Receptor Patching and Capping of Platelet Membranes
Induced by Monoclonal Antibodies

By S. Santos, U. Zimmermann, J. Neppert, and C. Mueller-Eckhardt

Redistribution of glycoproteins (GP) Ib, glycopcalicin, IIb, and IIIa on the surface of human platelets in response to stimulation with corresponding monoclonal antibodies (MoAb) and a polyclonal anticylgocalicin antibody was studied by immunofluorescence, immunoelectron microscopy, and a quantitative radioimmune assay. Immobilization of the antigens by prefixation with formaldehyde showed a uniform distribution over the surface of the platelet. Incubation of unfixed platelets with specific MoAb against various epitopes on GPIIb and/or IIIa resulted in a time-dependent patching, subsequent capping, and after prolonged exposure to the antibody/label complex, internalization of the complex, possibly by endocytosis. In contrast, GPIb could not be shown to cap. From these results we conclude that platelet GPIIb and/or IIIa undergo spatial rearrangement in a manner analogous to that observed in lymphocytes, whereas GPIb does not. Since both GPIb and GPIIb and/or IIIa seem to be transmembraneous GP associated with the cytoskeleton, a special, though unidentified, role of GPIIb/IIIa in the induction of lateral membrane mobility is postulated.

Antibodies. Designation, origin, and specificity of MoAb against GP of human platelets are given in Table 1. MoAb were purified from ascites by protein A-agarose 4B chromatography before use. MoAb were used at a concentration of 1 μg/mL and were free from aggregates as demonstrated by enzyme-linked immunosorbent assay and cross-immunoelectrophoresis (data not shown). Rabbit anticylgocalicin was kindly supplied by Dr. Clemetson, Bern, Switzerland. Goat antiserum (GAM) IgG (ab′2), fragments conjugated with fluorescein isothiocyanate (FITC) or tetrarhodamine isothiocyanate (RTIC), both from Cappel Laboratories, West Chester, PA, and FITC goat antirabbit IgG (Behringwerke, Marburg, FRG) served as secondary antibodies. These conjugates were used at a dilution of 1:50. An affinity-purified rabbit antimonise (RAM) IgG antibody and protein A-gold particles (diameter, 6 nm) were generously supplied by Dr. G. Griffiths, European Molecular Biology Laboratory, Heidelberg, FRG. Affinity-purified GAM IgG or rabbit antigoat (RAG) IgG (ab′2) fragments, both from Dianova, Hamburg, FRG, served as secondary or tertiary antibodies, respectively, and were labeled with Na 125I using the iodine monochloride method. Generally, 1 mg protein was labeled with 1 mCi Na 125I. The ratio of protein-bound 125I to free 125I was 98%.

Immunofluorescence microscopy. For labeling of GPIb or GPIIb/IIIa, 500 μL unfixed washed platelets (3 x 10^5/μL) were incubated with 50 μL MoAb or rabbit anticylgocalicin IgG, respectively, for ten or 30 minutes at 37 °C and then fixed with 1% formaldehyde (freshly prepared from paraformaldehyde) in TB. To demonstrate the distribution of the antigens on resting platelets, samples were first fixed, rinsed with TB, and then incubated with MoAb or rabbit anticylgocalicin for 30 minutes at 37 °C. Thereafter, all samples were rinsed and incubated with 30 μL RTIC-GAM for 30 minutes at 20 °C and finally washed twice with TB. Receptor internalization was studied by incubating unfixed platelets with MoAb for 30 minutes. Samples were then rinsed, incubated with RTIC-GAM for ten minutes at 20 °C, fixed, and finally washed.

MATERIALS AND METHODS

Platelets. Platelet-rich plasma (PRP) from the citrated blood of healthy donors was used. Platelets were washed using a Tyrod buffer system (TB, Huerzkow, Bremen, FRG) containing Ca2+ and prostaglandin (PGE1 [Serva, Heidelberg, FRG]) as described previously.

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343
selective filters, and a 40 x Plan-Neofluar and 63 x Plan-Apochromat objectives. Micrographs were made on Kodak Ektachrome 400 (commercially processed for 33 DIN [Deutsche Industrie Norm]) at final magnifications of 400 to 600 x.

**IEM.** Fixed or unfixed platelet samples that had been incubated with antibodies, as previously mentioned, were labeled with protein A–colloidal gold for 20 minutes at 20 °C. Prior to gold labeling, MoAb-treated samples were incubated with RAM for 30 minutes at 37 °C and rinsed in TB. This step was used to facilitate the binding of protein A. Samples were rinsed again, fixed with 2% OsO4 for 30 minutes at 4 °C, and dehydrated rapidly in acetone. Contrast was enhanced by treatment with 2% uranyl acetate for two minutes in a 4 °C bath, and contrast was further enhanced by treatment with 2% uranyl acetate and 2% lead citrate to enhance contrast and were observed and photographed with a Zeiss EM 10 CR electron microscope.

**Radioimmunoassay.** To demonstrate endocytosis quantitatively, 5 x 10^7 unfixed washed platelets were incubated with 50 μL MoAb for ten minutes at 20 °C. After washing with TB, the samples were incubated with 50 μL of either radiolabeled (20,000 cpm; series A) or unlabeled GAM (dilution, 1:1,000; series B) for 5, 10, 30, 60, and 90 minutes at 20 °C, were then fixed, and washed three times with TB. Series A samples were transferred to counting tubes, and the bound radioactivity was determined in a gamma counter (Abbot Laboratories, Eschborn, FRG). These values represented the total amount of GPIb or IIb/IIIa, respectively, detectable on the external surface of the platelets as well as after internalization. To discriminate between these two compartments, series B samples were tagged with 50 μL of 125I-2G12 (20,000 cpm) for ten minutes at 20 °C and were further assessed as previously described. These values equaled the amount of GP that had remained at the external surface prior to fixation. The difference of radioactivity between corresponding samples of series A and B was taken as a measure of internalization of the respective GP. All tests were run in duplicate.

### Table 1. Antibodies Used for Labeling of GPIb or GPIb/IIa on Human Platelets

<table>
<thead>
<tr>
<th>Designation of Antibody</th>
<th>Immunoglobulin</th>
<th>Specific for Various Epitopes on</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoAb Gi5</td>
<td>mouse IgG2a</td>
<td>GPIb/IIa</td>
<td>Santoso et al.</td>
</tr>
<tr>
<td>MoAb 2G12</td>
<td>mouse IgG2a</td>
<td>GPIb/IIa</td>
<td>Woods et al.</td>
</tr>
<tr>
<td>MoAb 3F5</td>
<td>mouse IgG1</td>
<td>GPIb/IIa</td>
<td>Woods et al.</td>
</tr>
<tr>
<td>MoAb C15</td>
<td>mouse IgG1</td>
<td>GPIIa</td>
<td>Tetteroo et al.</td>
</tr>
<tr>
<td>MoAb C17</td>
<td>mouse IgG1</td>
<td>GPIIIa</td>
<td>Tetteroo et al.</td>
</tr>
<tr>
<td>MoAb FMC 25</td>
<td>mouse IgG1</td>
<td>GPIb, IX</td>
<td>Zoli et al.</td>
</tr>
<tr>
<td>MoAb AN51</td>
<td>mouse IgG</td>
<td>GPIb</td>
<td>McMichael et al.</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>rabbit IgG</td>
<td>glycoprotein IV</td>
<td>Ali-Briggs et al.</td>
</tr>
<tr>
<td></td>
<td>rabbit IgG</td>
<td>glycocalicin</td>
<td>Ali-Briggs et al.</td>
</tr>
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twice with TB. Normal mouse IgG (Cappel Laboratories) served as a negative control.

For differential staining, GPIb and GPIb/IIa were investigated by double immunofluorescent labeling: platelets treated with MoAb against GPIb/IIa, according to the preceding procedure, were incubated with 50 μL MoAb against GPIb for 10 minutes at 20 °C and washed twice in TB. Finally, the platelets were labeled with 30 μL FITC-GAM for 30 minutes at 20 °C. Labeled sections were treated with 2% uranyl acetate and 2% lead citrate to enhance contrast and were observed and photographed with a Zeiss Universal microscope equipped with an epi-illuminator, FITC-, RITC-selective filters, and a 40 x Plan-Neofluar and 63 x Plan-Apochromat objectives. Micrographs were made on Kodak Ektachrome 400 (commercially processed for 33 DIN [Deutsche Industrie Norm]) at final magnifications of 400 to 600 x.

**RESULTS**

**Surface distribution of GPIb and GPIb/IIa on fixed platelets.** Immobilization of antigens by formaldehyde fixation before incubation with MoAb Gi5 allows the visualization of GPIb/IIa distribution in the resting state. As seen in Fig 1A, the fluorescence appeared rather diffuse and uniform over the platelet surface with only minute irregularities. The same distribution pattern was seen in double fluorescence (Fig 1E) using MoAb FMC 25 directed against GPIb. Colloidal gold labeling of GPIb/IIa in IEM (Fig 2A) also demonstrated a more or less uniform distribution of this antigen on the plasma membrane. The slight inhomogeneity in the distribution of GPIb/IIa on fixed platelets (Figs 1A and 2A) is probably a sequel of multiple platelet washing.

**Distribution of GPIb and GPIb/IIa after MoAb stimulation.** Incubation of unfixed platelets with MoAb Gi5 for 10 minutes resulted in clustering (patching) of the antigen as seen in fluorescence (Fig 1B) and in IEM (Fig 2B). If, after patching of GPIb/IIa, the platelets were counterstained with MoAb FMC 25 and FITC-GAM, GPIb remained uniformly distributed and did not coaggregate with the former antigen (Fig 1F). After 30 minutes’ incubation with MoAb Gi5, the majority of labeled platelets were capped, both in fluorescence (Fig 1C) and in IEM (Fig 2C), whereas the distribution of GPIb remained unchanged (Fig 1G). After 40 minutes’ incubation time, the antigen-antibody label complex was internalized, presumably by endocytosis. Both fluorescence (Fig 1D) and IEM (Fig 2D) demonstrated intracellular label and loss of the complex from the surface. In contrast, no change became visible in the fluorescence pattern of GPIb (Fig 1H). Control experiments with unfixed platelets incubated with MoAb FMC 25 for ten or 30 minutes did not reveal any capping (data not shown). No patching or capping was found by colloidal gold labeling of GPIb in IEM (Fig 3).

Identical results, not depicted in the Figs, were obtained by substituting 2G12, 3F5, C15, and C17 for Gi5 or AN51 and rabbit anticyclocalin IGG for FMC 25 (see Table 1).

**Internalization of GPIb/IIa (endocytosis).** Endocytosis was investigated by a quantitative radioimmune assay. The results of a representative experiment are shown in Fig 4. If unfixed metabolically active platelets were incubated either with MoAb Gi5 (open triangles) or with FMC 25 (open circles), respectively, and then with a radiolabeled secondary antibody (GAM), there was a moderate increase of radioactivity over the five minutes’ value (100%) with both MoAb that reached a plateau after approximately 30 minutes. Similar curves were obtained if fixed instead of unfixed platelets were used, indicating that the increase of radioactivity was due to binding kinetics of antibodies and not to a platelet-dependent process. The data also show that the total amount of GP-MoAb-GAM complexes remained constant.
during incubation, precluding the possibility of their release into the medium (shedding). The results were strikingly different if unfixed platelets were treated with unlabeled instead of radiolabeled secondary antibody (GAM; closed symbols) prior to fixation and exposure to tertiary radiolabeled antibody (RAG) under otherwise identical experimental conditions (for details see Materials and Methods). In these circumstances the radiolabeled tertiary antibody can only bind to those GAM molecules that are still accessible at the platelet surface. It is evident that there was a rapid, precipitous decrease of radioactivity after pretreatment of platelets with the GPIIb/IIIa-specific MoAb (Gi5), whereas no such change was discernible after pretreatment with the GPIb-specific MoAb (FMC 25). It therefore follows that a large portion of the GPIIb/IIIa-GAM complexes, but not of the GPIb-GAM complexes, had been transferred from the
PLATELET PATCHING AND CAPPING

Fig 4. Demonstration of internalization of GPIIb/IIIa (but not of GPIb) by a quantitative radioimmune assay. Washed platelets were first incubated with MoAb (ten minutes, 20°C), then with a radiolabeled (open symbols) or an unlabeled secondary antibody (GAM) for various periods of time, and were then fixed. Finally, unlabeled, fixed platelets were treated with a radiolabeled tertiary antibody (RAG; closed symbols). For details see Materials and Methods. Open symbols indicate the total amount of GPIb (○) or IIb/IIIa (△) present either on the surface and/or internalized; closed symbols indicate GP exclusively present on the platelet surface (GPIb, ○; GPIIb/IIIa, △). The differences between corresponding values of curves are a measure of GP internalization. It is evident that GPIb remained on the platelet surface while a large proportion of GPIIb/IIIa was internalized.

Fig 3. IEM of unfixed platelets labeled 30 minutes with MoAb FMC 25, followed by RAM and protein A gold particles. The distribution of GPIb remained randomly scattered. The marker ‡ indicates 1 μm.

In lymphocytes as well as in other cells it has repeatedly been demonstrated that the redistribution of cell surface macromolecules within the plane of the plasma membrane is caused by receptor-assembly aggregation in response to multivalent ligands. Lectins as well as specific antibodies have been shown to induce patching, a local clustering of receptors, followed by capping, an aggregation of the clusters to a single area on the cell surface. Whereas the former is a passive phenomenon, the latter is an active, energy-dependent process that requires an intimate association of the receptor to the intact cytoskeletal system. Following capping, the molecules can be seen to be internalized by endocytosis.

Our results show that similar events take place on the platelet membrane in response to MoAb directed against an epitope on GPIIb/IIIa, but not if the MoAb are directed against GPIb. Membrane reorganization occurred in a time-dependent fashion. After approximately 30 minutes, GPIIb/IIIa was largely capped at one polar site of the platelet, and after 40 minutes, was removed from the membrane by apparent internalization (Figs 1D and 1H). In double fluorescence labeling experiments it could be shown that, during capping and endocytosis of GPIIb/IIIa, GPIb remained evenly distributed on the platelet surface. This was further substantiated by a quantitative radioimmune assay using radiolabeled secondary and tertiary antibodies (Fig 4). In controls there was some minor inhomogeneity in GPIIb/IIIa distribution already on fixed platelets. However, the inhomogeneity did not progress upon prolonged incubation before fixation and most likely was due to mechanical stimulation of platelets by multiple washing.

Zucker and Nachmias have recently pointed out that it is basically difficult to prepare platelets in the absolute resting state, and Hourdille et al reported similar clustering phenomena with unactivated platelets.

The distinct behavior of GPIb as opposed to GPIIb/IIIa is difficult to interpret with regard to ligand-dependent reorganization on the platelet surface. From lymphocyte studies it is known that only molecules connected to the cytoskeleton can undergo membrane reorganization. By analogy one would have to assume that the same holds true for platelets. Since there is evidence that both GPIb and GPIIb/IIIa represent transmembrane GP associated to the cytoskeleton,
our results lend support for a special, as yet obscure, role of GPIIb/IIIa in the capping process.

Until now there is only sparse information as to the exact location of these GP within the platelet plasma membrane and their association to internal mechanical structures. It has been inferred from biochemical data that GPIIb/IIIa are integral, transmembrane proteins.\(^1\) More recent results by Painter et al\(^2\) have indicated that two forms of GPIIb and III exist, one of which binds directly to endogenous membrane actin and one that does not. It is conceivable that it is the actin-binding portion of the GPIIb/IIIa complex that transmits the glycoprotein-cytoskeleton interactions induced by Con A in the intact platelet.\(^2\) Since a similar effect can be elicited by MoAb against various epitopes on the GPIIb/IIIa complex, as shown here, it is likely that cross-linking of this particular GP constitutes the specific signal for lateral membrane mobility ultimately leading to capping and endocytosis. It furthermore follows that neither cross-linking of membranous GP as such nor their association with the cytoskeleton alone are sufficient prerequisites for cap induction. Such a concept is corroborated by our experiments using MoAb against GPIb and polyclonal antibodies against glycocalcin. Contrary to previous views, Solum and Olsen\(^2\) have recently provided evidence that GPIb traverses the plasma membrane whereas glycocalcin represents the terminal part of the GP at the outer surface. Okita et al\(^3\) have demonstrated that GPIb is tightly associated with actin-binding protein in an apparent transmembrane complex. Thus, if ligand-induced cross-linking of a transmembrane GP would suffice for cap formation, MoAb against GPIb should have produced the same phenomenon. This was not the case, however. Our data also suggest an indirect effect of thrombin in inducing cap formation\(^4\) since the MoAb FMC 25 directed against GPIb, which is assumed to carry the thrombin receptor, did not elicit cap formation. Our observation that patching, capping, and endocytosis can be triggered in platelets by GPIIb/IIIa-specific MoAb is noteworthy in another respect. Berke and Fishelson\(^5\) have suggested that in mouse L cells and leukemia EL 4 cells capping by Con A or antibodies could easily be induced in intact cells, but virtually not in nucleus-free cytoplasts enucleated with the aid of cytochalasin B. They concluded that capping of cell surface receptors depends on their interaction with a nucleus-associated structure. Since human platelets are released from the cytoplasm of megakaryocytes of the bone marrow and do not possess nuclei, our results clearly oppose such a hypothesis and favor the concept that cap formation is a nucleus-independent membrane process.

The findings presented here may provide a plausible explanation for some as yet poorly understood phenomena. If cap formation is regarded as a membrane-clearing process, one could hypothesize that platelet antibodies often directed against epitopes on GPIIb/IIIa may stimulate capping and subsequently be actively removed from the membrane in vivo by endocytosis, which in turn could assist the platelet to escape sequestration in the reticuloendothelial system. Such an assumption might explain why the concentration of platelet-associated IgG is normal in about 30% of patients with chronic autoimmune thrombocytopenia.\(^6\) On the other hand, circulating immune complexes bound to the Fc receptor on GPIb may be nonstimulatory for cap induction and therefore can remain detectable at high concentrations on circulating platelets. High levels of platelet-associated IgG is a frequent finding in patients with systemic lupus erythematosus, considered to be an immune complex disease, as well as in patients with sepsis or infectious conditions.\(^7,8\) Naturally, this hypothesis can only remain valid if GPIb does in fact contain the Fc receptor, which at this point is not certain.\(^9\)

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