular weight of 140,000 that is exposed on the cell surface only

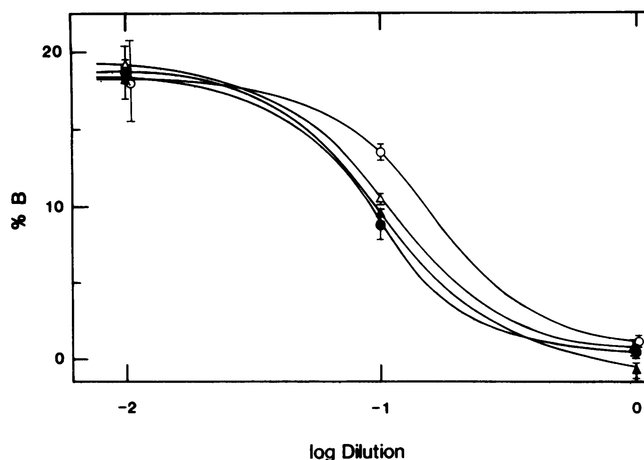

Figure 7. Demonstration that PADGEM protein is an integral membrane protein in resting platelets. Membranes prepared from resting and thrombin-activated platelets were washed sequentially with TBS or sequentially with TBS/10 mM EDTA, 1 M NaCl, 0.15 M NaCl (pH 10.0), and 0.15 M NaCl (pH 4.0). The presence of PADGEM protein in the membrane preparation was determined using a competition radioimmunoassay. The ability of membrane preparations (100 μl) to inhibit the binding of ^{125}I-labeled PADGEM protein (100 μl) to anti-PADGEM antiserum (100 μl; 1:100 dilution) was measured using a double antibody immunoassay. The inhibition of binding is shown by the decrease in the percentage of radiolabeled antigen bound to antibody (% B). Membranes from resting platelets exposed to TBS (●) or TBS/10 mM EDTA, 1 M NaCl, 0.15 M NaCl (pH 10.0), and 0.15 M NaCl (pH 4.0) (○); membranes from thrombin-activated platelets exposed to TBS (▲) or TBS/10 mM EDTA, 1 M NaCl, 0.15 M NaCl (pH 10.0), and 0.15 M NaCl (pH 4.0) (△).

after activation and secretion (8). We had hypothesized that this protein, an integral membrane protein, was a granule membrane protein that became incorporated into the external membrane during secretion (8). In the current work, five independent experimental approaches are consistent with the conclusions that PADGEM protein is localized to the alpha granule membrane of resting platelets and is translocated from the alpha granule membrane to the plasma membrane during activation and secretion: (*a*) immunofluorescent localization of antigen to the cell interior in permeabilized unstimulated platelets, (*b*) expression of PADGEM protein concurrent with alpha granule secretion at low thrombin concentrations, (*c*) cosedimentation of PADGEM protein with alpha granules during subcellular fractionation, (*d*) direct localization of PADGEM protein to the alpha granule by immunoelectron microscopy, (*e*) demonstration that PADGEM protein is an integral membrane protein in resting platelets. Our earlier work had shown that PADGEM protein is an integral membrane protein on the plasma membrane of activated, degranulated platelets (8). We have now also shown that this protein is an integral membrane protein in resting platelets based upon the evidence that the protein remains associated with the membrane fraction despite exposure to EDTA, high ionic strength, and extremes of pH. Since this protein is a platelet activation-dependent granule-external membrane protein, we have designated it PADGEM protein in consideration of these properties.

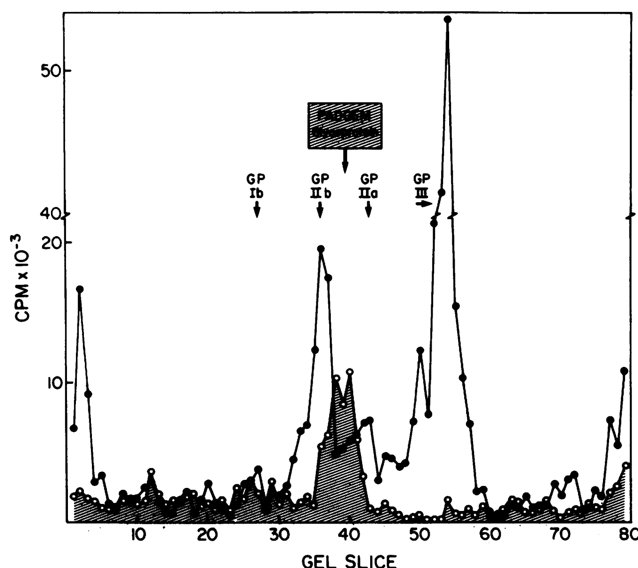

Figure 8. Comparison of the anodal migration of ^{125}I-PADGEM protein and ^{131}I-surface labeled platelets. ^{125}I-PADGEM protein and ^{131}I-surface labeled platelet glycoproteins were analyzed simultaneously by SDS polyacrylamide gel electrophoresis in 6% gels under nonreducing conditions. The ^{125}I and ^{131}I in each gel slice (1 mm) were determined and corrected for overlapping spectra.

In previous work by McEver and Martin (9, 10) a protein similar in size to PADGEM protein has been identified using the monoclonal antibody S12; this protein is also surface-expressed after platelet activation. On the basis of electrophoretic mobility, staining properties and availability for radiolabeling, they indicated that this protein is GPIIa, a protein of unknown function originally identified by Phillips and Agin (16). Since GPIIa is exposed on the surface of resting platelets, they suggested that the expression of the S12 antigen was associated with a conformational change in GPIIa (9). Further studies from their laboratory have shown that this protein is a constituent of alpha granules in resting platelets (10), implying that the protein is distinct from GPIIa. They have designated the protein GMP-140 (11). We have found that the S12 antibody binds to the PADGEM protein and McEver et al. have found that the KC4 antibody binds to the S12 antigen (11). The KC4 antibody and S12 antibody thus recognize the same alpha granule membrane protein. It would appear that PADGEM protein and GMP-140 are the same protein, and distinct from previously described platelet proteins.

The secretion of stored proteins in response to a secretagogue is a general function of many cells, including platelets. As part of the membrane flow hypothesis, Palade originally suggested that, during secretion, proteins of the granule membranes are incorporated into the plasma membrane (32). This hypothesis is generally accepted. However, since specific markers for secretory granule membranes have only recently been identified (33–35), secretion has classically been studied using granule content markers (36). The discovery of the PADGEM protein as an alpha granule membrane protein has provided evidence that a membrane fusion event occurs during platelet secretion. As it is unclear whether the alpha granules fuse with the open canalicular system or directly with the plasma membrane (37), PADGEM protein should prove an important marker of the movement of alpha granules to the membrane surface during platelet secretion. Furthermore, we are currently exploring the use of antibodies directed against PADGEM protein for the in vivo localization of activated platelets, including sites of bleeding and thrombi. The function of the PADGEM protein is currently unknown, and may involve an internal receptor for alpha granule coalescence, movement, or release, or may relate to the unique receptor and procoagulant activities of the activated platelet membrane.

Acknowledgments

This work is dedicated to the memory of Shu-Chun Hsu-Lin (1953–1984), who initiated this work and discovered PADGEM protein during her doctoral studies.

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