Antibody against a membrane inhibitor of the C5b-9 complex has been used to investigate regulatory control of the terminal complement proteins on blood platelets. Monospecific rabbit antibody (α-P18) was raised against the purified 18-kDa erythrocyte membrane inhibitor of C5b-9 (Sugita, Y., Nakano, Y., and Tomita, M. (1988) J. Biochem. (Tokyo) 104, 633-637). In addition to its interaction with erythrocytes, this antibody (and its Fab) bound specifically to platelet membranes. In immunoblots of cell membrane proteins prepared under non-reducing conditions, α-P18 bound specifically to an 18-kDa erythrocyte membrane protein and to a 37-kDa platelet membrane protein. Absorption of this antibody by platelet membranes competed its binding to the purified 18-kDa erythrocyte protein, suggesting that epitopes expressed by the erythrocyte 18-kDa C5b-9 inhibitor are common to the platelet. When bound to the platelet surface, the Fab of α-P18 increased C9 activation by membrane C5b-8, monitored by exposure of a complex-dependent C9 neo-epitope. Although α-P18 caused little increase in the cytolysis of platelets treated with C5b-9 (total release of lactate dehydrogenase <5%), it markedly increased the cell stimulatory responses induced by these complement proteins, including, secretion from platelet α- and dense granules, conformational activation of cell surface GP IIb-IIIa, release of membrane microparticles from the platelet surface, and exposure of new membrane binding sites for components of the prothrombinase enzyme complex. Prior incubation of C5b67 platelets with 100 μg/ml α-P18 (Fab) lowered by approximately 10-fold the half-maximal concentration of C8 required to elicit each of these responses (in the presence of excess C9). Incubation with α-P18 (Fab) alone did not activate platelets, nor did incubation with this antibody potentiate the stimulatory responses of platelets exposed to other agonists. These data indicate that a membrane inhibitor of the C5b-9 complex normally serves to attenuate the procoagulant responses of blood platelets exposed to activated complement proteins, and suggest the mechanism by which a deletion or inactivation of this cell surface component would increase the risk of vascular thrombosis.

In addition to mediating immune cytolysis, accumulating evidence suggests that the C5b-9 proteins of the complement system also elicit non-lytic stimulatory responses from certain cells through their capacity to selectively alter the ion conductance properties of the plasma membrane (1-6). In the case of human blood platelets assembly of the C5b-9 proteins has been shown to initiate a transient and reversible depolarization of the plasma membrane potential, a rise in cytosolic Ca++-metabolic conversion of arachidonate to thromboxane, and the activation of intracellular protein kinases (4-6). Concomitant with these electrochemical and metabolic changes, platelets exposed to the terminal complement proteins undergo shape change, secretory fusion of intracellular storage granules with the plasma membrane, and the vesiculation of membrane components from the cell surface (4-8). The generation of these membrane microparticles by platelets exposed to the C5b-9 proteins results in the exposure of membrane binding sites for the coagulation factor Va, initiating assembly of the prothrombinase enzyme complex and accelerating plasma clotting (8-10).

The capacity of the C5b-9 proteins to induce activation of human platelets while causing little or no cell lysis suggests that the functional properties of the complement pore are normally regulated by component(s) of the human platelet plasma membrane. Inhibitors of the C5b-9 complex have recently been identified in the human erythrocyte membrane, and there is evidence to suggest that these proteins are also distributed on other vascular and blood cell surfaces (11-15). In this paper, we present evidence for an inhibitor of the C5b-9 complex in the blood platelet membrane, and present data relating to its role in regulating cell activation-dependent responses initiated by the membrane-bound C5b-9 proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (globulin- and fatty acid-free), prostaglandin E1, apyrase, phorbol myristate acetate, and N-hydroxyisuccinimide biotin ester were obtained from Sigma; (p-amidino-phenyl)methanesulfonyl fluoride was from Med Cal; fluorescein-5-isothiocyanate (isomer I) was from Molecular Probes; phycerythrin-streptavidin conjugate was from Southern Biotechnology Associates, and Spectrozyme TH was from American Diagnostica. Iodo-Gen was from Pierce Chemical Co.; NaT from ICN Biochemicals, and [3H]serotonin was from Du Pont-New England Nuclear. Human complement proteins C5b6, C7, C8, and C9 were purified and analyzed for functional activity according to methods previously described (4). Bovine factors Va, Xa, prothrombin, thrombin, thrombomodulin, and the light chain of factor Va were gifts from Dr. Charles T. Esmon (Oklahoma Medical Research Foundation). All other chemicals were of reagent or analytical grade.

**Solutions**—Solution I = 145 mM NaCl, 4 mM KCl, 0.5 mM MgCl2,
erythrocyte membrane and its identity as an 18-kDa protein.

Ig Membranes—The 18-kDa human erythrocyte protein inhibitory for albumin, 5 mM PIPES, pH 6.8. Solution 11 corresponding to a single protein band at 18 kDa (Fig. 1).

Functional assay for C5b-9 inhibitory activity was then performed as described under "Experimental Procedures." Inset shows a silver-stained polyacrylamide gel of the applied material.

0.5 mM sodium phosphate, 0.1% (v/v) glucose, 0.1% bovine serum albumin, 5 mM PIPES, pH 6.8. Solution II was 137 mM NaCl, 4 mM KCl, 0.5 mM MgCl₂, 0.5 mM sodium phosphate, 0.1% glucose (v/v), 0.1% bovine serum albumin, 20 mM HEPES, pH 7.4.

Purification of the P18 Inhibitor of C5b-9 from Human Erythrocyte Membranes—The 18-kDa human erythrocyte protein inhibitory for C5b-9 lysis was isolated by modification of methods described by Sugita et al. (15). Following ion-exchange chromatography on DEAE, the active fractions were pooled, dialyzed into 0.1% (v/v) Nonidet P-40 (Sigma), 10 mM Tris, pH 8.4, and chromatographed on Mono-Q (Pharmacia LKB Biotechnology Inc.), using a linear gradient of 0–300 mM NaCl in this solution. The peak of activity, corresponding to a protein band of 18 kDa, eluted at approximately 150 mM NaCl. The fractions of peak activity were pooled, concentrated, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis without reduction using a 12% homogeneous polyacrylamide gel. The 18-kDa band was then eluted from the gel slice (Amicon Centricon). When incorporated into non-primate erythrocytes, this protein inhibited the hemolytic activity of the purified human C5b-9 proteins, due to inhibition of C9 activation by membrane C5b-8. All of the C5b-9-inhibitory activity of this protein was found to elute from a gel slice corresponding to a single protein band at 18 kDa (Fig. 1).

Hemolytic Assay for C5b-9 Inhibitory Activity in P18—The functional activity of the 18-kDa protein isolated from human erythrocyte membranes was assayed based on the capacity of this protein to inhibit C5b-9-mediated hemolysis when incorporated into non-primate erythrocytes (15). 1.5 × 10⁸ chicken red blood cells were incubated (10 min, 23 °C) with C5b6 (0.03 μg) plus C7 (0.01 μg) in a total volume of 1 ml. The concentration of Nonidet P-40 never exceeded 0.002% (v/v). The cells were then washed, and suspended with C8 (0.02 μg) plus C9 (0.03 μg) in a total volume of 1 ml. After 30-min incubation at 37 °C, cell lysis was determined. Comparison was made to identically matched C5b-9-treated chicken erythrocytes, omitting P18 protein, and data expressed as percent inhibition of lysis of this uninhibited C5b-9 treated control.

Antibodies—Murine monoclonal antibody S12, specific for the platelet α-granule membrane glycoprotein, GMP-140, was a gift from Dr. Rodger P. McEver (Oklahoma Medical Research Foundation, Oklahoma City) (16). Murine monoclonal antibody A1-P, specific for membrane glycoprotein GP Ib, was from Dr. Thomas J. Kunicki (Blood Center of Southwestern Wisconsin, Milwaukee, WI). Murine monoclonal FAC1 (17), specific for the activated conformation of the Gp lb complex, was from Dr. Mary H. Shachar (Blood Center of Southwestern Wisconsin, Milwaukee, WI). Murine monoclonal antibody MAC (8), specific for a neo-epitope in C9 exposed upon its incorporation into membrane C5b-9 or SC5b-9, was from Dr. John Tamerius (Cytotech Corp). Murine monoclonal antibody V237 recognizes an epitope in the light chain of factor Va (human or bovine) and binds to factor V and factor Va (8). Affinity purified goat antibody against rabbit IgG (absorbed against human IgG) was obtained from Sigma. Monospecific rabbit antibody against bovine thrombomodulin was a gift from Dr. Naomi L. Esmon (Oklahoma Medical Research Foundation). Monospecific rabbit antibody against the purified human erythrocyte 18-kDa protein (a-P18) was raised by repeated injection of the purified antigen (see above). The IgG fraction was isolated by absorption to immobilized Staphylococcus protein A, followed by gel permeation chromatography on Sephadex G-200 (Pharmacia). By Western blotting against detergent or butanol extracts of human erythrocyte membrane proteins, this antibody was specific for a single protein of 18 kDa that was common to this family of proteins. Reactivity of this antibody was lost upon disulfide reduction of the antigen.² Fab fragments of IgG were prepared by 2-h digestion at 37 °C with immobilized papain (Fierce Chemical Co.). The resulting Fab fragments were purified to homogeneity by absorption against immobilized human erythrocyte protein A and gel permeation chromatography (Sephadex G-150).

Fluorescence Labeling—For flow cytometry all antibodies were conjugated with FITC, except antibody A1-P, which was conjugated with N-hydroxysuccinimide biotin ester as described previously (8). Dye-to-protein ratios ranged from 3 to 5.

Protein Concentrations—Concentrations of unlabeled proteins were estimated assuming the following extinction coefficients (E₁%):

| Protein      | E₁% solvent (%) |
|--------------|-----------------|
| IgG (15)     | factor Va (15.1)|
| factor Va light chain (18.7) | factor Xa (12.4) |
| prothrombin (15.5) | C5b6 (10.0) |
| C7 (9.9)     | C8 (15.1)       |
| C9 (9.6)     |                 |

The concentrations of FITC-labeled proteins were determined by dye binding assay (Bio-Rad), using the respective unlabeled protein as standard. FITC concentration was determined assuming a molar extinction (492 nm) of 68,000.

Effect of α-P18 on Platelet Activation by C5b-9—Gel-filtered human platelets were prepared and collected into Solution I at 1–2 × 10⁹/ml as described previously (4, 5). To assemble membrane-bound C5b6 complexes, gel-filtered platelets (10⁷) were incubated for 5 min at 37 °C with C5b6 (15 μg/10⁶ platelets) and C7 (2.5 μg/10⁶ platelets). The C5b67 platelets were then incubated with the Fab fragments of α-P18 (0–125 μg/ml) for 10 min at room temperature. After dilution to 10⁶ platelets/ml in Solution II containing 2.5 mM CaCl₂, C8 (0–1 μg/10⁶ cells) and C9 (0 or 4 μg/10⁶ cells) were added, and the cells incubated at 37 °C without stirring for 10 min. Following incubation the platelets were fixed, washed and analyzed by flow cytometry. All analyses were complete within 30 min.

Preparation of Platelets for Flow Cytometry—5 × 10⁷ C5b-9-treated or control platelets were incubated in the dark in a total volume of 60 μl for 10 min at 23 °C in the presence of biotin-A1-P (1 μg/ml) and one or more of the following fluorescein-conjugated antibodies: FITC-α-P18 (IgG; 100 μg/ml); FITC-α-P18 (Fab; 50 μg/ml); FITC-MAC (30 μg/ml); FITC-PAC1 (30 μg/ml); FITC-SC12 (10 μg/ml), or FITC-V237 (20 μg/ml). To measure membrane binding sites for factor Va, cells were first incubated for 10 min at 23 °C with factor Va light chain (2 μg/ml) before addition of FITC-V237. Following incubation with the labeled antibodies, phycocerythrin-streptavidin was added (5 μl of a 1:20 dilution), and the cells incubated an additional 10 min. Then 0-5 μl aliquots of Solution II were added and B samples analyzed by flow cytometry. All analyses were complete within 30 min.

Flow Cytometry—Platelets were analyzed in a Becton Dickinson FACSCAN flow cytometer formatted for two-color analysis. The light scatter and fluorescence channels were set at logarithmic gain. In order to resolve platelet-dabeled microparticles from background light scatter, acquisition was gated so as to include only those particles distinctly positive for biotin-A1-P (detected by phycocerythrin fluorescence), using a fluorescence lower-limit threshold on the 585-nm

Fig. 1. Isolation of a C5b-9 inhibitor present in the human erythrocyte membrane and its identity as an 18-kDa protein. 200 μg of purified P18 protein (Mono-Q pool) was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. Horizontal 0.5-cm slices of the gel were prepared, and proteins eluted into 0.1% (v/v) Nonidet P-40, 10 mM Tris, pH 8.0. Functional assay for C5b-9-inhibitory activity was then performed as described under "Experimental Procedures." Inset shows a silver-stained polyacrylamide gel of the applied material.

Molecular Weight

| Mol. Weight | SDS-Page Gel Slice |
|------------|--------------------|
| 44          | 1                 |
| 21          | 2                 |
| 31          | 3                 |
| 42          | 4                 |
| 66          | 5                 |
| 97          | 6                 |

}[1] The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; α-P18, monospecific rabbit antibody against human 18-kDa erythrocyte membrane protein; GMP, granule membrane protein; GP, glycoprotein; FITC, fluorescein-5-isothiocyanate; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; EGT A, (ethyl-enediphosphatetetraacetic acid; ELISA, enzyme-linked immunosorbent assay.

[2] S. A. Rollins and P. J. Sim, manuscript in preparation.
channel that excluded background scatter. Thus, only those cells (and microparticles) expressing the platelet-specific membrane glycoprotein GP Ib, were included for analysis (8, 17). 10,000 phycoerythrin-positive particles from each sample were analyzed for forward and right angle light scatter and for FITC and phycoerythrin fluorescence intensities. To measure FITC-α-P18 binding to erythrocytes, the threshold for cell detection was set on forward scatter. All fluorescence data were corrected for cell or microparticle autofluorescence (generally, ≤2 arbitrary fluorescence units/particle). Where indicated, correction for nonspecific binding was made by incubation of cells with FITC-labeled antibody in the presence of a 20-fold excess of the unlabeled antibody (IgG or Fab).

Decoy and Cell Lysis Assays—Dense granule secretion was measured by the release of [3H]serotonin (4). α-Granule secretion was monitored by the surface expression of GMP-140, detected by binding of FITC-labeled monoclonal antibody S12 (8, 16). Cell lysis was monitored by release of cytoplasmic lactic acid dehydrogenase.

Prothrombinase Assay—Platelet prothrombinase activity was measured by modification of methods previously described, using the chromogenic substrate Spectrozyme TH (9). After activation, platelets were diluted (final concentration, 5 × 10^4/ml) in Solution II containing 1% albumin, 2.5 mM CaCl_2, 2 mM factor Va, and 2.7 μM prothrombin, and incubated at 37 °C. Prothrombin conversion was initiated by addition of factor Xa (2 nM final). The reaction was stopped at 0, 30, and 60 s by transfer of 1 volume of sample into 9 volumes of ice-cold buffer containing 1% albumin and 10 mM EDTA, and thrombin assayed as described previously (9).

Immunoblotting of Platelet and Erythrocyte Membranes—Washed human platelets or erythrocyte ghost membranes were suspended in solution I omitting albumin, but containing 10 mM EGTA, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin) and extracted for 60 min (23 °C) with 20% (v/v) n-butanol. After removal of the insoluble material and organic phase, the butanol-saturated aqueous phase was denatured (3 min, 100 °C) in 2% sodium dodecyl sulfate without reduction, and electrophoresed in a 12% polyacrylamide gel, using a Laemmli buffer system. Following transfer to nitrocellulose, immunoblotting was performed by overnight incubation with 100 μg/ml α-P18 (IgG), and developed with a radiolabeled affinity-purified antibody against rabbit IgG (Sigma). Radioactive bands were then visualized using an Ambis radioanalytic imaging system.

Cross-reactivity of α-P18 with Platelets—The expression of epitopes on the platelet surface common to those of the human erythrocyte P18 protein was evaluated by the capacity of platelets to absorb antibody specifically recognizing this protein. α-P18 IgG (15 μg/ml) was incubated (2 h, 23 °C) with either human platelets (0.5-3 × 10^6/ml) or human erythrocytes (0.5-3 × 10^6/ml). α-P18 remaining in the cell-free supernatants was then assayed by ELISA, using purified human erythrocyte P18 protein as the plate-coating antigen. To control for nonspecific absorption of antibody, platelets and erythrocytes were also identically incubated with rabbit IgG raised against bovine thrombomodulin, and the unabsorbed antibody assayed by ELISA using purified thrombomodulin as the plate-coating antigen. ELISA plates were developed with a peroxidase-conjugated affinity-purified goat antibody to rabbit IgG (Kirkegaard).

**RESULTS**

α-P18 Binds Specifically to the Platelet Plasma Membrane and Increases the Incorporation of Activated C9 into Membrane C5b-9—Recent evidence suggests that the hemolytic activity of the terminal complement proteins is regulated by two distinct proteins expressed on the surface of human erythrocytes: “homologous restriction factor” (also referred to as “C8-binding protein”) with an apparent mass of 64 kDa (11-14), and a newly described 18-kDa protein that does not exhibit C8-binding activity (15). Both of these membrane proteins have been shown to inhibit the cytoytic activity of the human terminal complement proteins, apparently by affecting the interaction of C9 with membrane bound C5b-8. Evidence has been presented that homologous restriction factor is normally bound to the cell surface by linkage to membrane phosphatidylinositol, and that this protein is deleted from the affected blood cells obtained from patients with the disorder paroxysmal nocturnal hemoglobinuria (13, 14, 18, 19). The distribution and potential function of the 18-kDa erythocyte protein on other vascular and blood cell surfaces remain unknown.

To determine whether this newly identified component of the erythrocyte membrane also plays a role in regulating the C5b-9 complex on the platelet surface, we have produced an antibody that binds to the 18-kDa erythrocyte protein and abrogates its C5b-9-inhibitory function (see “Experimental Procedures”). In addition to binding erythrocytes, this antibody was found to bind specifically to platelets (Table I). When bound to red cells, α-P18 (or, its Fab fragment) neutralized the C9-inhibitory activity of the 18-kDa membrane protein, and thereby increased the lytic susceptibility of the cells to purified C5b-9. To test the effect of this antibody on platelets, gel-filtered human platelets were first exposed to the Cs67 proteins, and then incubated with α-P18 (Fab fragments) before addition of C8 plus C9 (Fig. 2). As shown by these data, incubation with α-P18 increased the amount of activated C9 incorporated into plasma membrane C5b-9 complexes, suggesting that this antibody also neutralizes a C5b-9 regulatory function of the platelet membrane. In these experiments, the activated form of C9 was detected by use of a fluorescently labeled monoclonal antibody that recognizes

| Table 1 |
| --- |
| Binding of α-P18 to human platelets and red blood cells |
| Cell line of FITC-labeled α-P18 (IgG or Fab) was measured and corrected for nonspecific binding as described under “Experimental Procedures.” Data are expressed as fluorescence (arbitrary units)/10^6 cells. Mean ± S.D., n = 3. |
| | Erythrocytes | Platelets |
| --- | --- | --- |
| FITC-Fab | 86,100 ± 300 | 22,800 ± 200 |
| FITC-IgG | 415,100 ± 600 | 103,000 ± 600 |

**FIG. 2.** Effect of α-P18 on C9 incorporation into platelet membrane C5b-9 complexes. Cs67 platelets were incubated (15 min, 23 °C) with either 0 (hatched bars) or 100 μg/ml (solid bars) α-P18 (Fab) before addition of C9 (4 μg/10^6 cells) plus amounts of C8 given on the abscissa. After additional 10-min incubation at 37 °C, samples were stained with FITC-labeled monoclonal antibody against C9 neo-epitope (FITC-MAC) and processed for flow cytometry (see “Experimental Procedures”). Ordinate denotes total membrane-associated fluorescence (arbitrary units), corrected for nonspecific binding of FITC-MAC. Data for C9-free controls are also shown. Data from a single experiment, representative of three similar experiments performed on different days.
a neo-epitope in C9 that is expressed upon its incorporation into membrane C5b-9 (8).

Identity of the Platelet Antigen Recognized by α-P18—The capacity of α-P18 to potentiate assembly of C5b-9 complexes on the platelet surface suggested that this antibody recognizes and functionally inhibits a platelet plasma membrane protein that is analogous to erythrocyte P18. When platelet membrane proteins were analyzed by Western blotting (Fig. 3), α-P18 consistently detected a single band of approximately 37 kDa. In repeated experiments performed under a variety of conditions of membrane protein isolation and immunoblotting, we were unable to detect any binding of this antibody to platelet membrane proteins of 18 kDa. These data suggest either: 1) dimerization of an 18-kDa protein normally present in the platelet membrane; 2) the presence in platelet of an alternative molecular weight form of this protein, due to alternative splicing or processing of an extracellular domain sharing homology with erythrocyte P18; or 3) reactivity of this polyclonal antibody with epitopes expressed by a platelet membrane protein that is unrelated to erythrocyte P18. As illustrated by the data of Fig. 4, absorption of α-P18 by either erythrocytes or platelets resulted in the specific removal of antibody reactive with the purified erythrocyte P18 protein, suggesting that the IgG which binds to the platelet surface (Table I) and reacts with platelet membranes by immunoblotting (Fig. 3) recognizes epitopes that are also expressed by the erythrocyte-derived P18 protein. These studies also suggest that the amount of this antigen that is expressed on the surface of a platelet is approximately 5–20% of that expressed on an erythrocyte (cf. data of Table I and Fig. 4). Based on the respective surface areas of erythrocytes (1.5 × 10^{-10} m^2) and unstimulated platelets (2 × 10^{-11} m^2), these data suggest approximately equal surface density of P18 antigen on these two blood cells. Isolation of the platelet membrane protein recognized by α-P18 is now in progress.

Effect of α-P18 on C5b-9-induced Secretion and Activation of Cell Surface GPIIb–IIIa—The increased amount of activated C9 detected on the surface of platelets exposed to α-P18 suggested that this antibody might also affect the susceptibility of these cells to the stimulatory or cytolytic effects of the C5b-9 proteins. As shown in Figs. 5 and 6, incubation with α-P18 markedly potentiated the capacity of the C5b-9 proteins to induce secretion from both α- and dense granules, but caused little increase in the platelet’s susceptibility to lysis by these complement proteins. This potentiation of C5b-9-stimulated secretion of platelet storage granules increased in a dose-dependent fashion with the concentration of the antibody (Fig. 6A). At these concentrations, α-P18 alone, or, in combination with C5b-8 (in the absence of added C9), did not directly stimulate platelet secretion nor did this antibody potentiate the platelet’s secretory response to stimulation by low dose thrombin or phorbol ester (data not shown).

In addition to potentiating the platelet’s secretory response to C5b-9, α-P18 also caused a marked increase in the C5b-9-dependent binding of monoclonal antibody FITC-PAC1 to the platelet surface (Fig. 7). This monoclonal antibody recognizes a conformational neo-epitope expressed by activated cell surface GP IIb–IIIa (the platelet’s fibrinogen receptor) (17). As reported previously (20, 21), although C5b-9-stimulated the expression of the neo-epitope of GPIIb–IIIa that is recognized by PAC1, this conformational change in the receptor complex was not accompanied by increased fibrinogen binding, and platelet aggregation was not observed (data not shown).

Effect of α-P18 on the Procoagulant Activity of the C5b-9 Proteins—In addition to stimulating platelet secretion, the C5b-9 proteins have been shown to initiate the release of small membrane vesicles (~100 nm diameter) from the cell surface that incorporate plasma membrane glycoproteins Ib, IIb, and IIIa, and the α-granule membrane-derived glycoprotein GMP-140 (8). The release of these membrane microparticles from the platelet surface is directly coupled to the influx of Ca^{2+} into the platelet cytosol and results in exposure of membrane receptors for factor Va and expression of catalytic surface for the prothrombinase enzyme complex (7–10). As illustrated by Fig. 6B and the dot plots shown in Fig. 8, prior treatment with α-P18 greatly increased the number of microparticles formed upon C9 binding to membrane C5b-8. α-P18 alone did not induce vesiculation of the platelet surface (see Fig. 8B) nor did this antibody increase the number of microparticles detected when platelets were exposed to other agonists (data not shown). The distribution of cell surface components between platelets and the shed microparticles (re-
In platelets exposed to α-P18, the half-maximal concentration of C8 required for C5b-9-induced secretion (Fig. 5), exposure of the PAC1 epitope in GPIIb-IIIa (Fig. 7), exposure of factor Va binding sites (Fig. 9A) and expression of prothrombinase activity (Fig. 9B) decreased by more than 10-fold. This was accompanied by increased binding of activated C9 at each input of C8 (to pre-assembled plasma membrane C5b67; Fig. 2). Taken together, these data suggest that epitopes recognized by α-P18 include functional domains of a membrane component that inhibits formation of the comple-

In addition to increasing C9 incorporation and microparticle formation, α-P18 caused a 4–5-fold increase in the number of factor Va binding sites exposed upon C5b-9 binding to the platelet surface (Figs. 6C and 9A). This increase in total factor Va binding sites exposed on microparticles that were released from the surface of α-P18-treated platelets, combined with a 2-fold increase in the number of factor Va binding sites exposed on the cells themselves under these conditions (Table II). This increased exposure of factor Va binding sites due to the presence of α-P18 resulted in a comparable increase in the C5b-9-induced expression of catalytic membrane surface for the prothrombinase enzyme complex (Fig. 9B).

DISCUSSION

Our data suggest that the platelet plasma membrane contains an inhibitor of the terminal complement proteins that shares both functional and antigenic properties with the 18-kDa protein recently identified in the human erythrocyte membrane. In erythrocytes, this protein appears to serve a key role in restricting the cytolytic consequence of C5b-9 assembly (15). In addition to contributing to the normal resistance of human platelets to lysis by complement, our data suggest that this membrane component serves directly to attenuate the capacity of the C5b-9 proteins to induce procoagulant platelet responses.
blots of human platelets indicate that α-P18 recognizes a platelet GPIIb-IIIa complex in the absence of added C8 (Fig. 11), or, when saturating amounts of C9 were added to C5b-67 platelets exposed to α-P18 (Table 5), the addition of C8 and C9 (see Fig. 2). After incubation for 10 min at 37 °C, the cells were stained with monoclonal antibody AP1 (directed against GP Ib) and analyzed by flow cytometry. The red fluorescence threshold was set so that only GP Ib-positive particles were analyzed (see “Experimental Procedures”). Two rectangles are drawn on each dot plot to denote analysis gates of forward (abscissa, logarithmic scale) and side (ordinate, logarithmic scale) angle light scatter that were used to discriminate platelets (right-hand gate, depicted by solid rectangle) and microparticles (left-hand gate, depicted by dashed rectangle). Also shown are data for complement-free controls exposed to 0 (A) or 100 µg/ml α-P18 (B). In these experiments, the number of microparticles detected (as percentage of all GP-Ib positive particles analyzed) was 9.7% (A), 9.2% (B), 29% (C), and 74% (D). Data of a single experiment, representative of six similar experiments performed on separate days.

At the present time we do not know the relationship of this membrane inhibitor to the 64-kDa protein homologous restriction factor (11, 18) or C8-binding protein (12–14), previously identified in human erythrocytes. Although we cannot exclude the possibility of an undetected cross-reactivity of our polyclonal antibody with a 64-kDa component in either erythrocyte or platelet membranes, we note the following: (i) elution of protein from gel slices prepared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified antigen demonstrated C5b-9 inhibitory activity only in the 18-kDa protein band (Fig. 1). Similar results have been reported by Sugita et al. (15). (ii) Western blotting with α-P18 (IgG) of erythrocyte membranes, revealed a single band at 18 kDa (Fig. 3). These data suggest that the C5b-9 inhibitory activity neutralized by α-P18 resides in its effect on an 18-kDa erythrocyte protein (and not the 64-kDa homologous restriction factor). By contrast to these data for erythrocytes, immunoblots of human platelets indicate that α-P18 recognizes a platelet membrane protein of approximately 37 kDa. Although this suggests that a 37-kDa protein is responsible for the C5b-9-inhibitory activity of the platelet membrane (the function of which is blocked upon α-P18 binding), the actual molecular identity of the platelet C5b-9 inhibitor and its relationship to erythrocyte P18 awaits purification and biochemical characterization of this protein in the platelet membrane.

As shown in Fig. 5B, pre-treatment with α-P18 slightly increased the lysis of platelets exposed to C5b-9 (measured by release of lactic acid dehydrogenase). Nevertheless, this effect on the platelet's susceptibility to cytolysis by C5b-9 was quite small, and total cell lysis never exceeded 5% under our experimental conditions. This stands in contrast to the marked effect of this antibody on the sensitivity of platelets to the cell-stimulatory effects of the C5b-9 proteins. Most notably, α-P18 greatly increased both the number of membrane microparticles shed from the surface of C5b-9-treated platelets and the number of factor Va binding sites exposed on these membrane surfaces (microparticle and platelet). As described previously, this capacity of the platelet to shed cell surface components is integrally related both to the observed inactivation of functional C5b-9 pores (which restores the electrochemical integrity of the plasma membrane; Refs. 4 and 7) as well as to the exposure of membrane receptors for factor Va (which initiates the prothrombinase reaction; Ref. 8).
A, of 2 pg/ml of the ligand. Data plotted represent total membrane sites proteins under conditions described for Fig. 2, and then analyzed for lant activity. Platelets were incubated with a-Pl8 and the C5b-9 dures. Concentrations of a-Pl8 (Fab) were either shown.

Effect of a-Pl8 on microparticle formation

| No. of MICROPARTICLES a | FITC-MAC bound Platelets | FITC-V237 bound Platelets |
|-------------------------|-------------------------|-------------------------|
| C5b-9*                  | 24,580                  | 149,600                 |
|                         | 1,810                   | 68,600                  |
| C5b-8                   | 790                     | 0                       |
|                         | 790                     | 0                       |
| C5b67                   | 790                     | 0                       |
|                         | 790                     | 0                       |
| Control                 | 770                     | 0                       |

*Platelets incubated in presence (+) or absence (−) of 100 µg/ml a-Pl8 (Fab).

The capacity of this blocking antibody to potentiate the C5b-9-induced exposure of factor Va binding sites (and thereby increase platelet prothrombinase activity) suggests that a deletion or inactivation of the membrane epitopes recognized by this antibody would also potentiate the procoagulant response of platelets exposed to low levels of complement activation. In this context, we note the following: (i) affected red cells obtained from patients with the acquired stem cell disorder paroxysmal nocturnal hemoglobinuria have been shown to exhibit abnormal sensitivity to lysis by the C5b-9 proteins (22, 23). This has been attributed to the deletion of homologous restriction factor (18, 19). (ii) Platelets obtained from patients with paroxysmal nocturnal hemoglobinuria have been shown to be abnormally sensitive to fluid phase complement activation, and this disorder is characterized by an unusually high risk of venous thrombosis (14, 24, 25). Although we do not as yet know whether platelets obtained from patients with paroxysmal nocturnal hemoglobinuria are also deficient in this cell surface component, our data with a-Pl8 suggest a possible mechanism by which loss of the C5b-9 inhibitory activity from the platelet plasma membrane would directly give rise to the thrombotic episodes that are associated with this disorder.

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Note Added in Proof—Since submission of this manuscript, the full-length sequence of the erythrocyte 18-kDa C5b-9 inhibitor has been reported which establishes its identity to the leukocyte differentiation antigen CD59 (26, 27).

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