We have recently shown that several components from the platelet plasma membrane were also present at different rates in the alpha-granule membrane. This is the case for the glycoprotein (GP) IIb-IIIa (CD41), CD36, CD9, PECAM1, and Rap1b, while the GPIb-IX-V complex was considered to escape the rule. In this investigation, we studied the subcellular localization of GPIb, GPIX, and GPV in the resting platelets of normal subjects, patients with Bernard-Soulier syndrome, patients with Gray platelet syndrome, and human cultured megakaryocytes. Ultra-thin sections of the cells were labeled with antibodies directed against glycocalcin, GPIb, GPIX, and GPV. We have shown that a significant and reproducible labeling for the three GPs was associated with the alpha-granule membrane, accounting for approximately 10% of the total labeling. Furthermore, GPIb labeling appears colocalized with its alpha-granule-associated ligand, von Willebrand factor (vWF). After thrombin activation, vWF remained close to the limiting membrane of the open canaliculalar system (OCS), suggesting an early association of both receptor and ligand. Plasma membrane and alpha-granule labeling was virtually absent from the Bernard-Soulier platelets (characterized by a GPIb deficiency), thus proving the specificity of the reaction. In Gray platelets (storage granule deficiency syndrome), the small residual alpha-granules were also occasionally labeled for GPIb, GPIX, and GPV. Cultured megakaryocytes that displayed the classical GPIb distribution, eg, demarcation and plasma membranes, exhibited also a discrete labeling associated to the alpha-granules. In conclusion, this study shows that, evenly for these three GPs, the alpha-granule membrane mirrors the plasma membrane composition. This might occur through an endocytotic process affecting each plasma membrane protein to a different extent and could have a physiologic relevance in further presentation of a receptor bound to its alpha-granule ligand to the platelet surface.

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PLATELET alpha-granules represent a secretory compartment that releases its content after appropriate stimulation. They contain a wide variety of coagulation and adhesive proteins involved in hemostatic mechanisms. They are formed during megakaryocyte maturation, where they arise by a double mechanism: endogenous synthesis and endocytosis of plasmatic proteins. Furthermore, in recent studies, we have demonstrated that several plasma membrane receptors are also present in the alpha-granule limiting membrane. These receptors include glycoprotein (GP) IIb-IIIa, the fibrinogen receptor, CD56, the thrombospondin and collagen receptor, CD9; PECAM1; and Rap1b, a guanosine triphosphate (GTP)-binding protein. Therefore, most of the studied proteins seemed to follow the rule, except the GPIb-IX-V complex, which was considered until now to be restricted to the plasma membrane, possibly because of its cytoskeletal association. In this study, we have tried to document this statement using an immunoelectron microscopic approach performed on normal and pathologic platelets and megakaryocytes. We have been able to demonstrate that small amounts of GPIb, GPIX, and GPV are associated with the alpha-granule membrane. The presence of numerous plasma membrane receptors on the alpha-granule membrane suggests that the endocytotic process that directs plasmatic proteins into the alpha-granule also affects a large pattern of plasma membrane receptors, although to a different extent.

MATERIALS AND METHODS

Cells

Platelet samples were taken from normal healthy volunteers, from a patient with Bernard-Soulier syndrome, and from a patient with Gray platelet syndrome. Blood samples were harvested by venipuncture into plastic tubes containing AC-D-C buffer (6.8 mmol/L citric acid, 11.2 mmol/L trisodium citrate, 24 mmol/L glucose, pH 4.2). The platelet-rich plasma (PRP) was obtained by centrifugation for 10 minutes at 180g and 22°C. The isolated platelets were obtained by centrifugation of PRP for 10 minutes at 1,100g and 22°C and washed three times with Tyrode buffer (36 mmol/L citric acid, 5 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 103 mmol/L NaCl, 5 mmol/L glucose, pH 7.4) containing 3.5 mg/mL bovine serum albumin (Sigma Chemical Co, St Louis, MO). The washed platelets were resuspended and fixed with 1% glutaraldehyde (Ladd Research Inc, Burlington, UK) in 0.1 mol/L phosphate buffer.

Megakaryocytes used in the electron microscopic study were grown in liquid culture from bone marrow precursors obtained from normal adult graft donors, as previously described.

Antibodies

Different antibodies against glycocalcin, GPIb, GPIX, and GPV were used for immunoelectron microscopy study. An anti-human GPIb monoclonal mouse antibody purchased from Dakopatts (Glostrup, Denmark) was used at 1/100 dilution. An anti-human glycocalcin and GPV polyclonal rabbit antibodies, provided by Dr Michael Berndt, Prahran, Australia, were used, respectively, at 10 μg/mL and 30 μg/mL. An anti-human GPIb, provided by Dr Kenneth Clemetson, Bern, Switzerland, was used at 30 μg/mL. An anti-human GPIX monoclonal antibody, provided by Dr Michael Berndt, was used at 30 μg/mL. For double immunolabeling, an anti-human P-selectin, provided by Dr Michael Berndt, and an anti-human von Willebrand factor (vWF) purchased from Cappel Laboratory (Downington, PA) were used, respectively, at 30 μg/mL and 1/50 dilution. Gold-conjugated (10 and 15 nm) protein A purchased from the Department of Cell Biology, University of Utrecht (Utrecht, The Netherlands) were used, respectively, at 1/80 and 1/35 dilutions.

Characterization of the Polyclonal Antibodies

The specificities of the polyclonal antibodies were assayed by Western blotting of platelet lysate. Briefly, washed PRP was solubilized by
**Fig 2.** Biochemical characterization of the polyclonal antibodies used in this study by Western blotting of platelet lysates. (A) The anti-glycocalcin antibody recognizes a unique protein of approximately 145 kDa molecular weight. (B) The anti-GPV antibody recognizes a protein of approximately 80 kDa molecular weight, and (C) the anti-GPIX antibody recognizes a major protein of approximately 22 kDa molecular weight.

**Fig 1.** (A and B) Immunogold localization of GP Ib with a monoclonal antibody on thin frozen sections of resting platelets. Immunolabeling is found to be associated with the plasma membrane (pm) and open canalicular system (ocs). Some gold particles are also associated with the alpha-granule (a) membrane, while mitochondria (m) are devoid of labeling. Bars, 250 nm.
the addition of 2% sodium dodecyl sulfate (SDS) and 1 mmol/L EDTA and separated on SDS-polyacrylamide gel electrophoresis (PAGE) with or without a reducing condition with 5% 2-mercaptoethanol, using a 7% resolving gel and 3% stacking gel. Gels were electroblotted on nitrocellulose filter membrane by semidry transfer. The nonspecific binding was blocked in 5% low fat powder milk, and membranes were probed with polyclonal antibodies for 1 hour at room temperature. The labeled proteins were revealed after incubation with sheep anti-rabbit immunoglobulins bound to peroxidase.

**Electron Microscopy**

Normal platelets and megakaryocytes were prepared for immunoelectron microscopy as follows: they were fixed in 1% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 1 hour at 22°C, washed three times with the same buffer, embedded in sucrose, and freeze-dried in liquid nitrogen. Pathologic platelets were also embedded in glycol methacrylate according to the method described by Leduc and Bernhard; this technique permitted a more lasting storage for precious samples. Then, the immunohistochemical reactions were performed on thin sections collected on copper grids according to the method of Slot et al. Briefly, the sections were labeled by a first incubation with the antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma) for 20 minutes at 22°C, washed, and then incubated with protein A-gold (10 nm) for 20 minutes at room temperature. The sections were counterstained with 2% uranyl acetate, pH 7, and methyl cellulose uranyl.
Fig 4. Immunolocalization of GPIX (A) and GPV (B) with polyclonal antibodies on thin frozen sections of resting platelets. As expected for these GPIb-associated proteins, immunolabeling is found to be associated with the plasma membrane (pm), the open canalicular system (ocs) membrane, and also the alpha-granule membrane (a), while mitochondria (m) are devoid of labeling. For GPIX, the same results are obtained using a monoclonal anti-GPIX instead of the polyclonal antibody (A, inset). Bars, 250 nm.
For double immunolabeling, a short time fixation with 1% glutaraldehyde in phosphate buffer was realized after the incubation with the gold conjugate, and a second round of labeling with the second antibody was realized using a different size of gold particle protein A conjugate. Samples were observed on a Philips 450 CM 10 electron microscope.

Quantitative Estimation

Membrane labeling intensity was evaluated by counting the gold particles per micrometer of membrane. The alpha-granule pool was quantified by counting the gold beads associated with the alpha-granule membrane on one hand and the plasma membrane on the other: the ratio of intracellular versus plasma membrane pool per equatorial platelet section was calculated.

RESULTS

Resting Platelets

Immunolocalization of GPIb. To ensure the specificity of the observed immunolabeling reaction, a monoclonal antibody against GPIb was used as a first instance. Immunolabeling on thin sections allows marking of plasma membrane proteins as well as intracellular proteins. In this experiment, labeling for GPIb antigen with a monoclonal antibody was detected on the plasma membrane, and at the luminal face of the open canalicular system (OCS). Careful examination led to the observation that some gold particles were also bound on alpha-granule membranes, while other structures such as mitochondria were devoid of labeling (Fig 1A and B). Using a monospecific polyclonal antibody (Fig 2A) raised against purified glycolcalcin, we obtained a stronger labeling on the same structures; eg, plasma membrane, OCS, and alpha-granule membrane (Fig 3A).

Due to their high number, large size, and dark nucleoids, alpha-granules could be identified. In addition to morphologic criteria, double immunolabeling with both anti-glycolcalcin and an anti-P-selectin polyclonal antibody (Fig 3B) or an anti-vWF polyclonal antibody (Fig 3B, inset) as alpha-granule markers confirmed that the labeled granules were, indeed, alpha-granules because of the codistribution of the proteins.

Immunolocalization of GPIX and GPV. GPIb has been described to form a noncovalent complex in the platelet membrane with GPIX and GPV.25 We have investigated the localization of both proteins in normal resting platelets and found that their distributions were similar to GPIb (eg, plasma membrane, OCS) and that a small proportion of gold probes were also present on the alpha-granule membrane (Fig 4A and B).

The characterization of the polyclonal antibody anti-GPIX is shown in Fig 2C; we have also confirmed our results using a monoclonal antibody and have obtained a weaker labeling but an identical distribution (Fig 4A, inset).

Pathologic Platelets

Gray platelet syndrome is a rare congenital bleeding disorder21 in which the platelets are markedly deficient in morphologically recognizable alpha-granules.22 The cause of the abnormality affecting the alpha-granules is unknown, but it appears that the alpha-granule membrane is normally constituted because its proteic components, such as P-selectin,21 GPIIb-IIIa,7 and CD36,8 are present. On such platelets, immunolabeling for GPIb was found on the plasma membrane and also on abnormal alpha-granule-like structures (small residual alpha-granules and small or large vacuoles identified as empty pathologic alpha-granules; Fig 5A). Similar localization was also obtained for both GPIb-associated glycoproteins, GPIX and GPV (Fig 5C and E). These results suggest that this pool storage deficiency is not due to an alpha-granule membrane composition defect.

Bernard-Soulier syndrome is an inherited bleeding disorder characterized by a deficiency in the GPIb-IX-V complex.24,25 These platelets, embedded in glycol methacrylate or in sucrose, displayed a severely decreased immunolabeling for GPIb including the alpha-granule labeling. The same results were obtained for GPIX and GPV and attested for the specificity of the labeling observed on normal platelets (Figs 5B, D, and F and 6A).

Colocalization Between GPIb and vWF

To assess that alpha-granule GPIb could be associated with vWF, we performed a double immunolabeling for GPIb and vWF combined with quantitative estimations of associated labeling. We found that more than 80% of double-labeled alpha-granules showed an apparent association between GPIb and vWF labeling (Fig 3B, inset; Fig 6B, inset), whereas less than 10% presented this characteristic when P-selectin was used as the alpha-granule marker (Fig 3B).

Moreover, less than 20% of such an association between vWF and the other alpha-granule membrane-associated receptors, CD9 and PECAM1, was found (Table 1). After thrombin activation, most vWF labeling was located along the OCS membrane and was codistributed with GPIb labeling (Fig 6B).

Megakaryocytes

We further investigated the localization of GPIb in the platelet precursors, the megakaryocytes. Mature human cultured megakaryocytes displayed the classical membrane distribution for GPIb (eg, plasma and demarcation membranes), but also some alpha-granules showed immunolabeling for this glycoprotein (Fig 7). Similar results were found for GPIX and GPV (not shown).

Control

When the primary antibody was either replaced by a nonrelevant polyclonal antibody or omitted from the immunolabeling, staining was completely negative.

Quantitative Estimation

When quantitative measurements of the GPIb labeling were realized on normal platelets, they showed an association of approximately 10% of total labeling with the alpha-granule membrane (Fig 8). On Bernard-Soulier platelets, a decrease of 90% of total normal platelet labeling was observed. In these semiquantitative estimations, immunolabeling on glycol methacrylate-embedded Bernard-Soulier plate-
Fig 5. Immunolocalization of GPIb, GPIX, and GPV in glycol methacrylate-embedded pathologic platelets. In the Gray platelets, GPIb (A), GPIX (B), and GPV (C) are present and localized on the same structures, eg, plasma membrane (pm), small residual granules (a), and also vacuolar structure (a'), usually considered as empty granules. In the Bernard-Soulier platelets, which do not express the GPIb-IX-V complex, the immunolabeling for GPIb (D), GPIX (E), and GPV (F) is seriously decreased. This finding also attests for the specificity of the reaction. Bar, 250 nm.

Fig 6. Sucrose-embedded Bernard-Soulier platelets immunolabeled for GPIb: As in glycol methacrylate-embedded cells, membrane labeling for GPIb is virtually absent, close to the background staining. (B) Double immunolabeling for GPIb, 10 nm gold particles (arrowheads), and vWF, 15 nm gold particles (arrows). On resting platelets, double-labeled alpha-granules (inset, A) show frequently a close association of both labeling. After thrombin activation, most of the vWF labeling appears to be associated with the open canalicular system (OCS) membrane and codistributed with GPIb. Bar, 250 nm.
Table 1. Colocalization of GPIb and vWF

<table>
<thead>
<tr>
<th>Double Labeling</th>
<th>% of Alpha-Granules Showing Colocalization of Both Markers (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIb/vWF</td>
<td>82 (20)</td>
</tr>
<tr>
<td>GPIb/P-selectin</td>
<td>9 (15)</td>
</tr>
<tr>
<td>CD9/vWF</td>
<td>19 (15)</td>
</tr>
<tr>
<td>PECAM1/vWF</td>
<td>14 (20)</td>
</tr>
</tbody>
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On normal platelets, 82% of double-labeled alpha-granules show a colocalization of GPIb and vWF. When P-selectin is used as an alpha-granule marker, only 9% of double-labeled granules present such a colocalization. As a control, double labeling between vWF and two alpha-granule receptors, CD9 and PECAM1, show, respectively, 19% and 14% of marker association. These data suggest that GPIb and vWF are specifically associated.

DISCUSSION

The ultrastructural localization of membrane receptors in cell organization may reflect the dynamic feature of these membranes within secretory and endocytotic pathways. The alpha-granules are the main secretory organelles of platelets and megakaryocytes. They arise from a dual mechanism: endogenous synthesis occurring in megakaryocytes and endocytosis from the surrounding extracellular medium. The first mechanism involves many hemostatic factors, such as thrombospondin, beta-thromboglobulin, and vWF, whose corresponding mRNAs are present in megakaryocytes. The second pathway is the route of several plasmatic proteins such as immunoglobulins, albumin, and fibrinogen, which are acquired exclusively by endocytosis. Fibrinogen endocytosis appears to be a receptor-mediated process, under the control of GPIIb/IIIa. This fibrinogen endocytosis takes place at the end of megakaryocyte maturation and continues during platelet life.

The proteins of the alpha-granule membrane can also be categorized in two groups. On the one hand, some receptors...
are restricted to the alpha-granule limiting membrane and are absent from the plasma membrane, such as P-selectin,^30 osteonectin,^31 and GMP33. On the other hand, further studies have shown that some proteins that are normal components of the plasma membrane are also present on the alpha-granule membrane at different rates; eg, GPIbIIIa, with approximately 50% of the total platelet pool of GPIbIIa present in the alpha-granule membranes;^3 CD36, with 35%;^6 CD9 and PECAM1, with 25%; and the small GTP-binding protein Rap1b, with 15%.^10 Until now, GPIb, which is the platelet receptor that mediates the adhesion of unstimulated platelets to vWF,^33 was considered to be restricted to the plasma membrane of resting platelets, probably because of its cytoskeletal association. In the present report, we show that even for this membrane-associated protein, a consistent amount of GPIb, and also associated glycoproteins IX and V, is present in the platelet alpha-granule membrane.

In previous reports,^14,34 we proposed that GPIb was absent from the alpha-granule membrane because the observed scattered labeling associated with this structure was considered as background staining. Technical improvements—first, in the marked decrease of background staining using glycin as the saturation agent and phosphate buffer rather than Tris buffer, and second, using protein-A-gold instead of goat anti-rabbit gold—have increased technical sensitivity and permitted to demonstrate the observed labeling for GPIb associated with the alpha-granule membrane as a specific association. Moreover, this result has been definitely confirmed using a monoclonal antibody.

Labeling for the three glycoproteins Ib, IX, and V was dramatically decreased on platelets from a patient with Bernard-Soulier syndrome, attesting for the specificity of the reaction. On platelets from a patient with the storage disease Gray platelet syndrome, in which platelets lack normal alpha-granules, the residual pathologic granules were labeled for these three associated glycoproteins (Ib, IX, and V). This finding shows that GPIb-IX-V localization is unaltered in

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**Fig 8.** Distribution of the GPIb labeling on normal platelets (n = 28). On normal platelets, approximately 10% of total labeling is associated with the alpha-granules. Mitochondria represent the background staining.

**Fig 9.** Comparison of GPIb labeling on normal and Bernard-Soulier platelets. On Bernard-Soulier platelets, the total labeling appears seriously decreased.
these patients and confirms and supplements previous observations describing this pathology as soluble protein storage deficiency in which the alpha-granule membrane is normal.

Relative to previous findings in fibrinogen endocytosis that implicate GPIIB-IIIa,28 and also to the presence in the alpha-granule membrane of numerous plasma membrane receptors, these results raise the question of a protein alpha-granule targeting signal. A cDNA P-selectin transfection study in a pituitary cell line has led to the description of a 23-amino acid cytoplasmic domain of P-selectin responsible for its direct transport to secretory granules.35,36 However, concerning proteins like GPIIB-IIIa, CD36, or GPIb, which display a double localization in platelets and megakaryocytes, it appears more delicate to consider the existence of a specific signal leading the protein in two different compartments. On the other hand, the hypothesis of plasma membrane targeting followed by a specific and regulated endocytosis to the alpha-granules, as it has been proposed as targeting mechanism of a lysosomal protein,37 could appear more suitable. Numerous receptors, included some β-integrins, have been shown to contain in their cytoplasmic domain a specific endocytic motif, NPY.38-40 Other specific signals, such as the di-leucin motif and tyrosine-based motif, or secondary structures in the cytoplasmic tail of membrane receptors are also commonly proposed as endocytic and targeting signals.41-46 The description of such a specific signal governing a differential rate of endocytosis from the platelet plasma membrane to the alpha-granules, perhaps through differential coated pits affinity, could explain our results but remains to be determined. Such a process has been described for the transferrin receptor41,42 and for the low density lipoprotein receptor.43

Furthermore, a preliminary study of GPIIB-IIIa expression during megakaryocyte maturation shows the appearance of the first protein expression on the plasma membrane, on demarcation membranes, and then on the alpha-granule membrane. These observations hinge on the hypothesis of an indirect targeting, implicating first a plasma membrane expression.

Concerning the physiologic relevance of the presence of GPIb on the alpha-granule membrane, the observation of an apparent codistribution of GPIb and its ligand vWF on resting and thrombin-activated platelets raises the possibility for a specific role of GPIb in the redistribution and the presentation of functional adhesive complex (GPIb-vWF) during platelet activation. Such a phenomenon has already been proposed for GPIb-IIIa and fibrinogen43,44 and might be generalized to other alpha-granule receptors.

In conclusion, this report documents the original composition of the limiting membrane of alpha-granules, the platelet secretory organelles, which qualitatively mimics the plasma membrane, and proposes a functional interpretation for this composition.

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Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V

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