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A Platelet Alpha-Granule Membrane Protein (GMP-140) Is Expressed on the Plasma Membrane after Activation

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ABSTRACT We have previously characterized a monoclonal antibody, S12, that binds only to activated platelets (McEver, R. P., and M. N. Martin, 1984, *J. Biol. Chem.*, 259:9799–9804). It identifies a platelet membrane protein of M_r 140,000, which we have designated as GMP-140. Using immunocytochemical techniques we have now localized this protein in unstimulated and thrombin-stimulated platelets. Polyclonal antibodies to purified GMP-140 were used to enhance the sensitivity of detection. Nonpermeabilized, unstimulated platelets, incubated with anti-GMP-140 antibodies, and then with IgG-gold probes, showed very little label for GMP-140 along their plasma membranes. In contrast, thrombin-stimulated platelets exhibited at least a 50-fold increase in the amount of label along the plasma membrane. On frozen thin sections of unstimulated platelets we observed immunogold label along the alpha-granule membranes. We also employed the more sensitive technique of permeabilizing with saponin unstimulated platelets in suspension, and then incubating the cells with polyclonal anti-GMP-140 antibodies and Fab-peroxidase conjugate. Alpha-granule membranes showed heavy reaction product, but no other intracellular organelles were specifically labeled. These results demonstrate that GMP-140 is an alpha-granule membrane protein that is expressed on the platelet plasma membrane during degranulation.

Activated blood platelets undergo a complex series of biochemical and morphological changes that promote their hemostatic role at sites of vascular injury. Many of these changes occur at the platelet surface. For example, the local generation of thrombin stimulates platelet secretion and aggregation, as well as fibrin formation (1). The binding of fibrinogen, von Willebrand factor, thrombospondin, and fibronectin to the surface of the activated platelet promotes cell–cell interactions and adhesion of platelets to subendothelial surfaces (2). Factor XIIIa binds to thrombin-activated platelets, where it may be localized for efficient cross-linking of fibrin polymers into a stable clot (3). The molecular events responsible for these diverse interactions at the surfaces of activated platelets are incompletely defined but thought to involve changes in the conformation or rearrangements of platelet membrane glycoproteins.

We recently described a monoclonal antibody, S12, which reacts minimally with unstimulated human platelets but binds to ~10,000 sites/cell after platelets have been activated with thrombin (4). We isolated the protein recognized by S12 by affinity chromatography of Lubrol PX-solubilized human platelet membranes on S12-agarose. As analyzed by SDS PAGE, the unreduced protein has an M_r of 138,000, and the reduced protein has an M_r of 148,000. The slower mobility of the reduced protein presumably reflects unfolding of the polypeptide after cleavage of intrachain disulfide bonds. The molecule appears to be glycosylated, since it stains with periodic acid-Schiff.

In our earlier report (4), we suggested two possible reasons for the enhanced binding of S12 to platelets after thrombin activation. The first hypothesis was that the S12-binding protein is located in the plasma membrane of unstimulated platelets but is inaccessible to antibody. Thrombin activation "unmasks" the protein, either by a change in its conformation that allows expression of the antigen identified by S12 or by rearrangement of other membrane proteins that interfere with antibody access to the antigen. The second hypothesis was that the protein is localized to a secretory-granule membrane

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that becomes exposed on the platelet surface after thrombin-induced secretion from the platelet. We now report the localization of the S12-binding protein in unstimulated and thrombin-stimulated human platelets by three immunocytochemical techniques. Our data support the second hypothesis and indicate that the protein is localized exclusively in alpha-granule membranes in the unstimulated platelet. After thrombin stimulation and membrane fusion, the protein is redistributed to the surface-connected canalicular system (SCCS)[1] and plasma membrane. Because of its distinct localization in the unstimulated cell, we have given this protein the designation GMP-140, indicating that it is a granule membrane protein with a molecular weight of ~140,000.

MATERIALS AND METHODS

Preparation of Antibodies: Purified monoclonal S12 IgG was prepared as previously described (4). Platelet GMP-140, isolated by S12 affinity chromatography, was used to raise polyclonal antibodies in rabbits. GMP-140 was denatured by heating at 100°C for 5 min in the presence of 1% SDS. Rabbits were injected subcutaneously with 50 μg of either nondenatured or SDS-denatured GMP-140 emulsified in complete Freund's adjuvant. After 4 wk the rabbits were boosted subcutaneously with 50 μg of the same immunogen emulsified in incomplete Freund's adjuvant. They were bled 1 wk later.

The specificities of the polyclonal antibodies were examined by immunoblotting. 50-μg samples of platelet protein, solubilized in 2% SDS in the absence of reducing agent, were electrophoresed in parallel lanes of a 7–15% exponential polyacrylamide slab gel (5), then transferred to nitrocellulose paper (6). After the paper had been immersed in 10 mM $NaPO_4$, 0.15 M NaCl, pH 7.5, 0.05% (vol/vol) Tween 20, strips containing individual lanes of separated platelet proteins were incubated with nonimmune or immune sera (1:1,000 dilution in 10 mM $NaPO_4$, 0.15 M NaCl, pH 7.5, 0.05% (vol/vol) Tween 20) for 1 h at room temperature, then washed three times with 10 mM $NaPO_4$, 0.15 M NaCl, pH 7.5, 0.05% (vol/vol) Tween 20. Bound antibody was then detected with an enzyme-linked immunosorbent assay system (Vectastain, Vector Laboratories, Inc., Burlingame, CA) consisting of sequential additions of biotinylated goat anti-rabbit antibody, an avidin-biotinylated horseradish peroxidase complex, and a color-developing substrate, 4-chloro-1-napthol.

Triton X-114 Extraction: Platelet membrane proteins were extracted with Triton X-114 as described by Bordier (7). Crude platelet membranes prepared by freeze-thawing and sonication were lysed at 4°C in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, 1% Triton X-114. After the protein concentration was adjusted to 0.5 mg/ml, the aqueous and detergent phases were separated (7). The relative amount of GMP-140 in each phase was then determined by immunoblotting as described above, except that bound antibody was detected by incubating the nitrocellulose sheet with ^{125}I-protein A (90,000 cpm/ml). The nitrocellulose was then washed, dried, and subjected to autoradiography (4).

Preparation of Platelets for Immunocytochemical Studies: Platelets dripped into fixative were prepared from the blood of normal patients as described previously (8). Washed, unstimulated, and thrombin-stimulated (1 U/ml for 5 min) platelets were also prepared as described previously (9). EDTA (1 mM) was added to the platelet resuspension buffer to minimize cell clumping. Samples were fixed in 2% paraformaldehyde with 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 37°C.

Immunocytochemical Procedures: Immunocytochemical procedures on frozen thin sections and on suspensions of nonpermeabilized platelets using IgG-colloidal gold (5-nm) probes (Janssen Pharmaceutica, Beerse, Belgium) were performed as described previously (8, 9). When sections were incubated with polyclonal antibodies, the probe was colloidal gold conjugated with goat anti-rabbit IgG; for sections incubated with monoclonal antibody, the probe was colloidal gold conjugated with goat anti-mouse IgG. Immunocytochemical procedures on saponin-permeabilized platelets were performed as previously described (8, 9).

Control measures for all procedures included the substitution of buffer, preimmune rabbit serum, or purified mouse IgG for specific primary antibody.

RESULTS

Fig. 1*A* illustrates the reactivity of polyclonal antibodies prepared against nondenatured GMP-140 as assessed by immunoblotting of platelet proteins. Similar results were obtained with antibodies raised against SDS-denatured protein. To illustrate the specificity of the anti-GMP-140 antibodies, immunoblotting was also performed in parallel with antibodies to GP IIb, another membrane glycoprotein, which migrates near GMP-140 in SDS gels. Antibodies to GMP-140 reacted with a single protein of M_r 140,000, which migrates slightly faster than GP IIb under nonreducing conditions. The mobility of this protein relative to GP IIb is identical to that of purified GMP-140 (4). The monoclonal antibody S12 also reacted with a protein of identical mobility in the immunoblotting procedure (not shown). Therefore, both S12 and the polyclonal antibodies identify the same molecule. Neither S12 nor the polyclonal antibodies bound to GMP-140 after platelet proteins had been electrophoresed in the presence of reducing agent.

GMP-140 was initially isolated from a Lubrol (Sigma Chemical Co., St. Louis, MO)-PX lysate of platelet membranes (4), which suggests that it is a membrane protein. To define further the physical properties of GMP-140, we extracted platelet membrane proteins at 4°C with Triton X-114

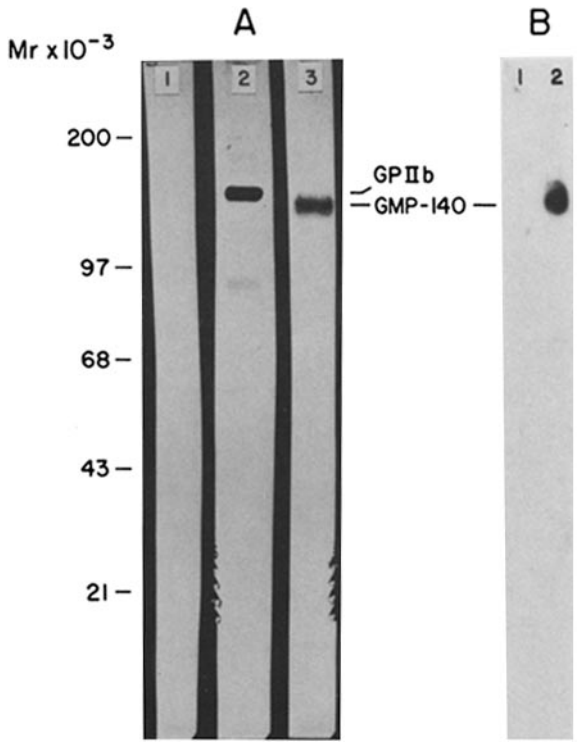
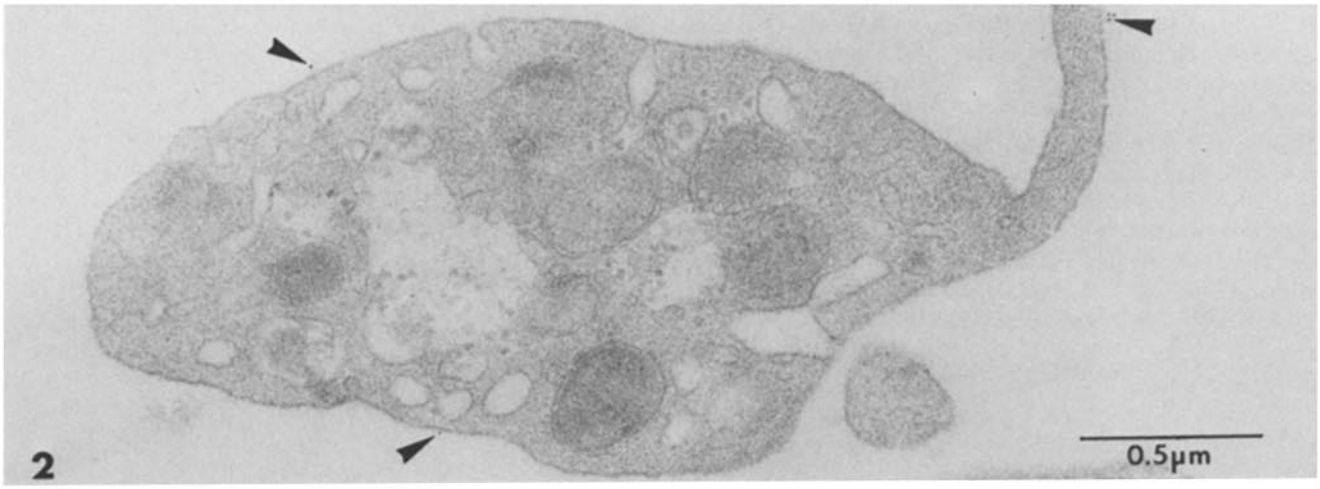


FIGURE 1 (*A*) Immunoblotting of platelet proteins with polyclonal antibodies to GMP-140. SDS extracts of platelets were electrophoresed in nonreduced SDS-polyacrylamide gels, transferred to nitrocellulose paper, and incubated with nonimmune or immune sera, and bound antibody was detected by an enzyme-linked immunosorbent assay. Lane *1*, nonimmune sera; lane *2*, anti-glycoprotein IIb antibodies; lane *3*, anti-GMP-140 antibodies. Antibodies to GMP-140 reacted with a single protein of M_r 140,000, which migrates slightly faster than glycoprotein IIb under nonreducing conditions. (*B*) Evidence for the amphipathic character of GMP-140. 40 μg platelet membrane protein was extracted with Triton X-114. The detergent and aqueous phases were recovered and analyzed by immunoblotting with anti-GMP-140 antibodies as in *A*, except that ^{125}I-protein A was used to detect bound antibody. Note that GMP-140 is markedly enriched in the detergent phase (lane *2*) and depleted in the aqueous phase (lane *1*).

[1] *Abbreviation used in this paper*: SCCS, surface-connected canalicular system.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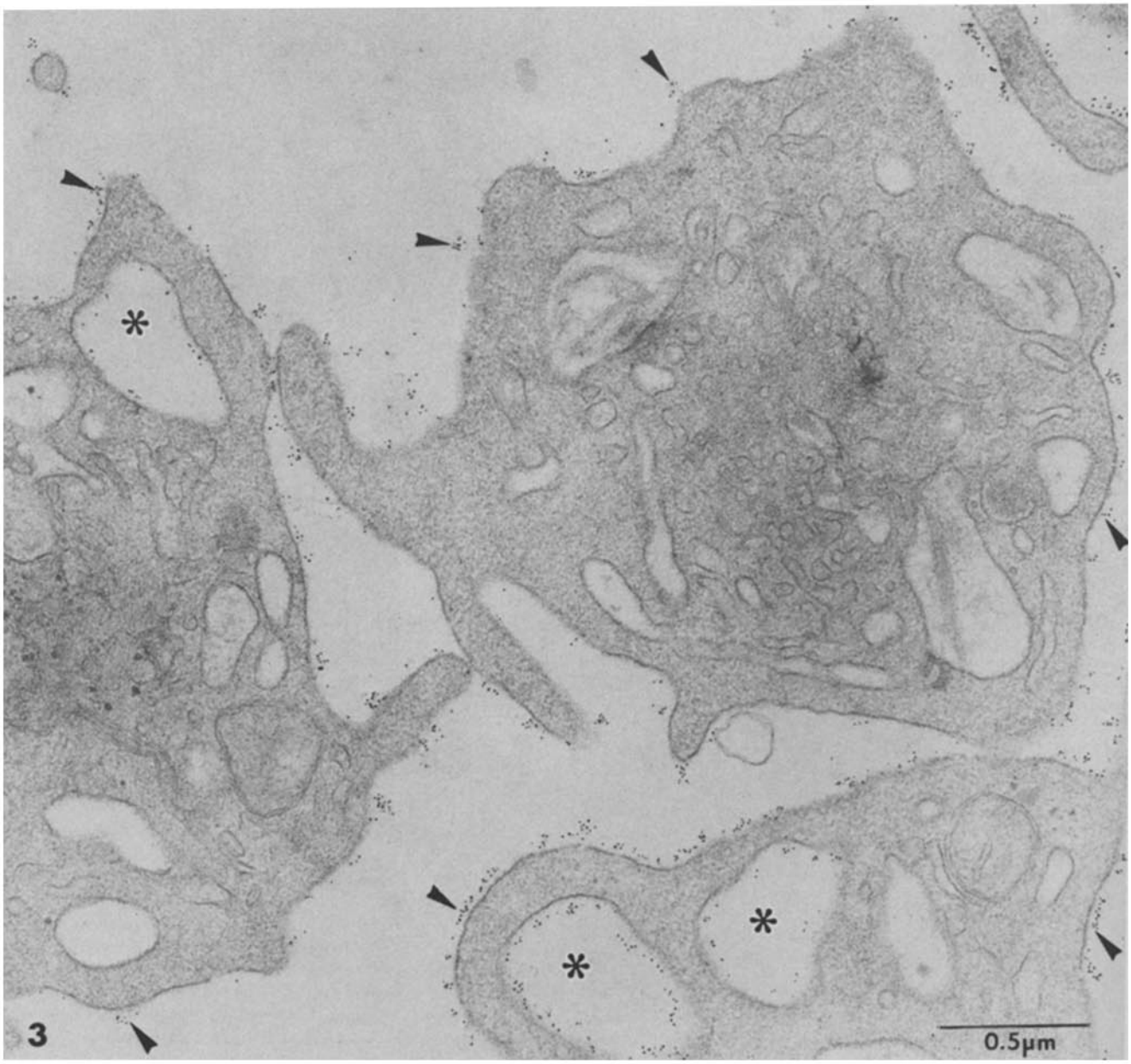
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(7), separated the detergent and aqueous phases at room temperature, then analyzed each phase by immunoblotting with anti-GMP-140 antibodies. As shown in Fig. 1 *B*, GMP-140 was highly enriched in the detergent phase and depleted in the aqueous phase. This indicates that GMP-140 has amphipathic properties and further suggests that it is an integral membrane protein.

In preliminary studies, we determined that the sensitivity of immunocytochemical detection of GMP-140 was much greater with the polyclonal antibodies than with the monoclonal antibody S12. This presumably reflects the binding of more than one polyclonal antibody to each GMP-140 molecule or greater affinity of some polyclonal antibody molecules for the platelet protein. We have therefore presented the polyclonal antibody data, although the S12 results were identical in all cases except for reduced labeling intensity.

Previous biochemical results (4) indicated that the binding of the monoclonal antibody S12 to platelets is dramatically enhanced after the platelets have been activated with thrombin. We first investigated this finding morphologically by incubating suspensions of nonpermeabilized platelets with antibodies to SDS-denatured GMP-140, and comparing the labeling with immunogold probes in unstimulated and thrombin-stimulated platelets. Unstimulated platelets, whether immediately fixed or washed, showed little label on the plasma membrane (Fig. 2). Thrombin-stimulated platelets, in contrast, showed at least a 50-fold increase in label on the plasma membrane (Fig. 3). Similar results were obtained with antibodies to nondenatured GMP-140 (not shown).

We next determined whether the increase in labeling of the protein on the platelet surface after thrombin stimulation reflected a conformational change in GMP-140; or rearrangement of other membrane proteins, either of which might allow expression of GMP-140; or the movement of the protein from an intracellular location to the plasma membrane. To answer this question, we used immunocytochemical procedures on frozen thin sections of unstimulated platelets, thus eliminating the problem of penetration of the cell by immunoreagents. Sections incubated with polyclonal antibodies prepared against denatured or nondenatured GMP-140 showed immunogold label along alpha-granule membranes (Fig. 4*a*). Occasionally the granule membrane bilayer is cleaved on frozen thin sections in a manner similar to freeze-fractured membranes. When this occurred, we could clearly detect the label within the inner granule membrane (Fig. 4*b*). Other intracellular structures were also labeled (Fig. 4*c*). These structures may be extracted alpha-granules. However, since the dense (serotonin-containing) granule cannot be identified on frozen sections (8, 9) we cannot rule out the possibility that its membrane also contains this antigen. Of a total of 92 platelets, we observed 73% of the immunogold particles over granules, 5% over plasma membrane, and 22% over the cell cytoplasm.

When we used the more sensitive technique of permeabilizing with saponin unstimulated platelets in suspension and then incubating the cells with polyclonal antibodies to nondenatured GMP-140 and Fab-peroxidase conjugate, we observed heavy reaction product along many alpha-granule membranes (Fig. 4*d*). No other intracellular organelles were specifically labeled; some alpha-granules showed no reaction product (Fig. 4*d*).

Since the technique used to examine the localization of antigen on thrombin-stimulated platelets did not allow visualization of internal sites (Fig. 3), frozen thin sections were also studied. These sections showed that 5 min after thrombin-stimulated degranulation had occurred, most of the GMP-140 had been redistributed from the alpha-granule membrane to the SCCS and plasma membrane (Fig. 5).

DISCUSSION

We have demonstrated by immunocytochemical procedures that a glycoprotein detected on the plasma membrane of thrombin-stimulated platelets is localized to alpha-granule membranes in unstimulated cells. This protein, when electrophoresed on SDS-polyacrylamide gels in the presence or absence of reducing agent, has mobilities similar to those previously reported for glycoprotein IIa, a platelet membrane protein of unknown function (10, 11). However, since more than one protein may have similar mobilities, depending on the gel system employed, we have given this protein (a granule membrane protein of M_r 140,000) the designation GMP-140 to indicate its distinct subcellular localization. GMP-140, which is localized in the membrane of alpha-granules, differs from secretory proteins such as fibrinogen and PF4, which are packaged within alpha granules (8, 9, 12). Shu-Chun et al. have described a monoclonal antibody named KC4, with properties similar to those of S12 (13). Subsequent experiments have indicated that KC4 and S12 identify different epitopes on the same platelet protein (Berman, C., B. Furie, B. C. Furie, and R. P. McEver, unpublished observations).

Other investigators have analyzed the protein composition of platelet secretory granule, or internal membranes by subcellular fractionation followed by SDS PAGE or crossed immunoelectrophoresis (14–18). Using human platelets, Gogstad et al. (14, 15) isolated an alpha-granule-rich fraction that contained a number of membrane-associated proteins, including two antigens thought to be unique to alpha-granules. Carty et al. (19) and Fishkes and Rudnick (20) have identified a proton-translocating ATPase in a dense granule fraction from porcine platelets, but the enzyme has not been isolated, nor

FIGURES 2 and 3 Fig. 2: Transmission electron micrograph of washed, unstimulated platelets, fixed and processed immunocytochemically for the extracellular distribution of GMP-140. Platelets were exposed to anti-GMP-140 polyclonal antibodies produced against SDS-denatured GMP-140, and then to immunogold conjugate. Note that there is only a small amount of label on the plasma membranes of these cells (arrowheads). Similar results were obtained with antibodies produced against nondenatured GMP-140. × 48,000. Fig. 3: Transmission electron micrograph of thrombin-stimulated (1 U/ml) platelets fixed 5 min after thrombin activation and examined immunocytochemically for the extracellular distribution of GMP-140 as in Fig. 2. A large amount of immunogold label is observed on the surfaces of these cells (arrowheads) as compared with the unstimulated platelets in Fig. 2. Note that the membranes of many vacuolar-appearing structures are also labeled (asterisks). However, since these cells have not been permeabilized, these structures must be continuous with the extracellular space, and probably represent dilated channels of the SCCS. Other such structures are unlabeled and are probably not yet continuous with the external space. EDTA (1 mM) was added to the platelet resuspension buffer to minimize cell clumping after activation with thrombin. × 48,000.

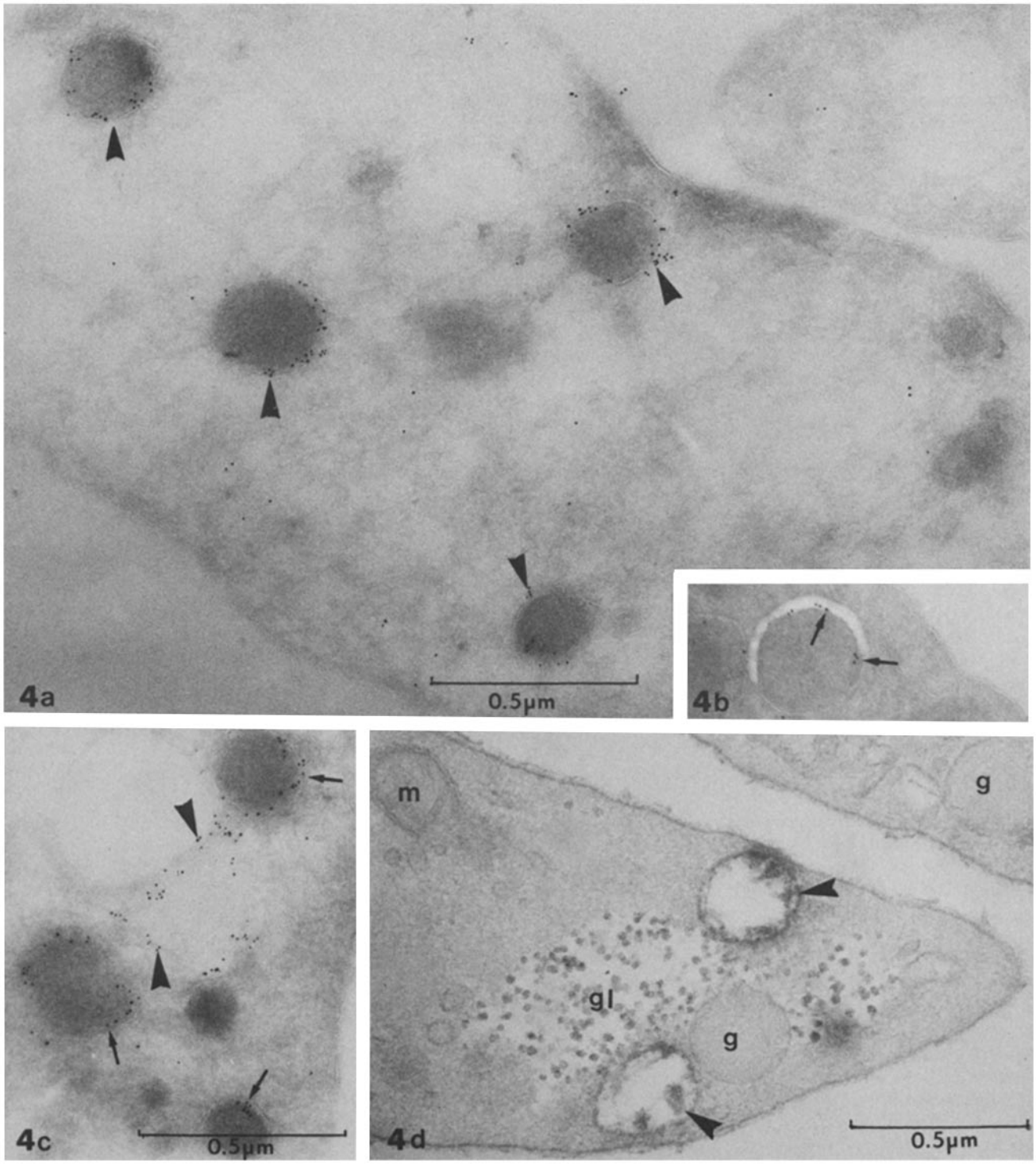

FIGURE 4 Frozen thin sections (*a–c*) of unstimulated platelets exposed to polyclonal antibodies to GMP-140, followed by immunogold conjugate. (*a*) Note the extensive label along alpha-granule membranes (arrowheads). Plasma membrane label is insignificant. The apparent cytoplasmic labeling may reflect granule membranes that are obliquely sectioned and not clearly visualized. × 70,000. (*b*) Note immunogold label along the inner granule membrane (arrows) which is cleaved on frozen thin sections in a manner similar to freeze-fractured membranes. × 70,000. (*c*) Label is present along both alpha-granule membranes (arrows) and along membranes of, as yet, unidentified structures (arrowheads), which may be α-granules with extracted content or dense bodies. × 70,000. (*d*) Unstimulated platelets, fixed, permeabilized with saponin, and exposed first to polyclonal antibodies to nondenatured GMP-140 and then to Fab-peroxidase conjugate. Some granules contain reaction product along their membranes (arrowheads), and others are unlabeled, (*g*) possibly because of incomplete permeabilization of the granule membranes by saponin. The matrix of these labeled granules is unreactive: apparently, GMP-140 is confined to the membrane of the alpha-granule. The labeled granules are designated as alpha-granules because of their typical size and number. We rarely observe dense granules in these frozen thin section preparations. Lysosomes and peroxisomes, which are revealed only by specific histochemical stains, are considerably smaller than alpha-granules. *gl*, glycogen; *m*, mitrochondrion. × 60,000.

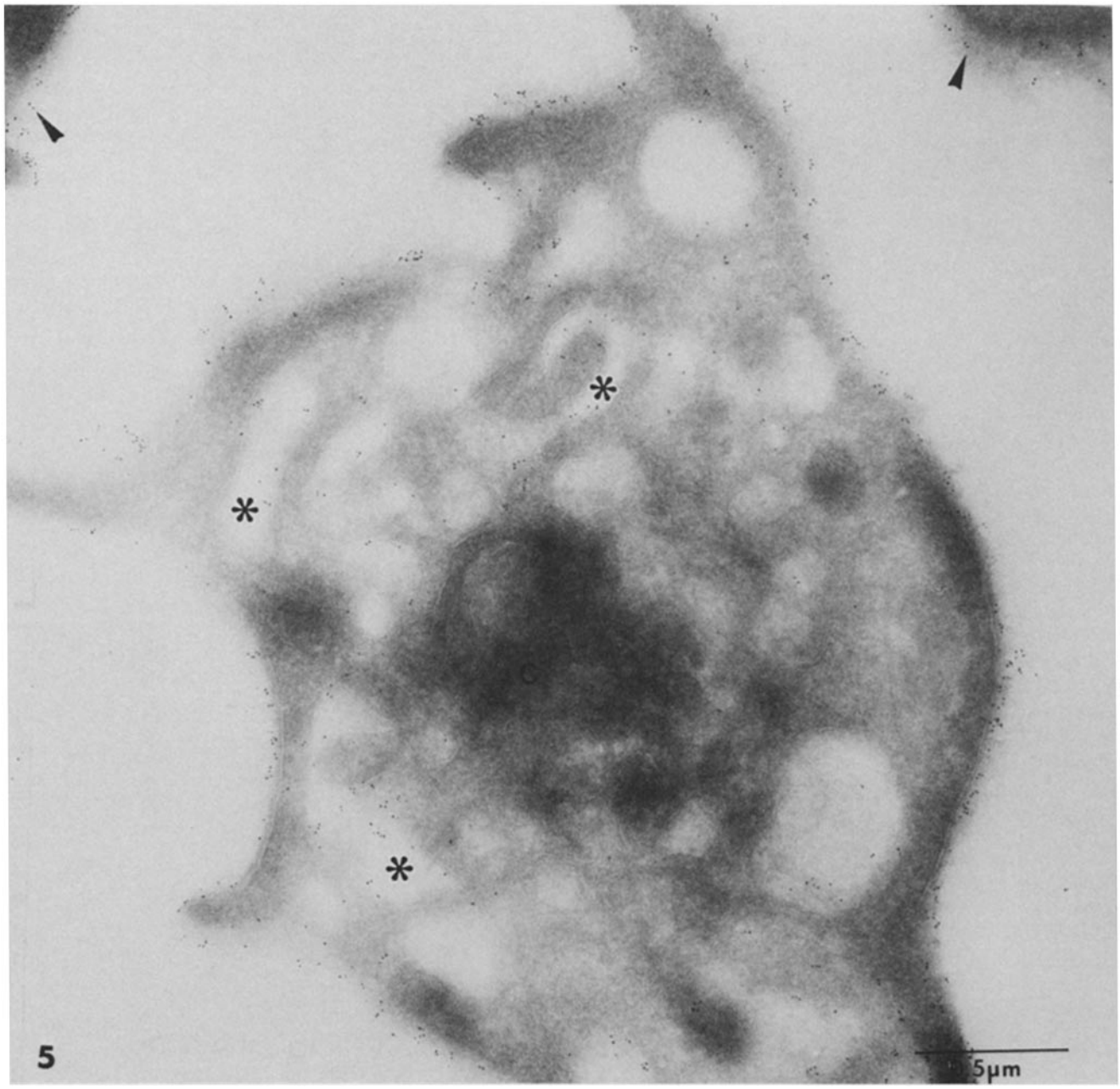

FIGURE 5 Frozen thin section of thrombin-stimulated (1 U/ml for 5 min) platelets exposed first to polyclonal antibodies to nondenatured GMP-140 and then to immunogold conjugate. By this time, degranulation of the alpha-granules into the SCCS has occurred and the center (c) of the cell is now occupied by a dense mass of cytoskeletal elements. Note the extensive label along the plasma membranes (arrowheads) and the widened channels of the SCCS (asterisks). × 48,000.

has SDS PAGE analysis of the dense granule membranes been reported. The technique of subcellular fractionation has the significant drawback that one membrane fraction may be contaminated with another. None of these studies involved immunocytochemical analysis: Therefore, we believe that we have provided the first well-documented example of a platelet glycoprotein confined to a secretory-granule membrane in the unstimulated cell. Recently, Buckley and Kelly (21) have characterized a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells.

After platelets have been stimulated with thrombin, GMP-140 is redistributed to the SCCS and plasma membrane. We showed previously that after thrombin stimulation, alpha-granules coalesce, then fuse with membranes of the SCCS (8). The proteins packaged in the granules are then released to the exterior of the cell, where at least some of the molecules bind to the platelet surface by Ca^{++}-dependent interactions (see diagram in reference 22). These membrane-bound proteins may play important roles in hemostasis (2, 8). Although the results reported here were obtained from platelet suspensions stimulated with thrombin for 5 min, studies are underway to examine shorter periods. This will enable us to determine the pattern of GMP-140 redistribution after stimulation from early time points to the point of maximal change. At low

thrombin concentrations GMP-140 becomes accessible to external S12 monoclonal antibody before significant release of [^{14}C]serotonin (4). This is consistent with the observation that release of alpha-granule proteins occurs at lower thrombin concentrations than does secretion of dense granule substances such as serotonin (23). GMP-140 presumably redistributes throughout the SCCS and plasma membranes after alpha-granule membranes fuse with SCCS membranes. We cannot exclude the possibility that GMP-140 clusters in discrete regions of the plasma membrane or the SCCS membranes. Resolution of this question will require immunocytochemical procedures that allow the visualization of large regions of the cell surface.

Membrane proteins in adrenal chromaffin granules, which are exposed on the cell surface after exocytosis, are subsequently internalized, presumably to be recycled into new secretory-granule membranes (24–26). Time-course studies of washed platelets indicate that the number of S12 binding sites present on the platelet plasma membrane remains constant from 10 to 60 min after thrombin stimulation at 37°C (George, J. N., and R. P. McEver, unpublished observations). This suggests that GMP-140 may not recycle after platelet secretion, although we cannot yet exclude such a possibility in platelets circulating in vivo.

The function of this platelet granule membrane protein is unknown. Since antibody binding studies indicate that there are ~10,000 molecules on the plasma membrane of each stimulated cell, GMP-140 appears to be a major component of platelet alpha-granule membranes and might play an important role in hemostasis. Even platelets from patients with the gray platelet syndrome have substantial amounts of immunoreactive GMP-140 (Rosa, J. P., R. P. McEver, and A. T. Nurden, unpublished observations). This may be explained by recent findings (27) that such platelets contain a population of abnormal small alpha-granules that are not identifiable by routine transmission electron microscopy. In addition to investigating the function of GMP-140, determining its localization in the human bone marrow megakaryocytes from which platelets are derived will be of great interest. Such studies may help to identify regions involved in membrane protein sorting and organelle formation in these unusual polyploid cells.

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