RAPID COMMUNICATION

Platelet α-Granule and Plasma Membrane Share Two New Components: CD9 and PECAM-1

By Elisabeth Martin Cramer, Gaëtan Berger, and Michael C. Berndt

CD9 (p24) and PECAM1 (CD31) antigens are well-defined components of the platelet plasma membrane. Both are integral glycoproteins (GPs) implicated in the adhesive and aggregative properties of human platelets. In the present report, we have investigated their subcellular localization using immunoelectron microscopy. The monospecificity of the two polyclonal antibodies used was confirmed by immunoblotting. On normal resting platelets, immunolabeling for CD9 and PECAM1 was found lining the plasma membrane and the luminal face of the open canalicular system. Some labeling was also consistently found on the α-granule limiting membrane. This was confirmed by double labeling experiments in which fibrinogen and von Willebrand factor (vWF) were used as α-granule markers. CD9 and PECAM-1 were found lining the membrane of the same granules that contained fibrinogen and vWF in their matrix. CD9 and PECAM-1 thus appear to have an intracellular distribution identical to GPIIb-IIIa, a major aggregation platelet receptor. To rule out a cross-reactivity of the GPIb-IX complex is found on the external plasma membrane and on the luminal face of the open canalicular system. Some labeling was also consistently found on the α-granule limiting membrane. This was confirmed by double labeling experiments in which fibrinogen and von Willebrand factor (vWF) were used as α-granule markers. CD9 and PECAM-1 were found lining the membrane of the same granules that contained fibrinogen and vWF in their matrix. CD9 and PECAM-1 thus appear to have an intracellular distribution identical to GPIIb-IIIa, a major aggregation platelet receptor. To rule out a cross-reactivity of the GPIb-IX complex, we studied PECAM1 and CD9 expression on the platelets from a patient with type I Glanzmann’s thrombasthenia whose platelets are devoid of GPIIb/IIIa. The same pattern of labeling was observed for both antigens as for normal platelets. Normal platelets were further observed after stimulation by agonists that either fail to induce (ADP) or induce granule secretion (thrombin). After treatment with ADP, platelets changed shape and centralized their granules; the plasma membrane immunolabeling remained unchanged; and gold particles were still found decorating the periphery of the centralized α-granules. After thrombin treatment, α-granules fused with the platelet membrane and secretion occurred. A significant increase of labeling was then observed on the platelet surface. From these results we conclude that the α-granule membrane contains two additional receptors in common with the plasma membrane. This suggests that α-granule membrane receptors may originate from a dual mechanism: direct targeting from the Golgi complex in megakaryocytes (for α-granule-specific receptors such as P-selectin) or by endocytosis from the plasma membrane (for proteins distributed in the two compartments).

© 1994 by The American Society of Hematology.

During hemostasis, platelet membrane glycoproteins (GPs) allow the platelet to react to and interact with its environment. Platelet adhesion is mediated by recognition of subendothelial bound von Willebrand factor (vWF) by a specific platelet receptor, the GPIb-IX complex. The GPIb-IX complex is also a critical component in maintaining platelet shape because it spans the membrane bilayer and is linked to a network of submembraneous actin filaments via its cytoplasmic association with actin-binding protein. Topographically, the GPIb-IX complex is found on the external plasma membrane and on the luminal face of the surface-connected open canalicular system (OCS). Other major membrane GPs, such as the platelet aggregation receptor GPIIb-IIIa and CD36 (GPIV), are present on both the platelet plasma membrane and on the α-granule membrane. In this study, we report the topographic distribution of two additional major platelet surface GPs, PECAM-1 and CD9.

CD9 is a major surface GP on platelets with a copy number (~40,000 per platelet) almost equivalent to the major surface GP, GPIIb-IIIa. CD9 has an apparent molecular weight of 24 kD and belongs to a family of membrane proteins termed tetraspanins because they transverse the membrane four times. The role of CD9 in platelet function is presently unclear. However, it is known to associate with GPIIb-IIIa after platelet activation, suggesting it may play a role in regulating the platelet aggregation response. PECAM-1 (platelet/endothelial cell adhesion molecule-1) or CD31 is a 130-kD integral membrane GP expressed on the surface of platelets, endothelial cells, monocytes, and neutrophils. There are approximately 7,000 copies of surface-accessible PECAM-1 on platelets. PECAM-1 is believed to be important in mediating endothelial cell-endothelial cell cohesiveness, but, like CD9, its function on platelets is unclear. However, the recent finding that PECAM-1 becomes phosphorylated and associates with the platelet cytoskeleton on platelet activation suggests that PECAM-1 may be a major mediator of postactivation events on platelets.

In this study, we have found that both CD9 and PECAM-1, like GPIIb-IIIa and CD36, are located not only on the plasma membrane and luminal surface of the OCS, but also in the α-granule membrane, with the α-granule content for each being approximately the same (~30% of total). The data are consistent with the membrane proteins of the α-granule arising in one of the two ways, i.e., by targeted localization to the α-granule membrane, as occurs with P-selectin, or by an endocytotic mechanism that delivers α-granule constituents such as fibrinogen and IgG to the granule as well as platelet plasma membrane GPs.
Platelets

Platelets were taken from three normal healthy volunteers and from two patients with Glanzmann's thrombasthenia type 1. Blood samples collected by venipuncture into plastic tubes containing ACD-C buffer (6.8 mM/L citric acid, 11.2 mM/L trisodium citrate, 24 mM/L glucose, pH 4.2). The platelet-rich plasma (PRP) was obtained by centrifugation for 10 minutes at 180g and 22°C. Isolated platelets were obtained by centrifugation of PRP for 10 minutes at 1,100g and 22°C and washed three times with Tyrode's buffer (36 mM/L citric acid, 5 mM/L KCl, 2 mM/L CaCl2, 1 mM/L MgCl2, 103 mM/L NaCl, 5 mM/L glucose, pH 7.4) containing 3.5 mg/mL bovine serum albumin (Sigma Chemical Co., St Louis, MO) and 7 mg/mL prostaglandin E1 (Sigma). The washed platelet samples were resuspended in Tyrode's buffer containing 2.9 mM/L CaCl2. When indicated, washed platelet samples were exposed to 0.05 U/mL human α-thrombin (Sigma) at 22°C for 5 minutes or 20 μM/L adenosine diphosphate (ADP; Sigma) at 37°C for 5 minutes, without stirring. The reaction was stopped by fixation with 1% glutaraldehyde (Ladd Research Industries, Burlington, UK) in Tyrode's buffer.

Purification of CD9 and PECAM-1

For CD9 purification, human platelet membranes were selectively extracted with isotypic and hypotonic buffers containing 0.1% Triton X-100, as previously described.23 After centrifugation of the hypotonic extract at 100,000g for 1 hour at 0°C, the supernatant was loaded onto a 10 × 1 cm column of FMC 56-Affigel 10 (2 mg FMC 56/mL of agarose). FMC 56 is a previously described murine monoclonal antibody directed against CD9.24 After thorough washing, CD9 was eluted with 0.1 M/L glycine, pH 2.4 (0.1% wt/vol), in Triton X-100. To prevent disulfide-dependent dimerization of the CD9 on neutralization, the CD9-containing fractions were made 1% glutaraldehyde in 0.1 M/L sodium phosphate buffer, pH 6.0 (containing 1 mM N-ethylmaleimide, 0.15 M sodium chloride, 0.1% [wt/vol] Triton X-100, 0.02% [wt/vol] NaNO2) and then against the same buffer, but without N-ethylmaleimide. CD9 was then rechromatographed on a second 10 × 1 cm column of FMC 56-Affigel 10 and processed in similar manner to the first FMC 56-affinity column. The purified CD9 gave a single 24-kD band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under both nonreducing and reducing conditions.

For PECAM-1 purification, the platelet membranes were extracted and the membrane extract chromatographed on concanavalin A-Sepharose 4B as previously described. Although the methyl-α-D-mannopyranoside eluate contained PECAM-1, the majority of the PECAM-1 did not bind to the concanavalin A-Sepharose 4B column. The flow through of this column was therefore then loaded onto a 10 × 1 cm column of WM 59-Affigel 10 (2 mg WM59/mL of agarose). WM 59 is a previously described monoclonal antibody directed against PECAM-1.17 After thorough washing, platelet PECAM-1 was eluted with 0.1 M/L glycine, 0.1% [wt/vol] Triton X-100, pH 2.4. The eluted fractions were immediately neutralized by the addition of one-fifth volume of 1 M/L Tris, pH 8.0, and then dialyzed against 0.01 M/L Tris, 0.15 M/L sodium chloride, 1 mM/L calcium chloride, 0.1% [wt/vol] Triton X-100, 0.02% NaN3, pH 7.4, and stored at -70°C. The purified protein on SDS-PAGE consisted of a protein band corresponding to PECAM-1, and a proteolytic degradation product of ~20 kD lower molecular weight.

Antibodies

Affinity-purified rabbit anti-CD9 and anti-PECAM antibodies were raised in New Zealand white rabbits and affinity-purified on the respective antigen coupled to Affigel 10/15, as has been previously described in detail for other platelet membrane GPS.3,4,5 Antifibrinogen and anti-vWF antibodies were purchased from Cappel Laboratories (Downington, PA) and Dakopatts (Copenhagen, Denmark), respectively, and used at a 1/100 dilution.

For immunoelectron microscopy, a 1/125 and 1/50 dilution of the affinity-purified polyclonal antibodies directed against PECAM-1 and CD9 were used, respectively. Goat antirabbit antibody (IgG) coupled to 10-nm colloidal gold particles (GAR-G10) were purchased from Amersham (Les Ulys, France) and used at a 1/50 dilution. Protein-A coupled to 10- and 20-nm gold particles was kindly provided by H. Heijnen (Cell Biology Department, University Hospital, Utrecht, The Netherlands).

Electron Microscopy

Cells were prepared for immunoelectron microscopy as follows. They were fixed in 1% glutaraldehyde in 0.1 M/L phosphate buffer, pH 7.2, for 2 hours at 22°C; washed three times in the same buffer; and embedded in glycol methacrylate (GMA) as described previously.23 The immunohistochemical reactions were then performed on thin sections by the method of Bendayan.26 Briefly, the sections were labeled by a first incubation with the polyclonal rabbit antibodies for 2 hours at 22°C in a humidified atmosphere. The incubation with GAR-G- or protein A-G was performed for 1 hour at room temperature and the sections were counterstained with uranyl acetate and lead citrate. Samples were observed on a Philips EM 300 or 450 CM 10 electron microscope.

To identify the labeled granules, a double-labeling was performed using the soluble proteins, vWF, and fibrinogen as α-granule markers. For the double-immunolabeling experiments, frozen sections were used as previously described.1 Bound anti-CD9 or anti-PECAM-1 were detected with protein A coupled to 5-nm gold particles. Anti-vWF or antifibrinogen were detected with protein A coupled to 15-nm gold particles.

Quantitative Study

Quantitative data were obtained by counting the gold beads associated with the various structures on an average of 15 platelet equatorial sections in each experimental condition.

Analytical Methods

Western Blot analysis of SDS-PAGE-separated platelet membrane GPs was performed as previously described.3

RESULTS

Specificity of the Polyclonal Antibodies

Figure 1 establishes the monospecificity of the affinity-purified polyclonal antibodies to human CD9 and PECAM-1 as evaluated by Western Blot analysis. Both antibodies reacted with a single major band, consistent with the reported molecular weight characteristics of CD9 (lane 3)10 and PECAM-1 (lane 2).17

CD9

Resting platelets. Immunogold staining for CD9 gave the following labeling pattern. Consistent labeling of the plasma membrane and of the luminal face of the OCS was observed as well as a definite immunostaining of the periphery of the α-granules (Fig 2a and b). α-Granules were identified due to their high number, large size, and dark nucleoids. Either because the reaction was close to the threshold of
sensitivity of this immunocytochemistry or because a heterogeneous population of α-granules exists as far as CD9 content is concerned, only 77% of the granules contained one or more gold particles (versus 19% of the mitochondria, which contained rarely more than 1 gold bead, representing the background staining as control structures). However, overall, ~28% of the total gold particles were found associated with α-granules.

**Thrombasthenic platelets.** The same qualitative and quantitative labeling pattern as for normal platelets was found in thrombasthenic platelets (Fig 2c). This result excludes a cross-reaction between the polyclonal anti-CD9 antibody and GPIIb-IIIa and confirms the monospecificity of the antibody as determined by the Western blot analysis. In addition, the data indicate that there is no difference in the subcellular localization of CD9 in Glanzmann’s thrombasthenia.

**ADP-stimulated platelets.** After treatment with ADP, which induced platelet shape change without secretion, immunolabeling for CD9 was found on the same structures as in resting platelets, eg, plasma membrane (including pseudopods), OCS, and the centralized α-granule limiting membrane (Fig 2d).

**Thrombin-stimulated platelets.** An increase in plasma membrane CD9-associated immunolabeling was observed after thrombin stimulation, coinciding with the fusion of the α-granules with the cell surface of the activated platelets. Indeed, the labeling associated to the surface increased of 40% compared with the initial level. No other cell organelle displayed any significant number of gold particles.

**PECAM 1**

**Resting platelets.** Normal platelets were examined after immunogold labeling of PECAM-1. The immunolabeling was overall weaker than for CD9. Gold particles could be observed outlining the outer face of the plasma membrane and the luminal side of the OCS. Moreover, labeling of the limiting membrane of the α-granules was consistently found (Fig 3a and b). Like CD9, an average of 27% of the total number of particles were found labeling the granules, whereas the remaining particles were associated with the plasma and OCS membranes. Forty-three percent of the α-granules were labeled with one or more gold particles against 14% of the mitochondria taken as control structures. Smaller granules of other types and the cytoplasmic matrix were not labeled.

**Thrombasthenic platelets.** No differences were detected in PECAM-1 immunolabeling between platelets from the...
normal subjects and from the patients with type I thrombasthenia (Fig 3c).

**ADP-stimulated platelets.** With ADP, platelets undergo shape change with centralization of α-granules. The peripheral actin-rich zone is deprived of gold labeling, which is concentrated in the central zone where the α-granules are located. Both the OCS and plasma membrane pseudopodia remain labeled (Fig 3d).

**Thrombin-stimulated platelets.** After thrombin stimulation, the α-granule membranes aggregate and fuse with the OCS membrane, where they discharge their content, creating secretory areas. On these platelets, immunolabeling for PECAM-1 was found on the plasma membrane, particularly on pseudopods; on the OCS membrane; and also in secretion areas membrane. On thrombin-stimulated platelets, which had undergone a virtually full degranulation, an average increase of 44% of the initial labeling was observed on the plasma membrane (Fig 3e).

**Control**

The primary antibody (anti-PECAM1 or anti-CD9) was replaced by an irrelevant polyclonal antibody, antihuman myeloperoxidase, and this led to a complete negativity of the staining. A similar negative result was obtained when the primary antibodies were omitted from the reaction.

**Double Immunolabeling**

To further confirm that both CD9 and PECAM-1 were associated with the α-granules, double-immunolabeling experiments were performed using antibodies against the known α-granule markers, vWF and fibrinogen. When platelets were double-immunolabeled for the α-granule protein, vWF, together with CD9, vWF was found eccentrically located in the α-granules, as previously described, and the same granules displayed labeling for CD9 on their periphery (Fig 4a). A similar result was obtained with the double-immunolabeling for fibrinogen and CD9, with fibrinogen more randomly distributed on the α-granule matrix than vWF (Fig 4b). The mitochondria were negative for the three markers. In a similar approach, labeling for PECAM-1 was present at the internal face of the same granules in which vWF and fibrinogen were detected, allowing for the identification of these structures as α-granules (Fig 5).

**DISCUSSION**

The α-granule of the platelet packages and stores a variety of proteins that are released on activation. Many of these proteins are endogenously synthesized and packaged within the megakaryocyte, eg, thrombospordin, β-thromboglobulin, and vWF. For these proteins, the corresponding mRNAs are present in megakaryocytes and the proteins can be immunodetected in transit during traffic in the Golgi sacculi. Other proteins in the α-granule, such as fibrinogen, albumin, and IgG, are stored via a different mechanism involving endocytosis from the surrounding extracellular medium. We have previously demonstrated that GPIIb-IIIa is not only found on the external face of the plasma membrane and the luminal face of the OCS, but also on the inner face of the α-granule membrane, and it is probable that the incorporation of fibrinogen into the α-granule is a receptor-mediated process dependent on fibrinogen binding to GPIIb-IIIa. Fibrinogen is present in platelet α-granules at higher concentration than other plasma-derived proteins such as albumin and IgG, and its presence in the granule is dependent on functional GPIIIa. Thus, fibrinogen is absent or markedly reduced in α-granule content in patients with Glanzmann’s thrombasthenia whose platelets lack or have dysfunctional GPIIb-IIIa. Alternatively, blockage of the capacity of GPIIb-IIIa to bind fibrinogen in vivo inhibits uptake of fibrinogen into the α-granule. Moreover, barbourin, a specific antagonist of GPIIb-IIIa, also inhibits the endocytic uptake of fibrinogen into the α-granules.

The GPIIb-IIIa–mediated endocytosis of fibrinogen into the α-granule appears to predominate in plasma over other proteins such as vWF that can also bind to GPIIb-IIIa through an RGD-dependent mechanism. The endocytosis of vWF has been ruled out. In an animal model in which a normal pig was engrafted with the marrow of a pig with severe von Willebrand’s disease, resulting in vWF-deficient platelets circulating in vWF-rich plasma, no α-granule vWF was detected. In this regard, the mechanism for α-granule packaging of megakaryocytic-derived proteins appears to be distinct to that for endocytically derived proteins. Thus, in the α-storage disease, Gray platelet syndrome, proteins such as vWF and β-thromboglobulin are essentially absent from the platelet, whereas the levels of albumin, IgG, and fibrinogen are only moderately reduced. Furthermore, GPIIb-IIIa can still be localized to the P-selectin–containing immature granules in Gray platelets, consistent with a relatively normal endocytic mechanism for plasma protein incorporation into the α-granule.

Recently, we reported that, in addition to GPIIb-IIIa, another major platelet surface GP, CD36 (GPIV), was also located in significant quantity on the inner face of the α-
Fig 4. Normal platelets double-immunolabeled for CD9 (5 nm gold) and either vWF or fibrinogen as α-granule markers (15 nm gold). (a) α-Granules contain vWF distributed along one of their poles. CD9 is detected along the periphery of the same granules (arrowheads) that display a large nucleoid (n), as well as on the plasma membrane (pm). (Original magnification × 101,000.) (b) α-Granules display labeling for both CD9 lining their membrane (arrowheads) and fibrinogen in their matrix. CD9 is also found along the OCS. Mitochondria (m) as control structures are negative for both antigens. (Original magnification × 190,000.)

In the present study, we have extended this observation to two additional major surface GPs, PECAM-1 and CD9. This finding for PECAM-1, although novel, is nevertheless consistent with the data of Metzelaar et al.19 who showed an increased expression of PECAM-1 on platelets after thrombin activation. The investigators suggest the existence of an intracellular pool of PECAM-1, without identifying its α-granular origin. Although there are technical limitations to quantitation in immunogold localization studies and potential differences in antibody accessibility with different antigens, the percentage of α-granule localization relative to total distribution is nevertheless in the range of 20% to 35% for all four surface GPs, GPIIb-IIIa, CD36, PECAM-1, and CD9 (this study, Berger et al8 and calculated from the data of Cramer et al7). Targeting of the α-granule-specific GP, P-selectin, to the α-granule membrane requires a specific signal localized within the cytoplasmic domain.39,40 P-selectin is only expressed on the plasma membrane of quiescent cells by default if the cell lacks storage organelles. In contrast, GPIIb-IIIa appears to be incorporated into the α-granule membrane as a consequence of the endocytosis of vesicles containing plasmatic fibrinogen (see above). We would suggest that the presence of three additional surface GPs in α-granules in similar relative amounts to GPIIb-IIIa argues that, when endocytosis occurs simultaneously to internalization of plasma proteins, plasma membrane may be internalized and many of its components become common to the storage granule membrane. This
Fig 5. Normal platelet double immunolabeled for PECAM-1 (5 nm gold) and either vWF or fibrinogen as α-granule markers (15 nm gold). (a) The antifibrinogen labels the matrix of the α-granules except for the dark nucleoid. Anti-PECAM-1 is present along the α-granule membrane (arrowheads). (Original magnification × 138,600.) (b) vWF is located in the electron-lucent zone of the α-granules next to the dark nucleoid (n), whereas PECAM-1 is detected at the inner face of the membrane of the same granules (arrowheads). Mitochondria (m) are not labeled. (Original magnification × 109,000.)

conclusion is supported by the finding that another major platelet membrane GP, GPIb-IX, has not been detected in α-granules. Regions of the plasma membrane containing GPIb-IX would be expected to be structurally ordered due to the association of GPIb-IX with the membrane network of actin-filaments associated with the maintenance of platelet shape and therefore less likely to undergo a process of pinocytosis. However, further evidence is clearly required to fully explain the presence and potential biologic relevance of surface membrane GP distribution between the plasma and α-granule membranes.

REFERENCES


Platelet alpha-granule and plasma membrane share two new components: CD9 and PECAM-1

EM Cramer, G Berger and MC Berndt