Glycoprotein Ic-IIa Functions as an Activation-independent Fibronectin Receptor on Human Platelets

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Abstract. Soluble fibronectin binds specifically to glycoprotein (GP) IIb-IIIa on thrombin-activated platelets, and this binding is not observed with platelets of patients with Glanzmann’s thrombasthenia (GT) which lack GPIIb-IIIa. Here we report that GT platelets retain the ability to interact with fibronectin-coated surfaces. Adhesion to fibronectin does not require platelet activation and is inhibited by soluble fibronectin, antibodies specific for fibronectin, peptides containing the sequence Arg-Gly-Asp and polyclonal antibodies specific for band 3 of the chicken embryo fibroblast fibronectin receptor (anti-band 3). Using anti-band 3, we have purified a second fibronectin receptor from human platelets, a heterodimer composed of glycoproteins previously designated GPIc and GPIIa. The GPIc-IIa complex is found on both GT and normal platelets and appears to be identical to the GPI38 kD–GPI60 kD complex recently immunopurified by Giancotti et al. (1986. Exp. Celt Res. 163:47–62) and by Sonnenberg et al. (1987. J. Biol. Chem. 268:10376–10383). In this report, we provide the first evidence that GPIc-IIa actually mediates adhesion of platelets to fibronectin-coated surfaces. GPIc-IIa thus represents a second functional fibronectin receptor, distinct from GPIIb-IIIa, that is largely responsible for the adhesion of nonactivated platelets to fibronectin-coated surfaces.

Platelets interact in vivo with the subendothelium of a damaged blood vessel to form a hemostatic plug. A chief component of the subendothelial matrix is the adhesive glycoprotein fibronectin (FN). In vitro observations concerning platelet–FN interactions have shown that human platelets will adhere to and spread on surfaces coated with FN (8, 13, 19). This process is similar to adhesion mediated by Arg-Gly-Asp (RGD)-specific cell surface receptors of other cells, e.g., fibroblasts or osteosarcoma cells (1, 6, 9, 11, 21, 30). It has also been demonstrated that soluble radiiodinated FN will specifically bind to the glycoprotein (GP) heterodimer GPIIb-IIIa on thrombin-activated platelets and that this binding can be inhibited by peptides containing the RGD sequence (22, 26). Because of these similarities between GPIIb-IIIa and receptors for adhesive glycoproteins, GPIIb-IIIa has been considered a likely candidate as the mediator of platelet adhesion to solid phase FN.

Recently, Giancotti et al. (7) demonstrated the presence of a glycoprotein complex, distinct from GPIIb-IIIa, which was immunopurified with a polyclonal antibody raised against the Fn receptor of mouse SR-BALB cells. The isolated complex consisted of two proteins with apparent molecular masses of 160 and 138 kD (nonreduced). Sonnenberg et al. (32) have used a rat monoclonal antibody raised against epithelial cells to immunopurify a similar complex from mice and human platelets. This group identified the complex components as platelet GPIc and GPIIa. Using a rabbit polyclonal antibody specific for band 3 of the chicken embryo fibroblast FN receptor (anti-band 3), we have immunopurified a complex of two proteins from lysates prepared from surface radiiodinated platelets. These proteins appear to be identical to the complex described by Giancotti et al. (7) and Sonnenberg, et al. (32). Analysis by two-dimensional nonreduced/reduced (NR/R) SDS-PAGE reveals that the purified proteins comigrate with platelet membrane glycoproteins Ic and IIa. We present evidence in this report that this heterodimer, present on platelets from normal subjects and patients with Glanzmann’s thrombasthenia (GT) (24), represents a functional FN receptor mediating the initial adhesion of nonactivated platelets to solid-phase FN.

Materials and Methods

Platelet Adhesion to FN

Platelet-rich plasma from normal volunteers or from patients with GT was obtained from acid/citrate/dextrose-anticoagulated whole blood by differen-
***Adhesion of Nonactivated Platelets to FN***

In our assay, 285,000 ± 70,000 (mean ± SD; n = 11) ⅛Cr-labeled, normal platelets adhere to a FN-coated microtiter well when incubated at 37°C for 30 min (Fig. 1). This value represents ~3% of the platelets added to each well. Increasing the incubation to 90 min increases the number of platelets adhering per well to ~15% of the total number of platelets.

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**Immunoblotting**

Normal platelets and platelets from patients with GT were lysed in 1% Triton X-100 in 50 mM Tris, pH 6.8, containing 10 mM benzamidine, 0.4 mM PMFS, 100 μg/ml leupeptin, and 004 μg/ml pepstatin A. The lysates were then centrifuged at 100,000 g for 45 min to pellet insoluble material. Soluble proteins in such lysates were prepared for electrophoresis by addition of 1% SDS with or without 5% N-ethylmaleimide, incubated at 56°C for 1 h and analyzed by two-dimensional NR/R SDS-PAGE as described (15). The resultant gels were stained, dried, photographed, and subjected to autoradiography.

**Results**

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**Adhesion of Nonactivated Platelets to FN**

Figure 1. Adhesion of normal platelets to FN-coated microtiter wells. ⅛Cr-labeled, normal platelets were preincubated for 1 h at room temperature. The beads were removed by centrifugation, and the pH of the eluted material in the supernatant was immediately neutralized by addition of 0.5 M Tris, pH 6.8.

The eluted, ⅛Cr-labeled antigens were mixed with 100 μg of nonradiolabeled total platelet protein, and the mixture was then made to 2% SDS plus 10 mM N-ethylmaleimide, incubated at 56°C for 1 h and analyzed by two-dimensional NR/R SDS-PAGE as described (15). The resultant gels were silver-stained, dried, photographed, and subjected to autoradiography.

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**Immunopurification and Two-Dimensional NR/R SDS-PAGE**

Normal platelets were surface-labeled with ⅛125I using the lactoperoxidase method (23) and solubilized by addition of 1% Triton X-100 in 50 mM Tris (pH 7.4), 200 mM NaCl, and 10 mM EDTA. Included in this lysis buffer were the proteolytic inhibitors leupeptin (100 μg/ml), benzamidine (10 mM), pepstatin A (002 μg/ml), and PMFS (4 mM). The mixtures were centrifuged at 100,000 g for 45 min at 4°C, and the supernatant containing soluble protein was aspirated and precleared by adsorption with nonimmune rabbit IgG conjugated to Sepharose CL-4B. All antibody-coupled Sepharose was prepared following the manufacturer's instructions. Generally, 200 μl of packed beads was added to tubes containing 10 ml of labeled platelet protein (12.0 mg). The slurry was incubated overnight at 4°C. After washing the beads six times with 10 mM Tris (pH 8.6), 2 mM EDTA, 1 M NaCl, plus 0.5% Triton (wash buffer), bound antigen was eluted by addition of 30 mM diethylamine (pH 11.2) plus 0.02% SDS and incubation for 15 min at room temperature. The beads were removed by centrifugation, and the pH of the eluted material in the supernatant was immediately neutralized by addition of 0.5 M Tris, pH 6.8.

The eluted, ⅛125I-labeled antigens were mixed with 100 μg of nonradiolabeled total platelet protein, and the mixture was then made to 2% SDS plus 10 mM N-ethylmaleimide, incubated at 56°C for 1 h and analyzed by two-dimensional NR/R SDS-PAGE as described (15). The resultant gels were silver-stained, dried, photographed, and subjected to autoradiography.

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the absence of antibody. (B) Effect of preincubation with the following antibodies: (1) anti-band 3 (n = 5, 24, and 3, respectively); (2) murine monoclonal AP-2 (n = 7,6,4); (3) anti-band 3 + AP-2 (n = 2,1); EDTA/37 not tested; (4) rabbit polyclonal anti-GPIlb + GPIIa (n = 2,1); (5) nonimmune rabbit IgG (n = 3,2); and (6) murine monoclonal AP-1 (n = 3,2,2). In B, the number of platelets adhering to FN-coated wells was determined and expressed as a percentage of normal adhesion, i.e., that observed in the absence of antibody (from A).

added. This increased value represents a solid monolayer of platelets covering the entire surface of the microtiter well when viewed by phase microscopy. Nonadherent platelets, when removed from one well after a 30-min incubation and placed into a second FN-coated well, adhere in a similar proportion, even when transfer is repeated up to four times. These findings indicate that adhesion to fibronectin, as reported in this article, is not the property of a subpopulation of platelets. The nonspecific adhesion to albumin-coated wells was routinely <1% of that obtained with FN (data not shown).

The total number of platelets adhering to FN was decreased to ~36% of control values if FN was included in the binding assay at concentrations >1.5 mg/ml. A similar inhibition was also noted when either of the peptides RGDS or GRGDSP (1.0 mM) was added, decreasing the number of platelets bound by 40% and 68%, respectively. Adhesion could also be inhibited by a monoclonal antibody (HFN 36.3, [American Tissue Culture Collection] ATCC No. CRL 1605) specific for the cell-binding domain of FN, but not by a second monoclonal antibody (HFN 7.1, ATCC No. CRL 1606) specific for a different domain of the FN molecule. A polyclonal rabbit antibody directed against fibrinogen (Dako Corp., Santa Barbara, CA) and a murine monoclonal antibody specific for von Willebrand Factor (AvWl, produced at The Blood Center of Southeastern Wisconsin) failed to inhibit adhesion.

When compared to untreated normal platelets, GT platelets or normal platelets treated with EDTA at 37°C (EDTA/37) exhibit enhanced binding to FN (Fig. 2). In each case, adhesion was inhibited by anti-band 3 (lane 1). Adhesion of normal platelets, but not GT or EDTA/37-treated platelets, was inhibited by the murine monoclonal AP-2, specific for the GPIIb-IIIa complex (lane 2). AP-2 plus anti-band 3 produced a synergistic effect, the combination inhibiting adhesion to a greater extent than either antibody alone (lane 3). Rabbit polyclonal antibody to GPIlb plus GPIIIa (lane 4) had no effect upon the binding of nonactivated normal platelets to Fn. Nonimmune rabbit IgG (lane 5) not only did not inhibit but actually enhanced the adhesion of control or GT platelets to Fn. AP-1, a murine monoclonal antibody specific for platelet GPIlb, also had no effect upon platelet adhesion to FN (lane 6).

Identification of Antigen(s) Recognized by Anti–Band 3

Proteins in lysates prepared from normal or GT platelets were separated by one-dimensional SDS-PAGE, transferred to nitrocellulose, and incubated in buffer containing anti-band 3 or a rabbit polyclonal antibody specific for GP IIb plus GPIIIa (Fig. 3). Protein bands that had been bound by antibody were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG. Anti–band 3 bound to a protein in lysates of either normal or GT platelets with an apparent molecular mass of 125 kD (nonreduced). The mobility of this protein is identical to that of GPIIa. In contrast, antibody specific for GPIlb plus GPIIIa reacted with different protein bands representing GPIlb and GPIIIa, respectively. The anti–GPIlb + GPIIIa antibodies reacted only faintly with a protein band in the GT platelet lysate most likely representing an altered form of GPIIIa. GT platelets, lacking GPIIb–IIIa, thus possess that component of the activation-independent receptor recognized by anti–band 3, namely, GPIIa.
Figure 4. Immunopurification of anti-band 3 antigen(s) and characterization by two-dimensional NR/R SDS-PAGE. $^{125}$I-labeled platelets were solubilized in Triton X-100 and incubated with anti-band 3-conjugated Sepharose CL-4B. The eluted material was mixed with 100 μg of a nonlabeled total platelet protein and subjected to two-dimensional NR/R SDS-PAGE. The autoradiograph derived from the gel is shown in A. The positions occupied by GPIIbα and GPIIIa, as determined in the corresponding silver-stained gel (B), are demarcated on the autoradiograph in A. Electrophoresis in the first dimension (nonreduced) was from right to left; in the second dimension (reduced), from top to bottom.

Immunoprecipitation and Two-Dimensional SDS-PAGE

The immunopurified, radiiodinated antigens isolated by anti-band 3 affinity chromatography from lysates of normal platelets were mixed with soluble protein from nonlabeled platelets and analyzed by two-dimensional NR/R SDS-PAGE (Fig. 4). The apparent molecular masses of the radiiodinated antigens visualized in autoradiograms (A) were then deduced by direct comparison to the mobilities of known glycoproteins visualized in the same gel by silver stain (B). Material immunopurified by anti-band 3 is composed of three radiolabeled proteins. The most prominent has an apparent molecular mass (NR/R) of 125 kD/150 kD. This radioactive protein comigrates with GPIIa on the silver-stained gel. The second and third components are subunits of a protein that has an apparent molecular mass nonreduced of 155 kD. Upon reduction, this protein is dissociated into two subunits, the larger with an apparent molecular mass of 130 kD, the smaller with an apparent molecular mass of 25 kD. These two subunits comigrate with platelet GPIcα and GPIcβ, respectively. Since anti-band 3 binds solely to GPIIa in an immunoblot assay (Fig. 3), it is thus reasonable to conclude that the immunopurified material is a noncovalently associated complex composed of GPIc and GPIIIa.

Discussion

The results of binding assays summarized in this report confirm that adhesion of nonactivated platelets to FN-coated surfaces is a specific interaction, mediated by the cell recognition sequence of the FN molecule (RGD) (13). The adherent platelets do not appear to represent a platelet subpopulation as evidenced by the repeated ability of nonadherent platelets, gently removed from assay microtiter wells and added to another set of FN-coated microtiter wells, to adhere again at a level identical to that of the initially adherent platelets.

The conditions used in the assay maintain platelets in a quiescent state. Ill et al. (13) have demonstrated, in assays similar to those presented here, that platelets loaded with $[^{14}C]$serotonin do not secrete the label upon adhesion to Fn. By enzyme-linked immunoassays using the monoclonal antibody S12 (5), we have determined that platelets adhering to FN also do not exhibit any increase in expression of GMP140, an alpha granule membrane glycoprotein (GMP) whose surface expression can be shown to increase upon platelet activation (data not shown). Furthermore, the fact that nonadherent platelets, when repeatedly removed from assay wells and added to fresh FN-coated wells, continue to
adhere at the same level as fresh platelets, suggests that the adherent platelets do not represent a subpopulation of platelets which were activated in the wash procedure. These results confirm that platelets adhering to FN in our assay are not activated, at least as monitored by assessing platelet secretion and expression of alpha granule membrane antigens.

The adhesion of nonactivated platelets to FN-coated surfaces cannot be explained solely by the interaction of GPIIb-IIIa with FN. First, GPIIb-IIIa is an activation-dependent receptor, and platelet stimulation by, e.g., thrombin is required in order that soluble, radioiodinated FN is bound (26). Secondly, platelets from patients with GT, lacking GPIIb-IIIa, still adhere to FN as well as or better than normal platelets. This result can be mimicked by treating normal platelets with EDTA at 37°C for 15 min, a treatment previously shown, by crossed immunoelectrophoresis, to irreversibly alter the GPIIb-IIIa complex and to destroy its ability to bind fibrinogen (17).

If GPIIb-IIIa is not required for the adhesion of nonactivated platelets to FN, then additional receptors may be involved. Giancotti et al. (7) have shown that a complex of two platelet membrane proteins cross-reacts with polyclonal antibodies raised against the FN receptor of mouse SR-BALB cells. Sonnenberg et al. (32) have confirmed the existence of a platelet glycoprotein heterodimer composed of GPIc and GPIIa. We have immunopurified a glycoprotein complex from human platelets by anti-band 3 affinity chromatography which is apparently identical to that described by Giancotti et al. (7) and Sonnenberg et al. (32). On two-dimensional NR/R SDS-PAGE gels, the immunopurified complex comigrates with platelet membrane glycoproteins designated GPIc and GPIIa. Since anti-band 3 binds only to GPIa in an immunoblot assay, it is reasonable to conclude that GPIc and GPIIa exist as a noncovalently associated complex.

Our results confirm and extend the observations of Giancotti et al. (7) and Sonnenberg et al. (32). We present evidence, utilizing in a functional assay, that GPIc-IIa is the receptor responsible for the adhesion of nonactivated platelets to FN.

Platelets thus possess two distinct FN receptors, one activation-dependent (GPIIb-IIIa) and another activation-independent (GPIc-IIa). Although specific for GPIIb-IIIa, AP-2 routinely decreases the extent of platelet binding and acts synergistically with anti-band 3, further decreasing the number of platelets bound. AP-2 recognizes an epitope on the GPIIb-IIIa complex that probably is proximal to the ligand binding site, as evidenced by the ability of AP-2 to inhibit platelet aggregation and fibrinogen binding (25). There are a number of explanations why AP-2 might exert this effect upon the adhesion of nonactivated, normal platelets to FN. First, it is conceivable that AP-2 may cross-react with GPIc-IIa. This would seem a likely conclusion since both GPIc-IIa and GPIIb-IIIa are RGD-dependent FN receptors and the epitope recognized by AP-2 may have been conserved through the evolution of these proteins. If this were the case, however, AP-2 would have inhibited the adhesion of both GT platelets and normal platelets. It seems more likely, therefore, that AP-2 affects adhesion via its binding to GPIIb-IIIa. Whether this means that a portion of GPIIb-IIIa molecules on nonactivated platelets serve as receptors for solid-phase FN or that the binding of AP-2 to GPIIb-IIIa in some manner interferes with the adhesion mediated by GPIIb-IIa remains to be determined. Thus, although not absolutely necessary for the adhesion of nonactivated platelets to FN, GPIIb-IIIa must exert an important effect upon this adhesion.

The adhesion of nonactivated platelets to FN-coated surfaces is a two-step process: platelets first attach and then they spread. If platelets were to attach to FN but show a reduced capacity to spread, a larger area of exposed FN would be available for the attachment of additional platelets. This would result in a greater number of platelets eventually able to cover the FN surface. This might explain why enhanced numbers of GT and EDTA/37 platelets adhere to FN. One can hypothesize that GPIIb-IIIa may mediate spreading on solid-phase FN. This theory finds support in the recent observation of Lawrence and Gralnick (20) that antibodies specific for IIb-IIIa block the spreading of platelets on subendothelium without inhibiting the initial binding. Along these lines, the inhibition of platelet spreading by AP2 might decrease the overall attachment of normal platelets to FN-coated surfaces. An assessment of the ability of thrombas-thenic and EDTA-treated platelets to spread onto FN-coated surfaces, and the effects of anti–GPIIb-IIIa and anti–GPIc-IIa agents on the attachment and spreading of normal platelets are the subjects of current studies in our laboratory.

GPIIb-IIIa is a member of a family of receptors that mediate adhesion of cells to extracellular matrices. Others include integrin, lymphocyte function-associated antigens (LFAs) and the lymphocyte antigens expressed very late after T-cell activation (VLAs) (for a review, see Hynes [12]). It is clear that the platelet heterodimer GPIc-IIa must also be included in this family. Both GPIc-IIa and GPIIb-IIIa are RGD-specific and composed of two components, the electrophoretic mobilities of which change in a characteristic fashion upon reduction. GPIIb-IIIa is fairly unique in that it requires cell activation for ligand binding, and it can bind a number of different adhesive glycoproteins, including fibrinogen (2), fibronectin (4, 26, 27), von Willebrand factor (27, 28, 31), and vitronectin (29).

The amino acid sequences of LFA-1, the beta chain of the chicken fibronectin receptor (integrin), and GPIIa have been published and appear quite homologous (3, 14). Despite this homology, it is GPIa and not GPIIIa that is immunologically cross-reactive with antibodies raised against the fibroblast FN receptor used in this study. It is therefore reasonable to expect that even greater homology will be found between GPIa and the beta chain of the FN receptor.

VLA antigens are a series of heterodimer adhesion receptors composed of an identical beta subunit and one of at least five different alpha subunits (10, 33, 34). Antibodies directed against the beta subunit block human fibroblast binding to both FN and vitronectin and are cross-reactive with chicken integrin (33). Since GPIa appears to be immunologically related to the beta subunit of integrin (band 3), it may represent the platelet analogue of the VLA beta chain. Our data indicate that platelet GPIc is the alpha subunit of the platelet activation-independent FN receptor. GPIc may be homologous to one of the VLA alpha subunits since antibodies specific for the VLA-3 alpha subunit crossreact with an alpha chain of chicken integrin (33). Furthermore, VLA-3 alpha has an apparent molecular mass (nonreduced/reduced) very similar to that of platelet GPIc.
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