Ultrastructural Demonstration of CD36 in the α-Granule Membrane of Human Platelets and Megakaryocytes

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

CD36 (glycoprotein [GP] IV) is a membrane GP of 88 kD found on monocytes, endothelial cells, and platelets. It may serve as a receptor for collagen and is also able to bind thrombospondin (TSP), because a monoclonal antibody to CD36 inhibits TSP binding to thrombin-stimulated platelets. In the following study, we investigated the subcellular distribution of CD36 within normal resting platelets, thrombin-stimulated platelets, and in cultured megakaryocytes (MK) by an immunogold staining technique and electron microscopy. We used an affinity-purified monospecific polyclonal antibody showing a single major band of immunoreactivity on Western blot analysis. CD36, homogeneously distributed along the platelet plasma membrane and in the luminal side of the open canalicular system (OCS). Moreover, some labeling was found around the α-granules along the inner face of their limiting membrane. An average of 70% of granules were labeled. The granule-associated pool of CD36 was estimated at approximately 25% of the total cell content. To exclude the possibility of a cross-reaction with GPllb-IIIa, platelets from a patient with type I Glanzmann’s thrombasthenia (which completely lack GPllb-IIIa) were studied and showed a similar subcellular distribution of CD36, including α-granule membrane labeling. In activated platelets, CD36 was shown to be redistributed to the OCS and pseudopods of the plasma membrane. Platelets from a patient with the Gray platelet syndrome expressed CD36 on their plasma membrane, and some immunolabeling was also found within small abnormal α-granules. In cultured MK, CD36 immunolabeling was detected in the Golgi saccules, associated vesicles, immature α-granules, and demarcation membranes. In conclusion, this study shows the existence of a significant intragranular pool of CD36 in platelets that may play a critical role in the surface expression of α-granule TSP during platelet activation.

© 1993 by The American Society of Hematology.

From the INSERM U.348 and Institut des Vaisseaux et du Sang, Hôpital Lariboisière, Paris, France; and the Baker Medical Research Institute, Prahran, Victoria, Australia.

Submitted March 9, 1993; accepted July 19, 1993.

Supported in part by the Association pour la Recherche sur le Cancer.

Address reprint requests to Elisabeth Martin Cramer, MD, Laboratoire d’Hématologie, Hôpital Lariboisière, 2 rue Ambroise Paré, 75010 Paris, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

Blood, Vol 82, No 10 (November 15), 1993: pp 3034-3044
Fig 1. (a) and (b) Immunolabeling for CD36 on thin section of a resting platelet by a post embedding immunogold staining technique is shown. The plasma membrane (pm) is labeled as well as the luminal face of the open canalicular system (ocs). Moreover, some gold particles are found at the periphery of α-granules (A) (original magnification × 60,000). Inset: The same results were obtained with a polyclonal antibody obtained from a different source. (c) At a higher magnification, α-granule immunostaining for CD36 is observed circumferentially at the inner face of their limiting membrane (original magnification × 140,000). (d) In contrast, thrombospondin immunolabeling is scattered within the matrix of the α-granule (A) (original magnification × 60,000).
Fig 2. Platelet cryosectioned, double-labeled for CD36 and Fg followed by immunogold coupled to 10- and 15-nm particles, respectively, is shown. CD36 (arrowheads) is found on the plasma membrane (pm) and around the α-granules (A) which also display labeling for Fg (arrows) (original magnification × 60,000). Inset: Fg is found scattered in the matrix of the α-granule (A), whereas CD36 lines the α-granule membrane (arrowheads) as well as the plasma membrane (pm) (original magnification × 110,000).
Fig 3. Immunoblotting of the polyclonal anti-CD36 antibody on a platelet lysate after electrophoresis shows a single band at a molecular weight of about 88-kD, between the 68- and 94-kD markers, confirming the monospecificity of the antibody.

Blood samples were harvested by venipuncture into plastic tubes containing acid-citrate-dextrose (ACD) buffer (6.8 mmol/L citric acid, 1.2 mmol/L trisodium citrate, 24 mmol/L glucose). The platelet-rich plasma (PRP) was obtained by centrifugation at 1,100g for 10 minutes at room temperature and were washed 3 times with Tyrode's 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 albumin (Sigma Chemical Co, St Louis, IL) and 7 ng/mL prostaglandin E₁ (Sigma). The washed platelets were resuspended in Tyrode’s buffer containing 2.9 mmol/L CaCl₂. Washed platelet samples were alternatively exposed to 0.05 U/mL human a-thrombin (Sigma) at 22°C for 6 minutes and to 20 μmol/L adenosine diphosphate (ADP) (Sigma) at 37°C for 6 minutes, without stirring. The reaction was stopped by fixation with 1% glutaraldehyde (Ladd Research Industries, Burlington, UK) in Tyrode’s buffer.

MK. MK were grown in liquid culture from bone marrow precursors obtained from normal adult graft donors. As previously described, light density cells were separated by density centrifugation on ficoll-metrizoate (d = 1.077). Adherent cells were eliminated by adherence to plastic. The remaining cells were cultured in suspension in Iscove’s modified Dulbecco’s medium supplemented with 10% aplastic plasma from patients who were markedly thrombocytopenic at the time of blood collection. Cell culture was performed for 11 days in culture flasks at 37°C in a 5% CO₂ fully humidified atmosphere.

Antibodies

Purification of CD36. Human platelet membrane CD36 was purified to homogeneity by immunoaffinity chromatography using the anti-CD36 MoAb, VM 58. Briefly, human platelet membranes, prepared as previously described, from 50 U of platelet concentrate, were suspended in 150 mL of 0.02 mol/L Tris buffer, 0.15 mol/L sodium chloride, 0.001 mol/L calcium chloride, 1%
Fig 5. Platelet from a patient with the GPS reacted for CD36 shows some very small granules (A1), and numerous intracellular vacuoles the size of normal α-granules (A2), both of which are lined by gold particles. Although the plasma membrane (pm) of this platelet is poorly labeled, surrounding platelets display membrane labeling equivalent to the control (arrowheads) (original magnification × 55,000).

(Wt/vol) Triton X-100, pH 7.4. After 30 minutes at 4°C, the suspension was made 100 μg/mL in leupeptin, 0.2 mmol/L in phenylmethylsulfonyl fluoride (PMSF), and 10 U/mL in aprotinin, and then ultracentrifuged at 100,000g for 60 minutes at 4°C. The supernatant was immediately loaded at 40 mL/h onto a 1.5 × 25-cm column of concanavalin A-Sepharose 4B equilibrated with 0.02 mol/L Tris buffer, 0.15 mol/L sodium chloride, 0.001 mol/L calcium chloride, 0.1% (wt/vol) sodium azide, 0.2 mmol/L PMSF, 10 U/mL aprotinin, pH 7.4 (Buffer A). After thorough washing, bound membrane GP were eluted with 0.2 mol/L methyl-α-mannopyranoside in Buffer A.

After dialysis against Buffer A, the GP fraction was loaded at 20 mL/h onto a 1 × 10-cm column of VM 58 coupled to Affigel-10 (2 mg of VM 58 IgG per milliliter of agarose). After thorough washing with Buffer A and with Buffer A made with 1 mol/L in sodium chloride, platelet CD36 was eluted with 0.1 mol/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 mol/L Tris, pH 8.0 and were then dialysed against Buffer A for storage at −70°C. The purified CD36 was homogeneous as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions.

Affinity-purified rabbit anti-CD36 antibody. Rabbit polyclonal anti-CD36 antisera prepared by injecting New Zealand white rabbits with purified CD36 were affinity-purified on a column of CD36 coupled to a 1:1 mixture of Affigel 10 and 15. After thorough washing, bound antibody was eluted with 0.1 mol/L glycine, pH 2.4, and immediately neutralized by the addition of one-fifth volume of 1.0 mol/L Tris, pH 8.0. The affinity-purified antibody was dialysed against 0.01 mol/L Tris, 0.15 mol/L sodium chloride, pH 7.4 and stored at −70°C before use. The polyclonal antibody antihuman CD36 was used at 30 μg/mL for immunoelectron microscopy.

Another antihuman CD36, a polyclonal rabbit antibody (IgG purified), used at 10 μg/mL was generously given by Dr. G.A. Jameson (Rockville, MD). Anti-human TSP, a polyclonal rabbit antibody, used at 30 μg/mL was generously given by Dr. A. Nurden (Pessac, France). Anti-human fibrinogen (Fg) (IgG fraction) was purchased from Cappel Laboratories Inc (Downington, PA) and used at a 10−2 dilution. Sheep antirabbit antibody (IgG) conjugated with peroxidase was purchased from Sigma and used at a 10−1 dilution. Goat antirabbit antibody (IgG) coupled to 10-nm colloidal gold particles (GAR-G10) was purchased from Amersham (Les Ullys, France) and used at a 2 × 10−2 dilution.

Electron Microscopic Study

Cells were fixed in 1% glutaraldehyde in 0.1 mol/L phosphate buffer pH 7.2 for 2 hours at 22°C, washed 3 times in the same buffer, and embedded in glycol methacrylate (GMA) as previously described.23 The immunochemical reactions were then performed on thin sections according to the method of De Mey.24 Briefly, the sections were labeled by a first incubation with a polyclonal rabbit antibody for 2 hours at room temperature in a humidified atmosphere; next, a second incubation with GAR-G10 was performed for 1 hour at 22°C, and the sections were counterstained with uranyl acetate and lead citrate. Samples were observed on a Philips EM 300 and Philips 450 CM 10.

Double immunolabeling. To confirm that CD36 was localized in the α-granules, we performed a double-immunogold labeling on
Fig 6. (a) In ADP-stimulated platelets, immunolabeling for CD36 is observed on the centralized α-granule membranes (A). Labeling is also present on the plasma membrane (pm) (original magnification × 48,000). (b) In thrombin-stimulated platelets, gold particles line the plasma membrane and particularly the pseudopods’ (p). They are also present in degranulation areas (D) formed by the fusion of α-granules with the ocs (original magnification × 50,000) (c) When thrombin-treated platelets have fully secreted, virtually all labeling is redistributed to the plasma membrane and namely on pseudopods (arrowheads) (original magnification × 28,000).
Fig 7. (a) Human cultured MK (original magnification × 7,000) immunolabeled for CD36. Specific labeling is observed: (b) on the Golgi sacculles (G), arising vesicles (v), and forming α-granules (A) (original magnification × 48,000) (c) at the luminal side of the demarcation membranes (DM) and at the inner face of the α-granule (A) limiting membranes (N, nucleus) (original magnification × 55,000).
frozen tissue sections for Fg and CD36 according to Slot et al.28 Platelets were fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany), cryo-sectioned, and CD36 labeling was shown by protein A coupled to 10-nm gold particles. Subsequently Fg was shown by protein A coupled to 15-nm gold particles. Before incubation in the anti-Fg antibody, a 1% glutaraldehyde treatment was used to prevent any interference between the different antibodies-gold particle complexes on the section. 

Quantitative study. Membrane labeling intensity was evaluated by counting the gold particles per micrometer of membrane. Alternatively, the intracellular pool was quantified by counting the gold beads associated to the open canalicular system (OCS), α-granule membranes and on the plasma membrane; the ratio of intracellular versus plasma membrane pool per equatorial section of 10 platelets was calculated. In activated platelets, plasma membrane immunolabeling was counted per equatorial section of platelet and compared with control platelets. Again, 10 platelets were analyzed for each experimental conditions.

Control
When the first antibody (anti-CD36) was omitted and replaced by nonimmune rabbit serum, no gold labeling of any cell structures was obtained.

Biochemical Study
The specificity of the antihuman CD36 rabbit polyclonal antibody was assayed by Western blotting of platelet lysates. Briefly, washed platelets were solubilized by addition of 2% SDS and 1 mmol/L EDTA and separated on SDS-PAGE under reducing condition with 5% 2-mercaptoethanol, using a 7% resolving gel and 3% stacking gel. Gels were electroblotted onto a nitrocellulose filter and probed with antihuman CD36 for blocking with 5% low-fat powder milk and membranes were probed with the anti-CD36 polyclonal antibody and display immunolabeling on OCS plasma membrane and intracytoplasmic vacuoles. Moreover, the cytoplasmic, abnormal, small α-granules are also labeled (Fig 5). Compared with normal platelets, the ratio of intracellular versus extracellular labeling is unchanged.

RESULTS

CD36 Localization in Resting Platelets
In resting platelet sections, labeling for CD36 antigen was detected on the plasma membrane and at the luminal face of the OCS. Of particular interest, some gold labeling was also found bound on α-granule membranes (Figs 1a and b). Furthermore, this new localization was also detected with an alternative anti-CD36 polyclonal antibody (from Dr Jamieson; Fig 1b, inset). At a higher magnification, the α-granule immunolabeling was observed at the inner face (Fig 1c), contrasting with the scattered distribution of α-granules in the matrix of α-granules (Fig 1d), suggesting that the antibodies were specific for membrane CD36. Under these conditions, an average of 70% of the α-granules were immunolabeled with 2 or more gold particles, against only 5% of mitochondria serving as control structures and representing the background staining. The CD36 immunolabeling associated with the α-granule membrane represents an average of 30% ± 8% of the total platelet labeling. Double immunolabeling for Fg and CD36 confirmed that the labeled granules were indeed α-granules, Fg being detected scattered in the granule matrix whereas CD36 was found along its membrane (Fig 2).

Specificity of the Polyclonal Anti-CD36
Figure 3 establishes the monospecificity of the antihuman rabbit polyclonal antibody to CD36 used for the present immunoelectron microscopic study as evaluated by Western blot analysis. A major band can be seen at approximately 88 kD corresponding to the normal SDS-PAGE migration position of CD36 between the 68- and 94-kD molecular weight markers.

CD36 Localization in Pathologic Platelets
Glanzmann’s thrombasthenia. The localization for CD36 in normal platelets in the present study is reminiscent of the localization of the major platelet GP, GPIIb-IIIa (50,000 molecules/platelet).22 The study of the platelets of a patient with Glanzmann’s thrombasthenia type I with total GPIIb-IIIa deficiency21 permitted the exclusion of a possible cross-reaction between the polyclonal antibody to CD36 and platelet GPIIb-IIIa. The distribution of CD36 in this patient had the same localization as observed in normal platelets (Fig 4).

GPS. This syndrome is characterized by thrombocytopenia and large platelets lacking normal α-granules that are replaced by small immature α-granules and empty α-granules.30 It has been reported that in platelets from the GPS labeled with an anti-P-selectin (GMP-140), the α-granule membrane is normally present even when the granules are empty.31 Here we show that these Gray platelets also react with the anti-CD36 polyclonal antibody and display immunolabeling on OCS plasma membrane and intracytoplasmic vacuoles. Moreover, the cytoplasmic, abnormal, small α-granules are also labeled (Fig 5). Compared with normal platelets, the ratio of intracellular versus extracellular labeling is unchanged.

CD36 Localization on Activated Platelets
ADP-stimulated platelets. In response to this agonist, platelets undergo a shape change with a 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. OCS and plasma membrane are also labeled (Fig 6a).

Thrombin-stimulated platelets. After thrombin stimulation, specific granie membranes combine and fuse with the OCS membrane where they discharge their contents, creating degranulation areas. In these platelets, immunolabeling for CD36 lines the plasma membrane, particularly pseudopods, and the OCS membrane. It is also present in the degranulation area limiting membrane (Fig 6b). On fully degranulated platelets, an increase in CD36 surface expression was found, corresponding to the exportation of the intracellular pool (OCS + α-granules) and accounting for a twofold increase (110%) of the initial labeling. The mean gold-particle number per unit of membrane on pseudopods (28 ± 7.9) was significantly higher than on the rest of the plasma membrane (8.7 ± 0.2) excluding pseudopods.

CD36 Localization in Human Cultured MK
In maturing culture MK, immunolabeling for CD36 is found in the Golgi sacules and some associated vesicles, at
their function is still unclear. CD36, an 88-kD plasma membrane and has been implicated in platelet-leukocyte interaction. In the secretion process, the storage granule membrane combines with the plasma membrane while their content (e.g., Fg, von Willebrand factor, and TSP) is secreted or redistributed via their receptors (e.g., GPIIb-IIIa, GPIb, and CD36).

Some receptors have been described on the α-granule membrane; GPIIb-IIIa, evenly present on plasma and OCS membrane, is the Fg receptor. P-selectin, a specific α-granule membrane protein, is transferred onto the plasma membrane and has been implicated in platelet-leukocyte interaction. Other proteins such as GMP-140 and osteonectin have been localized in the α-granule membrane, but as yet their function is still unclear. CD36, an 88-kD plasma membrane protein acts as a TSP receptor in activated platelets and is involved in platelet-collagen adhesion. In this study, we have examined the subcellular localization of CD36 by immunoelectron microscopy and have identified it on this plasma membrane. Furthermore, we have shown the presence of an intracellular pool of CD36 associated with the OCS membrane and with the inner face of the α-granule membrane. This α-granule pool of CD36 contains an average of 25% of the total platelet content. A previous preliminary electron microscopic report using an anti-CD36 MoAb (OKM5) failed to observe this α-granule localization. Using our postembedding immunogold technique, we were not able to obtain any labeling with OKM5; this was probably because of the alteration of the recognized epitope by the embedding medium, GMA. However, in the current study it is possible that the use of a polyclonal antibody allowed the antigenic recognition of some sites which were inaccessible to the monoclonal OKM5. Furthermore, the present results were directly confirmed with another anti-CD36 polyclonal antibody. The same phenomenon has been described for the localization of GPIIb-IIIa, where the α-granule pool was only shown by means of a polyclonal antibody. The monospecificity of the polyclonal antibody to CD36 used in this study was confirmed by several additional criteria. First, immunoblotting of platelet lysate after electrophoresis showed a single band with a molecular weight of approximately 88 kD, similar to that of CD36. Second, GPIIb-IIIa is a major platelet protein found both at the platelet surface and on the α-granule membrane. This localization is similar to that which we described for CD36. To exclude any possibility of cross-reaction between the anti-CD36 polyclonal antibody and GPIIb-IIIa, we investigated the platelets from a patient with Glanzmann’s thrombasthenia type I, whose platelets were totally devoid of GPIIb-IIIa, and found the same result suggesting that the α-granule localization was indeed caused by CD36. The similarity of CD36 with the lysosome-integral membrane protein LIMP II raises the question whether the labeled granule pool of CD36 was indeed α-granules. Nevertheless, the role of this granule-associated CD36 remains to be elucidated; it may mediate exocytosis and membrane expression of TSP during platelet secretion in a manner similar to that of GPIIb-IIIa and Fg and may contribute to the consolidation of the platelet aggregate.

**DISCUSSION**

The platelet plasma membrane mediates platelet activation by binding agonists such as ADP, thrombin, and catecholamine. It contains GP receptors favoring cell-to-cell and cell-to-subendothelial matrix adhesion; both are major events in hemostasis. After adhesion and shape change, platelets secrete their α-granule content and irreversibly aggregate. In the secretion process, the storage granule membrane and cell-to-subendothelial matrix adhesion; both are major events in hemostasis. After adhesion and shape change, platelets secrete their α-granule content and irreversibly aggregate. In the secretion process, the storage granule membrane combines with the plasma membrane while their content (e.g., Fg, von Willebrand factor, and TSP) is secreted or redistributed via their receptors (e.g., GPIIb-IIIa, GPIb, and CD36).

We have also explored the intracellular localization of CD36 in a patient with the GPS, a disease characterized by the presence of empty α-granules with normal α-granule membranes. The intracellular distribution of P-selectin in Gray platelets shows the presence of small granules, possibly corresponding to immature α-granules. Furthermore, the Gray platelets also contain many intracytoplasmic vacuoles, possibly corresponding to α-granule–like structures. The presence of CD36 within the abnormal α-granule–related structures suggests that the CD36 intracellular pool is present in this pathology and that the targeting disorder does not involve this α-granule membrane component, nor P-selectin nor GPIIb-IIIa. Thus, the reduced TSP level found in this disorder does not seem to be related to a CD36 deficiency and remains to be explained in relationship with the general soluble α-granule protein defect.

During platelet activation, Kieffer et al have shown the redistribution of CD36 in the plasma membrane characterized by a predominant labeling on the pseudopods of spread platelets. In the present study, we clearly observe CD36 immunolabeling on centralized α-granules after ADP stimulation. A previous biochemical report was inconsistent with the existence of an intracellular pool of CD36, because no increase in plasma membrane expression was observed after thrombin stimulation. However, the study was performed with an MoAb that might not recognize CD36 after activation. Within thrombin-stimulated platelets, immunolabeling is observed around the degranulation areas and on the plasma membrane where the degree of labeling increases by approximately 30%. The observed increase may possibly correspond to the unfolding of the OCS and externalization of the α-granule pool of CD36. Nevertheless, the role of this granule-associated CD36 remains to be elucidated; it may mediate exocytosis and membrane expression of TSP during platelet secretion in a manner similar to that of GPIIb-IIIa and Fg and may contribute to the consolidation of the platelet aggregate.

Cultured human MK also contain CD36 in the Golgi saccules, the demarcation membrane system, and on the α-granule membranes, suggesting that CD36 synthesis occurs at the MK level. However, the presence of CD36 within α-granule membranes suggests that it may possess a unique targeting signal either directly or via endocytosis to the secretion granules. Thus, it would be of interest to investigate whether a specific signal exists that can direct the protein to...
the compartment of regulated secretion as previously shown for another α-granule component, P-selectin.\textsuperscript{43,44}

In conclusion, this study shows the presence of a significant intragranular pool of CD36 in platelets, which may play a critical role in the surface expression of TSP during platelet activation, thus contributing to the consolidation of the aggregate.

ACKNOWLEDGMENT

The authors acknowledge Dr G.A. Jamieson for providing an anti CD36 polyclonal antibody, Drs Chantal Legrand and Jean-Philippe Rosa for useful comments, Dr Paul Harrison for editorial help, Mrs Danièle Tenza for expert technical assistance, Elisabeth Savariau for photographic work, and Muriel Kindelberger for secretarial help.

REFERENCES

33. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti


Ultrastructural demonstration of CD36 in the alpha-granule membrane of human platelets and megakaryocytes

G Berger, JP Caen, MC Berndt and EM Cramer