Orientation and Specificity of Fibrin Protofibril Binding to ADP-Stimulated Platelets

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We have investigated the molecular basis of platelet:fibrin binding by studying interactions between platelets and protofibrils, soluble two-stranded polymers of fibrin, which are intermediates on the fibrin assembly pathway. The specificity of these interactions was examined with transmission electron microscopy (TEM), which clearly showed thin fibers with lengths to 150 nm attached to the cell surface of normal, stimulated platelets. Immunogold electron microscopy using rabbit anti-human fibrinogen as the first stage antibody verified the identity of the surface-bound molecules, and the immunogold distribution paralleled that observed with the fibrin/fibrinogen molecules alone. Contacts between the ends of the fibers and the platelets were frequently observed, but lateral contacts were also evident. Given the diameter at the point of fibrin contact (18.2 ± 1.3 nm), it is possible that several glycoprotein receptors were involved in binding each protofibril.

A DHESION of fibrin to the surface of human blood platelets is a critical event in the formation of a hemostatic plug at the site of vascular injury. Exposure of circulating platelets to physiologic activators such as ADP or thrombin not only causes a rapid change in morphology, but it also enables the circulating plasma protein fibrinogen to bind to receptors formed by the glycoprotein IIb/IIIa complex (GP Ib/IIa) on the surface of the stimulated cells. Fibrinogen appears to be the major protein involved in linking activated platelets into larger aggregates, which in turn provide the catalytic surface required for rapid activation of the proteases of the coagulation cascade. The resultant serine protease thrombin cleaves the fibrinopeptides from fibrinogen, triggering the multistep fibrin assembly sequence that leads to the formation of an insoluble fibrin gel.

Biophysical and electron microscopic studies have shown that soluble, two-stranded polymers of fibrin, termed protofibrils, which reach lengths in excess of 600 nm before they self-associate to form the highly interconnected network of the fibrin clot, are obligatory intermediates in this process. Radiolabeled ligand binding studies with model systems composed of fibrin polymers (maintained in a soluble form by either the anticoagulant peptide Gly-Pro-Arg-Pro or the fibrinogen degradation product, fragment D) have demonstrated that fibrin protofibrils, like fibrinogen, bind to the GP Ib/IIa complex on ADP-stimulated platelets through specific regions of the fibrin α- and γ-chains. Similarly, fibers of the three-dimensional fibrin network adhere to stimulated platelets through contacts between the fibrin α-chains and the GPIIb/IIIa receptor complex.

These biochemical studies have been complemented by several ultrastructural investigations that demonstrate the association of both endogenous (α granule) and exogenous fibrinogen with the platelet surface following thrombin or ADP stimulation. Analogously, fibers of the three-dimensional fibrin network develop close contacts with pseudopods and bodies of stimulated platelets, and fine filamentous bridging has been described between the cell surface and thicker fibers of retracted platelet-fibrin clots. The studies in the present report extend the observations cited above by addressing, through high-resolution transmission electron microscopy, the following questions related to interactions of soluble fibrin polymers and platelets: Do fibrin protofibrils bind to the platelet GPIIb/IIa complex? Which domains of the multinostril fibrin molecules are involved in binding? What is the orientation of platelet-bound fibrin oligomers with respect to the cell surface?

EXPERIMENTAL PROCEDURES

Materials

Human fibrinogen (Kabi, grade L) and plasmin (Kabi) were purchased from Helena Laboratories (Beaumont, TX). Human fibrinogen purchased from IMCO (Stockholm, Sweden) was used in some experiments, and processed similarly. Bovine serum albumin (Pentex, grade V) was purchased from Miles Laboratories (Elkhart, IN). Aprotinin, ADP (grade IX), and HEPES were purchased from Sigma Chemical Co (St Louis). Phe-Pro-Arg-Chloromethylketone (PPACK) was purchased from Calbiochem-Behring Corp (San Francisco, CA). Materials were purchased from Sigma Chemical Co (St Louis). Phe-Pro-Arg-Chloromethylketone (PPACK) was purchased from Calbiochem-Behring Corp (San Francisco, CA).
The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the distribution of radioactivity was determined with a Bio-Scan System 200 Imaging Scanner (Biocom, Washington, DC). The peaks corresponding to GPIIb and GPIIIa were identified by their characteristic molecular weights (mol wt), using coomassie blue-stained standards. The area of the radiolabeled GPIb peak was used as an internal standard, and the areas of the GPIIb/GPIIIa peaks were expressed relative to that value for both normal and patient samples. As the latter peaks were not fully resolved, the combined area was determined for GPIIb/GPIIIa. The platelets obtained from J.B. were found to contain 17% ± 6% of the normal quantity of GPIIb/GPIIIa.

Flow Cytometric Analysis of GPIIb/IIIa Antigen

The quantities of immunoreactive GPIIb/IIIa complex present on the surface of normal and thrombasthenic (J.B.) platelets were determined by flow cytometry, as described by Jennings et al19 with the following modifications. Gel-filtered platelets (0.7 to 1 x 10^8/mL) in HEPES-albumin-Tyrode's buffer pH 7.4, containing 2 mmol/L Ca^{++} were stimulated with 10 μmol/L ADP, then incubated with either monoclonal antibody HPI-1D (directed against the GPIIb/IIIa complex20) or control antibody SS-18 (directed against placentarial alkaline phosphatase and used here to correct for nonspecific binding) for 30 minutes at room temperature. Fluorescein-labeled antimouse IgG (moles fluorescein/moles protein = 3.6) was added to final concentrations of 38 and 73 μg/mL and incubated for ten minutes.

Flow cytometric analyses were carried out with an Ortho Cytofluorograph 50H flow cytometer/cell sorter with excitation provided by the 488-nm line of a Lxel 4-W argon laser and emission detection in the 515-530-nm range (Ortho Diagnostic Systems, Westwood, MA). The peak fluorescence intensity that resulted from specific binding of HPI-1D to the Glanzmann's thrombasthenic platelets was 9.0% ± 0.5% of that observed with normal platelets.

Microfluorimetry

The extent of adhesion of fluorophore-labeled fibronectin to normal and Glanzmann's thrombasthenic platelets was quantified by microfluorimetry using a Leitz Laborlux 11 phase contrast/epifluorescence microscope equipped with a Leitz MPV Compact microscope photometer (Leitz, Rockleigh, NJ). Fluorescein-fibrinogen (moles fluorescein/moles protein = 2.1) at 1 mg/mL was clotted by the addition of Brotrobolin (final concentration, 0.5 U/mL; Pentapharm, Basel, Switzerland) to suspensions of normal and Glanzmann's thrombasthenic platelets (1.1 to 1.2 x 10^8/mL), which were stimulated with 10 μmol/L ADP and maintained at 37°C in HEPES-Tyrode's-albumin buffer, with 2 mmol/L Ca^{++} at pH 7.2. The extent of nonspecific adhesion was determined with formaldehyde-fixed platelets under the same conditions. Details of fluorescein-fibrinogen preparation and fluorescence intensity measurements are described in detail elsewhere.21

Preparation of Fibrin Proteofibrils

Stable solutions of fibrin protofibrils were prepared by addition of thrombin (final concentration, 1 U/mL) to solutions containing fibrinogen (0.3 to 1 mg/mL) and a 35-fold molar excess of fragment D in HEPES-Tyrode's buffer, pH 7.2. Following an incubation period of ten minutes at room temperature, thrombin activity was irreversibly inhibited by addition of excess PPack (30 μmol/L). Further details of the characterization of fragment D-inhibited fibrin protofibrils and their interactions with platelets are described elsewhere.8

Platelet Isolation Procedures

Normal donors. Blood was drawn by venipuncture from healthy, adult volunteer donors (who had taken no aspirin for at least 2 weeks) into acid-citrate-dextrose (ACD) anticoagulant. The procedures used were fully examined and approved by the Clinical Research Practices Committee of the Bowman Gray School of Medicine. J. Kiffin Penry, MD, Chairman. Details of the procedures for preparation of platelet-rich plasma and gel-filtered platelets have been published previously.17 Platelet counts were determined with an Electrozone/Celloscope particle counter.

Glanzmann's Thrombasthenic Platelets

Clinical studies. Platelets from an individual (J.B.) with Glanzmann's thrombasthenia were obtained at Bowman Gray School of Medicine. Coagulation studies carried out at the time of his birth in 1978 included a platelet count, prothrombin time, factor VIII and IX assay, and fibrinogen determination; all were within a normal range. Qualitative platelet function studies, however, revealed complete absence of primary and secondary aggregation to high and low range. Qualitative platelet function studies, however, revealed complete absence of primary and secondary aggregation to high and low density collagen. Normal platelet aggregation was observed with ristocetin. These findings were felt to be diagnostic for Glanzmann's thrombasthenia. Subsequently, the patient has demonstrated a long bleeding time (>30 minutes by modified Ivy technique) and reduced platelet count, prothrombin time, factor VIII and IX assay, and fibrinogen determination; all were within a normal range.
Electron Microscopy

Samples containing ADP-stimulated gel-filtered platelets (0.5 to $1 \times 10^8$ cells/mL) and either fibrinogen or fibrin protofibrils (0.1 to 0.5 mg/mL), or no exogenous fibrinogen in HEPES-Tyrode’s-albumin buffer, 2 mmol/L Ca$^{2+}$, were incubated for 15 minutes at 37°C for 30 minutes at 23°C to allow binding. The reaction was then stopped by diluting the samples with an equal volume of 2.5% glutaraldehyde. In one series, the platelet/fibrin(ogen) samples were diluted 20-fold into 2.5% glutaraldehyde to control for aldehyde conjugation of protein to platelet surfaces. Following a 30-minute fixation period, the platelet/fibrin(ogen) samples were pelleted by centrifugation, postfixed with OsO$_4$, and embedded in epoxy resin for transmission electron microscopy. Morphometric analyses to determine specificity, orientation, and distribution of fibrin(ogen) at the platelet surfaces was carried out on electron micrographs of platelets sectioned at a thickness of 70 nm. Selected platelet samples for these studies were fixed in the presence of tannic acid (1%) to enhance molecular definition and delineate the plasma membrane-fibrinogen interface.

Ultrastructural immunocytochemistry to verify fibrinogen and fibrin identification on platelet surfaces was by a two-stage indirect method with colloidal gold as the electron opaque marker. Platelets for immunoelectron microscopy, subsequent to the binding of fibrin, fibrinogen, or in buffer control, were immediately fixed for five minutes at room temperature in 2.5% glutaraldehyde, buffered to pH 7.2 with 0.1 mol/L phosphate. Subsequent to the brief fixation, the cells were washed in Gey’s balanced salts containing 0.1% glycine to quench the free aldehydes. The washed cells were then resuspended for 15 minutes in the first stage antibody, rabbit antihuman fibrinogen (Cooper), which was diluted 1:25 with balanced salts. The primary immunolocalization was followed by extensive washing and then resuspension for 15 minutes in the second stage antibody, rabbit antimouse conjugated to 15-nm gold colloid particles. Comparision of the number and size of the two gold particles on platelets of each treatment provided a measure of immunospecificity.

RESULTS

Transmission electron microscopy of platelet/fibrin(ogen) specimens. Activation of gel-filtered platelets with ADP in the presence of either fibrin or fibrinogen resulted in specific association of the molecules with the plasma membrane. This association, however, occurred independently of aggregation, for the experimental conditions prohibited complete fibrin polymerization and aggregation of the cells did not occur. Generally, the platelets in the samples remained dispersed, yet they were often associated through a delicate macromolecular network. This network enshrouded complete platelets and in the case of experiments with fibrin protofibrils often interconnected the cells (Fig 1, A through D). Given the variability of cell surface contour and the randomness of the sectioning process, precise molecular orientation at the cell surface could not be determined; however, several organizational patterns of platelet-associated fibrin(ogen) were consistently observed. As illustrated in Fig 1C, platelet activa-
ADP stimulation but in the absence of exogenous fibrinogen. Contrast these relatively clean membranes to those illustrated in (C). (Original magnification × 39,500.) (G) Platelets from the Glanzmann’s patient following ADP activation in the presence of fibrin protofibrils. Platelets have undergone shape change, but unlike normal cells illustrated in (A), these Glanzmann’s samples lack the delicate molecular layer external to the plasma membrane. (Original magnification × 7,300.) (H) High magnification micrograph showing plasma membranes from two Glanzmann’s platelets stimulated with ADP in the presence of fibrin protofibrils. The crisp, clean membranes resemble control normal cells illustrated in (F) and are in dramatic contrast to stimulated normal platelets illustrated in (D). (Original magnification × 43,000.) (I) Plasma membranes of normal platelets following ADP stimulation in the presence of fibrin protofibrils and the monoclonal antibody (anti-GP IIb/IIIa) AP-2. A small amount of fibrillar material is present on the surfaces, but the degree of fibrinogen binding is significantly less than control. (Original magnification × 41,000.) (J) Plasma membranes of normal platelets following ADP stimulation in the presence of fibrin protofibrils and the monoclonal antibody (anti-GP IIb/IIIa) HP1-1D. As in the case of monoclonal AP-2, the cell surfaces are devoid of fibrin molecules. (Original magnification × 47,500.)
tion leading to fibrinogen binding resulted in the deposition of a delicate molecular layer at the surface. When this molecular layer was observed at high magnification, the constituent fibrinogen molecules were predominantly oriented perpendicularly to the underlying plasma membrane, indicating platelet binding through a single outer domain of the fibrinogen molecule. This configuration was not singular, for numerous examples of oblique orientation could be found, which suggested the possibility of multiple fibrinogen domain involvement in the binding (Fig 1C). A similar multiplicity of interactions was noted with platelets and fibrin, but the greater diameter of the fibrin protofibrils (18.2 nm ± 1.3 nm for fibrin ν 6.0 nm ± 0.4 nm for fibrinogen) at the platelet surface precluded clear identification of the fibrin binding sites (Fig 1D). Given the diameter at the point of fibrin contact, it is possible that several glycoprotein receptors were involved in binding of each fibrin protofibril. Typically, the fibrin protofibrils projected from the surfaces for distances of 40 to 50 nm, after which they became enmeshed in an anastomosing network of protofibrils (Fig 1D). The binding of both fibrinogen and the fibrin protofibrils was platelet specific, for although leukocytes and erythrocytes were occasionally observed in the samples (about 1 in 10,000), neither fibrin nor fibrinogen consistently interacted with these cells (Fig 1E).

**Ultrastructural specificity and morphometric analysis.** The ultrastructural specificity of cell surface characteristics following molecule binding to platelets was experimentally verified through the use of normal platelets activated in the absence of exogenous fibrin/fibrinogen (Fig 1F) and through the use of platelets from the Glanzmann’s patient, which were studied following ADP stimulation in the presence of fibrin protofibrils (Fig 1G and 1H). Molecular projections of a consistent nature were not observed with any of these samples. However, platelet surfaces are characterized by an extensive glycocalyx, and confusion of this macromolecular complex with fibrinogen or fibrin protofibrils was possible. Therefore, the length of molecules projecting from platelet surfaces was quantitated. As summarized in Fig 2, the plasma membrane of normal platelets stimulated in the absence of fibrinogen had an external molecular layer that extended to a length of 24.6 nm ± 1.2 nm (mean ± SD) from the membrane bilayer. The molecules on these control platelet surfaces were predominantly in the range of 20 nm to 25 nm, and they were sparsely distributed on the surface. Glanzmann’s platelets stimulated by ADP in the presence of fibrin protofibrils had an external layer with a mean molecular length of 20.9 nm ± 1.0 nm. As in the case of normal control platelets (stimulated, in the absence of added fibrinogen), these molecules were sparsely distributed (Fig 1G and 1H). In marked contrast to both the normal control cells and the Glanzmann’s cells, normal cells stimulated by ADP in the presence of fibrinogen had an extensive extracellular molecular layer in which the mean molecular length was 31.1 nm ± 1.3 nm. Most significantly, 60% of the molecules on these platelets had lengths >32.5 nm; and 33% had lengths in the range of 40 nm to 47.5 nm. As noted above, neither fibrinogen nor fibrin protofibrils bound to the surface of thrombasthenic platelets.

This lack of binding to the thrombasthenic controls was extended using a recently developed quantitative fluorescence microscopic technique to include fibers of the three-dimensional fibrin network. Clots formed by addition of Batroxobin to suspensions of ADP-stimulated platelets and fluorescein-fibrinogen were examined by epifluorescence microscopy, and the intensity of fibrin-platelet complexes was determined by microfluorimetry (Fig 3). While nearly all normal platelets were present as small aggregates, the thrombasthenic platelets were sparsely distributed on the surface. Glanzmann’s thrombasthenic platelets stimulated by ADP in the presence of fibrinogen had an external layer with a mean molecular length of 31.1 nm ± 1.3 nm. Most significantly, 60% of the molecules on these platelets had lengths >32.5 nm; and 33% had lengths in the range of 40 nm to 47.5 nm. As noted above, neither fibrinogen nor fibrin protofibrils bound to the surface of thrombasthenic platelets.
aggregates with substantial adherent fluorescein-fibrin, the thrombasthenic platelets were seen as single cells that demonstrated little or no interaction with the fibrin network. The mean fluorescence intensity of normal platelets in fluorescein-fibrin clots was fivefold greater than the background value measured with formaldehyde-fixed cells, whereas the thrombasthenic platelets exhibited a mean fluorescence intensity corresponding to a low level of nonspecific adhesion.

The distribution of fluorescence intensity values in Fig 3 illustrates this point, in that the histogram obtained with control platelets exhibited a normal distribution, as confirmed by a nonlinear regression analysis, with a mean value of 0.61 (SD = 0.37) arbitrary intensity units, while the data obtained with thrombasthenic platelets all fell in the lowest range, exhibiting a mean value equal to 0.04. The corrected fluorescence intensity parameter obtained with thrombasthenic platelets was zero, indicating no specific adhesion of fibrin to these receptor-deficient platelets. While the normal platelet clots retracted fully in approximately one hour, no evidence of retraction was found with the thrombasthenic platelets, even after four hours. Finally, consistent with the binding data, normal platelets stimulated by ADP in the presence of fibrin protofibrils and either of the two monoclonal antibodies directed against the GPIIb/IIIa complex (ie, HP1-1D and AP-2) lacked the molecular layer on the surface (Fig 11 and 1J).

Ultrastructural immunochemistry. Identity of the molecular layer on the fibrin-treated cells and the delicate macromolecules on the fibrinogen-treated cells was verified through immunoelectron microscopy using an antifibrinogen antisera. As shown in Fig 4, the fibrin-treated cells were heavily labeled with the antibody, resulting in gold particles distributed along protofibrils and in association with the short fibrin molecules on the cell surface. The density of 5-nm particles on the fibrin-activated cells was approximately 750 particles/μm of cell membrane. This density was 4.2 times higher than that found on the control cells; and the number of 5-nm particles, indicating specif

Fig 4. Immunocytochemical localization of fibrin and fibrinogen. (A) Cells activated in the presence of fibrin have surfaces and associated protofibrils extensively decorated with the specific (5 nm/L gold) goat antirabbit antibody. Nonspecific association of goat IgG is indicated by the relatively low density of the nonsense (15-nm gold) goat antimouse antibody. (Original magnification x 75,000.) (B) Cells activated in the presence of fibrinogen have a delicate label often characterized as three to five gold particles projecting from the cell surface (compare to Fig 1C). (Original magnification x 75,000.) (C) Control platelets stimulated in the presence of buffer alone. Fewer immunogold particles are evident, and in this experiment the nonsense antibody (15 nm) composed a greater percentage of the particles. (Original magnification x 75,000.)

Inhibition of fibrin protofibril binding by synthetic peptides. The final series of studies was concerned with the role of specific inhibitory peptides in fibrinogen/fibrin binding to platelets. The three peptides used at similar concentra-
DISCUSSION

Fibrinogen binding following platelet activation is now universally recognized as a prerequisite to normal platelet aggregation leading to hemostasis, and patients lacking either fibrinogen or its primary receptor, glycoprotein IIb/IIIa, are clinically characterized by hemorrhagic diathesis of varying severity. Similarly, hemorrhagic diathesis has been reported for patients with GPIIb/IIIa-specific platelet autoantibodies. When studied in vitro, fibrinogen associates with the platelet surface immediately following agonist stimulation, and at the ultrastructural level, fibrinogen binding has been related to a nonspecific flocculence on the extracellular face of platelet plasma membranes and to the presence of delicate molecular strands extending between the plasma membranes of closely apposed cells.

Consistent with these previous investigations, filamentous proteins at the surface of ADP-activated platelets are described in the present report. When stimulated by ADP in the presence of fibrinogen, the filaments had a mean length of 31 nm with a large component having lengths in the range 40 to 45 nm. These latter values are consistent with the molecular dimensions of fibrinogen, and the data suggest perpendicular orientation for a significant portion of the platelet-bound molecules. Perpendicular orientation was also observed when the cells were stimulated in the presence of fibrin protofibrils. However, it is clear from the protofibril studies and the fibrinogen morphometrics that perpendicular orientation is not exclusive. In the case of fibrin protofibrils, obliquely oriented molecules interconnected in an anastomosing network were often observed. Such an oblique orientation of the protofibrils is consistent with the fibrinogen morphometry data, for molecules oriented other than perpendicular to the surface would have projection lengths less than the true molecular length of fibrinogen. The presence of obliquely oriented molecules would, therefore, explain the mean macromolecular length of 31 nm found for platelets stimulated in the presence of exogenous fibrinogen.
interpretations of these observations are graphically illustrated in Fig 6.

Although platelet surfaces in the control experiments were largely devoid of fibrinogen, a surface coat having a thickness in the range 20 to 25 nm was observed with both normal platelets and the thrombasthenic cells. This molecular layer is consistent with the early observations of Behnke and others, and it probably encompasses numerous endogenous surface glycoproteins. Interestingly, the endogenous surface coat was relatively homogenous in thickness; whereas the poststimulation fibrinogen molecular layer was heterogenous in thickness with areas of long fibrinogen strands (45 nm) interspersed with regions devoid of fibrinogen and having thickness similar to the control surface. This surface coat/fibrinogen heterogeneity is consistent with recent reports, which indicate that both fibrinogen and its receptor are clustered following platelet activation either in suspension or by adhesion. In the present study, focal clumping of bound molecules was evidenced following immunoelectron microscopy, but it was also dramatically demonstrated in experiments involving fibrin protofibril binding to the activated cells. This observation provides support for the suggestion that fibrinogen receptor clustering may provide the molecular basis for fibrin anchoring to platelets. Such clustering of receptors would afford multiple binding opportunities for fibrin protofibrils and ultimately contribute to stability necessary for clot retraction subsequent to polymerization of the fibrin network.

Clearly, the interaction of fibrinogen and fibrin protofibrils with the platelet surface is more complex than the model of end-on association; for, as noted above, oblique orientation of molecules was common. Conceivably, this multiplicity of orientations results from different portions of the fibrinogen molecule interacting with GPIIb/IIIa. This possibility is substantiated by recent studies demonstrating that platelet/fibrinogen interactions involve both the outer, D-domains of fibrinogen, and the RGD(S) sequence, which is found in two distinct regions of the alpha-chains of fibrinogen. The relative contributions of these different RGD-containing sequences to platelet/fibrin(ogen) interactions has yet to be fully determined. In the present studies, a series of peptides GHRP, RGDS, and HHLGGAKQAGDV were used to explore interaction. Hypothetically, RGDS would have been competitive for both end-on and laterally associated fibrinogen, whereas HHLGGAKQAGDV would have been most competitive for “D” domain binding and GHRP would not have been competitive. Consistent with the hypothetical model, GHRP had little or no effect on organization at the platelet surface, and the RGDS peptide was most effective in competing for the surface of the platelets. The final competitor, HHLGGAKQAGDV, did reduce the amount of protein at the platelet surface, but the reduction was for both end-on and obliquely oriented molecules, suggesting that in the ultrastructural experiments, selectivity for a particular orientation of fibrinogen was not found. Whether the lack of selectivity was related to specificity of the competitor peptides or due to complexities of fibrinogen interactions with the platelet surface remains to be determined.

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