Effector Cell Protease Receptor-1, a Platelet Activation-dependent Membrane Protein, Regulates Prothrombinase-catalyzed Thrombin Generation*

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Platelets, through their adhesive properties in concert with their ability to contribute to and regulate thrombin generation, are central to the (patho)physiological processes of hemostasis, thrombosis, and atherosclerosis. In normal hemostasis, subsequent to adherence at a site of vascular injury, platelets play a pivotal role by localizing, regulating, and modulating catalytic activities within the vascular system, which result in thrombin generation and the formation of a platelet-fibrin plug at the injured site. Activated platelets promote the catalysis of two sequential, homologous, and Ca²⁺-dependent reactions in coagulation: the proteolytic conversion of factor X to factor Xa catalyzed by the intrinsic tenase complex, a membrane-bound complex consisting of the nonenzymatic cofactor factor VIIIa and the serine protease factor IXa; and the proteolytic conversion of prothrombin to thrombin catalyzed by the prothrombinase complex, a membrane-bound complex consisting of the nonenzymatic cofactor factor Va and the serine protease factor Xa (1). The high degree of homology between the intrinsic tenase and prothrombinase complexes is underscored by the observations that the serine proteases factor IXa and factor Xa (2) and the procofactors factor VIII and factor V (3) are structurally homologous, as well as the observations that both factor VIIIa (4) and factor Va (5) are essential to complex assembly and function by virtue of their membrane-binding capabilities to form at least part of the receptor for their respective serine proteases at the human platelet surface. Binding of the protein components of each enzymatic complex to an appropriate membrane surface is an obligate requirement for physiologically relevant catalysis (1). It is well known that synthetic phospholipid vesicles support the assembly of both complexes with functional activities equal to those provided by the complexes bound to activated platelets (6). However, activated platelets appear to express high affinity (≤1 × 10⁻⁸ M), saturable binding sites that are specific for the various protein components (5, 7–15). To date, studies detailing the direct binding of factor VIIIa to platelets have not been performed. Furthermore, since platelets contain a large reserve of factor V, which is released and activated to factor Va subsequent to thrombin-induced platelet activation (16), direct factor Va binding measurements are difficult to interpret. In contrast, extensive studies have been performed detailing the binding of factor IXa (7–9) and factor Xa (13–15) to the activated platelet surface. Ahmad and colleagues have demonstrated that factor IXa binds to approximately 600 specific binding sites/activated platelet (7). Prothrombin and factor X, at a 450-fold molar excess, minimally displaced factor IXa binding (15% and 35%, respectively) (7). Similar results have been obtained in analyses of factor Xa binding to platelets. In these studies, factor X, factor IXa, and prothrombin did not compete with factor Xa for

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platelet binding sites (13). These combined studies strongly suggest that factor IXa and factor Xa interact with discrete binding sites on thrombin-activated platelets.

Previous studies from our laboratory using analyses of kinetics of prothrombin activation with both normal and factor V-deficient human platelets indicate that both factors Va and Xa bind to ~3,000 functional sites on the surface of thrombin-activated platelets (17) through formation of a 1:1 stoichiometric complex, governed by a $K_d \approx 10^{-10}$ M and expressing a $k_{cat}/$site $= 30$ s$^{-1}$. Furthermore, factor Xa binding is absolutely dependent upon prebound factor Va (15, 17) indicating that factor Va forms at least part of the receptor for factor Xa at the activated platelet membrane surface.

Altieri and colleagues (18–20) have demonstrated that sub-populations of peripheral blood leukocytes express EPR-1, a 65-kDa membrane-spanning receptor for factor Xa. EPR-1 expressed at the leukocyte surface appears to bind factor Xa specifically in a Ca$^{2+}$-dependent reaction (n = 150,000, $K_d$ = 10–30 nM) (18). More recent studies have shown that EPR-1 is also expressed by human endothelial cells and smooth muscle cells (21), as well as cells expressed during Hodgkin’s disease (22). Since factor Xa binding to EPR-1 expressed by these cell populations occurs independently of factor Va, this binding interaction appears to be important in initiating the intracellular signaling responses that are important in cell proliferation (20, 21) and perhaps the immune response (23).

Most recently we have shown that EPR-1 plays an important role in mediating prothrombinase-catalyzed thrombin generation at the membrane surface of pericytes derived from normal, human brain microvessels (24). In the current study, we demonstrate that expression of EPR-1 plays an important role in regulating prothrombinase complex assembly and function on the surface of activated platelets. Based on published data from several groups of investigators, we propose a model for assembly of the functional complex on the activated platelet membrane in which the platelet receptor for factor Xa assembly into the prothrombinase complex consists minimally of EPR-1, membrane-bound factor Va, and the surrounding phospholipid environment.

**EXPERIMENTAL PROCEDURES**

**Materials—**L-α-Phosphatidyl-L-serine (bovine brain) (PS), L-α-phosphatidylcholine (egg yolk) (PC), and Arg-Gly-Asp-Ser (RGDS) peptide were purchased from Sigma. HEPES was purchased from J.T. Baker. Recrystallized bovine serum albumin was from ICN ImmunoBiologics. The fluorescent α-thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was obtained from Haemalogic Technologies. Phospholipid vesicles composed of 75% (w/w) PC and 25% (w/w) PS were prepared as described (25).

**Isolation and Modification of Proteins—**Coagulation proteins were isolated from human fresh-frozen plasma as described previously (26). Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to and following reduction with β-mercaptoethanol as described by Laemmli (27), and visualized using Cooamassic Blue R-250. Protein concentrations were determined by absorbance at 280 nm using the following molecular weights and extinction coefficients, $\varepsilon_{280}^{\text{nm}}$: factor V, 330,000, 9.6; factor Xa, 50,000, 11.6; prothrombin, 72,000, 14.2; thrombin, 37,000, 17.4; and mouse IgG, 150,000, 14.0. Factor Xa, the specific monoclonal antibody directed against human factor V/Va, α-HFV#2, and a nonspecific mouse IgG2a were labeled with $^{32}$P by the IODGEN transfer technique as described previously (26).

1 The abbreviations used are: EPR, effector cell protease receptor; PS, phosphatidylycerine; PC, phosphatidylcholine; PE, phycoerythrin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; HTA, HEPES-buffered Tyrode’s solution containing bovine serum albumin; dansyl, 5-dimethylaminoanthracene-1-sulfonly; DAPA, dansylarginine N-3-ethyl-1,5-pentanediylamidyl. **Platelet and Megakaryocyte Preparations—**Washed platelets were routinely prepared from anticoagulated whole human blood as detailed previously (28). Platelets were suspended and maintained in 5 mM HEPES-buffered Tyrode’s solution (0.137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.36 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM dextrose), pH 7.4, containing 0.35 mM Ca$^{2+}$/site, serum 0.35 mM and expressing a $k_{cat}/$site $= 30$ s$^{-1}$. Furthermore, factor Xa binding is absolutely dependent upon prebound factor Va (15, 17) indicating that factor Va forms at least part of the receptor for factor Xa at the activated platelet membrane surface.

Megakaryocytes were isolated from human bone marrow aspirates as described previously (29). The megakaryocyte-like cell lines CMK (30), MV (31), HIEL (32), and CHRP-288 (33) were cultured as described previously using a centrifugation through oil technique (37).

**Prothrombinase Activity Measurements—**Prothrombin activation mixtures consisting of purified components were monitored to determine how increasing concentrations of thrombin as agonist affected platelet factor Va release and as expression of platelet prothrombinase activity. Subsequent to platelet isolation and storage, platelet suspensions (2 × 10$^8$/ml) were incubated with varying concentrations of thrombin (2 min, 22 °C, without stirring) followed by addition of the thrombin inhibitor, DAPA (3 μM) to quench the reaction. Expression of platelet prothrombinase activity was monitored as described in detail previously (17, 37). Briefly, subsequent to thrombin-induced platelet activation, equivalent concentrations of factor Va and factor Xa (10 nM) were added and allowed to incubate for 2 min. The reaction was initiated by the addition of 1.39 μM prothrombin. The initial rate of thrombin generation was monitored continuously using the reversible thrombin inhibitor DAPA (3 μM), which displays enhanced fluorescence upon binding to thrombin (38).

Alternatively, platelet releasates were prepared in analogous protocols, and released and activated platelet factor Va was separated from the platelets and assayed for cofactor activity as described in detail previously (39). Briefly, aliquots of platelet factor Va releasates (50 μl containing less than 1 nM factor Va) were assayed for cofactor activity using 20 μM PCPS, 5 nM factor Xa, 1.39 μM prothrombin, and 3 μM DAPA in 20 mM HEPES, 0.15 mM NaCl, 5 mM Ca$^{2+}$, pH 7.4. The factor Va concentration in each sample was determined by comparing the initial rate of thrombin generation with the initial rates obtained using known concentrations of factor Va.

For anti-EPR-1 antibody inhibition studies, washed platelets (1 × 10$^9$/ml in HTA containing 2 mM Ca$^{2+}$) were preincubated with buffer and various dilutions of B6 ascites (1:50–1:10), or a nonspecific mouse ascites (30 μl, 22 °C). Platelets were then activated with thrombin (30 nM, 22 °C) and assayed immediately using 1.39 nM factor Xa and 1.39 μM prothrombin. No exogenous factor Va was added so that platelet-released factor Va was the sole factor Va source. Since small reaction volumes were used, platelet prothrombinase activity (rate of thrombin generated) was determined by discontinuous, two-stage assay as described previously (40).

**Radiolabeled Ligand Binding Assays—**Ligand binding assays were used to determine how increasing concentration of thrombin as agonist affected factor Va and factor Xa binding to the activated platelet surface. Again platelets (1 × 10$^9$/ml in HTA containing 5 mM Ca$^{2+}$) were activated with thrombin as detailed above. Factor Va binding to the activated platelet surface was determined in the presence of 10 nM each added factor Va and factor Xa, and 1.39 μM prothrombin using 100 nM $^{125}$I-α-HFV#2, which in separate experiments was determined to be a saturating concentration. Likewise, direct $^{125}$I-factor Xa binding (10 nM) was determined in the presence of 10 nM Ca$^{2+}$ and 1.39 μM prothrombin. Nonspecific binding/entrapment was determined using an equilibrium concentration of $^{125}$I-labeled isotype-matched monoclonal antibody (IgG2a), or using a 50 μM excess of unlabeled factor Xa or 10 mM EDTA. Specific binding of the various ligands was determined as described previously using a centrifugation through oil technique (37). Similar protocols were used to assess factor Va and factor Xa binding to
FIG. 1. Effects of thrombin-induced platelet activation on prothrombinase complex assembly and function. Washed human platelets (2 × 10^8/ml), suspended in HTA containing 5 mM CaCl₂ (pH 7.4), were activated with thrombin (0–200 nM, 2 min, 22 °C), followed by the addition...
of 3 μM DAPA to inhibit added thrombin. A, in parallel, yet separate reaction mixtures, the binding of factor Va, as assessed using 125I-factor Xa (10 nM, \( \square \)) and 125I-factor Xa (10 nM, \( \square \)), to the platelet surface were directly compared as a function of the concentration of thrombin used to activate the platelets as detailed under “Experimental Procedures.” All binding measurements were made with prothrombin present at 1.59 μM and factor Va and factor Xa (labeled or unlabeled) present at 10 nM each. B, factor Va binding (\( \bullet \)) was also compared with functional platelet factor Va release (\( \lambda \)) from α-granules as detailed under “Experimental Procedures.” C, factor Xa binding (\( \Box \)) was directly compared with expression of platelet prostasinase activity (\( \bigcirc \)), i.e. rate of thrombin generated/platelet at saturating enzyme (10 nM factor Va and factor Xa) and a plasma concentration of substrate (1.39 μM prothrombin) as detailed previously (17, 40) and under “Experimental Procedures.” Thrombin generation was monitored continuously by enhanced fluorescence of DAPA-thrombin complex. All data represent the mean + S.E. (n = 8 different platelet donors).

**Fig. 2.** Western blotting analyses of isolated platelets, isolated megakaryocytes, and megakaryocytic cell lines with anti-EPR-1 monoclonal antibodies. Washed platelets, megakaryocytes, and megakaryocytic cell lines were prepared for Western blotting analyses as described under “Experimental Procedures.” A, EPR-1 was detected in cell lysates derived from platelets (4 separate donors) and megakaryocytes using the anti-EPR-1 monoclonal antibodies B6 (1:50 dilution) and 2F1 (25 μg/ml). Blotting with a nonspecific mouse IgG or mouse ascites used at the same dilutions as B6 indicated that with the exception of the 65-kDa protein all other reactivity was nonspecific (data not shown). B, detection of EPR-1 in cell lysates derived from the megakaryocytic cell lines CMK, Mo7, HEL, and CHRF-288 were compared with platelets, using the anti-EPR-1 monoclonal antibody B6 (1:50 dilution). Blotting with a nonspecific mouse ascites at the same dilution as B6 indicated that, with the exception of the 65-kDa protein all other reactivity was nonspecific (data not shown).

**Fig. 3.** Amplification of EPR-1 mRNA from megakaryocytic cell lines. Total RNA was isolated as described under “Experimental Procedures.” Following reverse transcription using oligo(dT), two successive rounds of PCR were performed using EPR-1 sequence-specific primers: forward T5/27 (161–184, 5′-CGTGTGGAGAACGTGACAGATGTTG-3′) and reverse G5/27 (739–718, 5′-AATGTTGCTGGACCTCATGTTG-3′). cDNA from HEL cells (lane 1) or CMK cells (lane 2) was amplified using EPR-1-specific primers. A control reaction using no template is shown in lane 3. M denotes the molecular size markers in base pairs. Products were run on a 1.25% agarose gel and detected by ethidium bromide staining.
RESULTS AND DISCUSSION
The Binding of Factors Va and Xa to the Activated Human Platelet Surface Is Independently Regulated—Initial experiments were done to determine how the binding of factor Va and factor Xa to human platelets was affected by increasing concentrations of thrombin (0–200 nM) as a platelet agonist. Because platelets contain stores of factor VVa, which contribute significantly to prothrombinase complex assembly and function (16), a radiiodinated monoclonal antibody directed against the light chain of human factor Va (α-HFV#2), which does not affect complex assembly and function and binds with equal avidity to both platelet-released and plasma forms of factor Va, was used to indirectly monitor factor Va/platelet binding interactions. Factor Xa was radiiodinated directly. Incubation of excess 125I-α-HFV#2 with platelets, with or without increasing concentrations of thrombin as agonist, and in the presence of saturating, equimolar concentrations of factor Va and factor Xa (10 nM), indicated that little, if any, factor Va bound to unactivated platelets, whereas maximal binding (~6000 molecules/platelet) was achieved following platelet activation with 0.5 nM thrombin (Fig. 1A). As might be expected, factor Va binding to platelets correlated directly with its release from α-granules (Fig. 1B). However, the amount of platelet-derived factor released was not sufficient to saturate the available platelet binding sites (data not shown), which required addition of exogenous plasma factor Va as seen in Fig. 1A. In marked contrast to observations made with factor Va binding, 20 times more thrombin (10 nM) was required to elicit a platelet activation state resulting in maximal 125I-factor Xa binding (~2800 molecules/platelet; Fig. 1A) in the presence of saturating concentrations of factor Va. These data indicate that saturating the surface of an activated platelet with factor Va alone is not sufficient to mediate a subsequent factor Xa/platelet binding interaction. Furthermore, when maximum binding of each ligand was achieved, approximately twice as much factor Va as factor Xa was bound. Since kinetic studies indicate that a functional enzyme consists of a 1:1 complex (17), only 50% of the bound factor Va molecules will function in this capacity. As would be expected, a factor Xa/platelet binding interaction correlated most closely with the functional expression of thrombin-induced platelet prothrombinase activity (Fig. 1C) achieved with saturating concentrations of both ligands. These combined data indicate that platelet activation is absolutely required for the assembly and function of the prothrombinase complex at the human platelet surface and that the extent of factor Va and factor Xa binding is regulated independently by the agonist concentration used to effect activation, as well as the "level" of activation achieved (Fig. 1). We hypothesize that their independent binding interactions are most likely mediated through discrete platelet membrane receptors.

Flow Cytometric Analyses—Metabolically inhibited or thrombin-activated platelets (5 × 10^7/ml, 50 µl) were incubated with B6 or nonspecific ascites (5 µl, 45 min, 22 °C). Thrombin activated platelets were initially preincubated with the RGDS peptide (1 mM, 1 h, 37 °C) to prevent thrombin-induced aggregation. The platelets were washed by dilution in HTA (1:10) followed by centrifugation (1000 × g, 3 min), and then incubated with 50 µl of goat anti-mouse IgG phycoerythrin (PE) conjugate (1:150 dilution, 45 min, 22 °C). The platelets were again washed in HTA followed by incubation with 50 µl of non-specific mouse IgG (10 min, 22 °C), and an anti-glycoprotein Ib/IIa monoclonal antibody, HP1–1D, conjugated to fluorescein isothiocyanate (FITC) (333 ng, 45 min, 22 °C). The platelets were washed three times and resuspended in HTA. Fluorescence from 1 × 10^4 platelets was analyzed on a Coulter EPICS Elite Flow cytometer (Hialeah, FL).

**Fig. 4.** Immunohistochemical staining of bone marrow aspirates with anti-EPR-1 monoclonal antibodies. Immunohistochemical analyses of bone marrow were performed using the anti-EPR-1 monoclonal antibody 9D4 (5 µg/ml) (A) as detailed under "Experimental Procedures." Identical results were obtained using the anti-EPR-1 primary antibody as a control.
EPR-1 mRNA transcripts in HEL cells. Likewise, as shown in Fig. 3, we confirm the expression of an EPR-1 transcript in HEL cells and demonstrate its presence in CMK cells using RT-PCR with specific EPR-1 primers. A band of the expected molecular size (579 base pairs) was amplified from total mRNA extracted from both cell lines, but not in control incubations in the absence of template cDNA. The cDNA sequences of the PCR products shown were identical to that of the cloned EPR-1 transcript (data not shown). Our ability to demonstrate the coexpression of EPR-1 mRNA and protein in megakaryocyte-like cell lines is consistent with the immunohistochemical staining of normal human bone marrow with a third specific anti-EPR-1 monoclonal antibody, demonstrating that megakaryocytes and platelets were the only cell types to stain positively in situ (Fig. 4).

The expression of EPR-1 on the surface of thrombin-activated platelets was demonstrated using flow cytometric analyses (Fig. 5, A and B). Platelet activation was required for B6 immunoreactivity, since metabolically inhibited platelets, which could not be activated by thrombin addition (Fig. 5A) exhibited dramatically reduced binding of the anti-EPR-1 antibody (<10%) when compared with thrombin-activated platelets (Fig. 5B). Two-color fluorescence was used to demonstrate that the anti-EPR-1 antibody was binding to platelets by analyzing the coexpression of the platelet-specific marker, glycoprotein IIb/IIIa (43). The requirement of platelet activation for EPR-1 membrane expression is consistent, with EPR-1 potentially playing an important role in prothrombinase complex assembly.

Platelet-expressed EPR-1 Regulates Prothrombinase Complex Assembly and Function—Additional flow cytometric analyses demonstrated that preincubation of activated platelets with equimolar concentrations of factors Va and Xa (20 nM) inhibited a B6/platelet interaction by 50% (Fig. 5C), suggesting that B6 and a preformed prothrombinase complex share a common EPR-1 epitope. This hypothesis is consistent with the observations of Ambrosini and Altieri (35), who epitope-mapped B6 to the factor Xa binding site on EPR-1 expressed by transfected Chinese hamster ovary cells and human umbilical vein endothelial cells.

A role for platelet EPR-1 expression in regulating factor Xa binding and the function of the platelet prothrombinase complex was confirmed by addition of B6 to platelets prior to their thrombin-induced activation and assay for prothrombinase activity. In the absence of added plasma factor Va (i.e. relying on platelet-released factor Va) and the presence of limiting factor Xa (0.1 nM), the expression of platelet prothrombinase activity was inhibited by B6 in a dose-dependent manner (Table I), whereas control antibodies were without affect. The inhibitory effect of B6 on the expression of the activity of the prothrombinase complex was variable and dependent on the platelet donor. Furthermore, 100% inhibition was never achieved. Our inability to effect total inhibition of platelet prothrombinase activity with B6 is most likely due to competition of a factor Va/factor Xa complex for EPR-1 since flow cytometric analyses clearly demonstrated that the preformed complex effectively precludes, thereby limiting, anti-EPR-1 antibody binding (see Fig. 5C).

**Fig. 5.** EPR-1 expression on activated platelets analyzed by flow cytometric techniques. Metabolically inhibited (A) and thrombin-activated (B) platelet suspensions were analyzed by two-color flow cytometry to measure the coexpression of glycoprotein IIb/IIIa (FITC) and EPR-1 (PE) as detailed under “Experimental Procedures.” Horizontal axes, FITC fluorescence related to specific binding of the anti-glycoprotein IIb/IIIa monoclonal antibody, HP1–1D (333 ng). Vertical axes, PE fluorescence related to specific binding of the anti-EPR-1 monoclonal antibody, B6 (1:10 dilution). In each scatter plot, the upper right quadrant represents positive staining for both EPR-1 and GPIIb/IIIa, the lower right quadrant represents positive staining for GPIIb/IIIa only, the upper left quadrant represents positive staining for EPR-1 only, and the lower left quadrant represents double negatives. The values shown in the upper left quadrant of each scatter plot indicate the number of PE (B6) positive events. In some experiments (C), activated platelets were incubated with 20 nM each factor Va and factor Xa prior to the addition of B6.
An EPR-1 contribution to prothrombinase complex assembly and function was supported further by kinetic analyses of prothrombin activation on various megakaryocytic cell lines. These data indicate that CMK cells generate thrombin at an initial rate equal to 2.6 ± 0.72 × 10^6 molecules thrombin/min/cell, whereas the initial rate of thrombin generation expressed by CHRF-288 cells equals 3.6 ± 0.35 × 10^6 molecules thrombin/min/cell. Assuming that the prothrombinase complex assembled on these cell lines expresses the same catalytic efficiency/site as that expressed by platelets (30 s⁻¹) (17), then CMK cells possess approximately 70 times more sites/cell than CHRF-288 cells (1.4 × 10^5 versus 2.0 × 10^3 sites/cell, respectively). While it is difficult to quantify proteins by Western blotting analyses, comparison of the blots of the megakaryocytic cells (see Fig. 2B) indicates that CMK cells express substantially more EPR-1 than CHRF-288 cells, on a per cell basis, which is consistent with the functional data. Likewise, HEL cells, which also expressed substantially more EPR-1 than CHRF-288 cells by Western blotting analyses, were shown to bind 1.2 ± 0.05 × 10^5 molecules of ^125I-factor Xa in the presence of added factor Va (data not shown). Thus, HEL cells appear to express EPR-1 sites/cell at a level nearly identical to that of CMK cells and approximately 60 times greater than that of CHRF-288 cells.

Collectively, these data indicate that activated human platelets and their precursors express EPR-1 on their membrane surface. Functional and immunological studies demonstrate that this platelet molecule forms at least part of a membrane receptor for factor Xa, which mediates its assembly in the prothrombinase complex. These observations document the first description of a membrane protein, which regulates coagulant enzyme complex assembly on the surface of human activated platelets.

Our data, when considered with that of other investigators (12–15), suggest that EPR-1 is most likely only one component of a complex platelet receptor for the prothrombinase complex. We also observed that saturation of the platelet factor Va binding sites, in the absence of factor Xa, inhibited B6 binding 45% (data not shown) indicating that platelet-bound factor Va may sterically hinder a B6 interaction with platelet EPR-1. Interestingly, Nesheim and colleagues (44) have detected a 65-kDa protein in platelet lysates using Sepharose-immobilized factor Va. These data, combined with our observations, suggest that platelet EPR-1 and bound factor Va may form a heterodimer that functions as a factor Xa receptor. Furthermore, several studies using synthetic vesicles of defined phospholipid content have demonstrated that anionic phospholipids are important for both the assembly and function of the prothrombinase complex (45, 46). The exposure of anionic phospholipids, primarily phosphatidylserine, subsequent to agonist-induced platelet activation has likewise been correlated to the expression of prothrombinase activity at the platelet surface (47). In support of this notion, Jenny and Mann (10) demonstrated that the anionic phospholipid-binding protein, annexin V, inhibited assembly and function of the intrinsic tenase and prothrombinase complexes on the activated platelet surface. However, cofactor-cofactor competition studies indi-

### Table I

**Inhibition of platelet prothrombinase complex function with the anti-EPR-1 monoclonal antibody, B6**

| Experiment               | Prothrombinase activity | nm thrombin/min |
|-------------------------|-------------------------|-----------------|
| Control ascites         | 159.9 ± 27.3            | 104 ± 3.8       |
| 1:50 B6                 | 129.4                   | 32.9            |
|                         | (80.9)°                 | (31.5)°         |
| 1:25 B6                 | 93.4                    | 33.0 ± 3.0      |
|                         | (58.4)°                 | (88.2)°         |
| 1:10 B6                 | ND°                     | 33.2 ± 4.0      |
|                         | (65.2)°                 |                 |

| Experiment               | Prothrombinase activity | nm thrombin/min |
|-------------------------|-------------------------|-----------------|
| Control ascites         | 20.8                    | 17.7 ± 7.4      |
| 1:50 B6                 | 20.8                    | 32.6 ± 0.6      |
|                         | (19.9)°                 | (47.3)°         |

° % prothrombinase activity remaining.

*Not determined.*

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**Fig. 6. Hypothetical model for the prothrombinase complex receptor expressed by activated platelets.** Unactivated platelets are not able to support the binding of either factor Va or factor Xa to their membrane surface. Subthreshold stimulation of platelets results in platelet factor V/Va release and expression of a putative factor Va receptor, which is competent to bind both plasma and platelet factor Va. Saturation of the platelet surface with factor Va (~6000 sites/cell) is not sufficient to facilitate factor Xa binding; however, maximal platelet stimulation results in expression of EPR-1 to effect maximal factor Xa binding (~2800 sites/cell). In addition to EPR-1, maximal factor Xa binding is also regulated by prebound factor Va and anionic phospholipid exposed as a result of platelet activation. The factor Va receptor and EPR-1 would form discrete, but overlapping binding sites on the platelet membrane. Although not indicated here, another membrane component, in addition to bound factor Va and factor Xa, may be mediating prothrombin binding (49).
culated that the binding of factor Va and factor VIIIa to activated platelets showed more specificity than on synthetic phospholipid bilayers (10), suggesting that activated platelets may express specific factor Va and factor VIIa receptors as well.

More recently, London and colleagues (48) demonstrated that annexin V differentially inhibited factor IXa binding and function on activated platelets when compared with a model system comprising synthetic PC vesicles containing PS. In addition, in the absence of factor VIIa, the affinity of factor IXa for the activated platelet surface was 25-fold higher than its affinity for synthetic phospholipid bilayers, suggesting that components in addition to phospholipids in the activated platelet membrane govern factor VIIa-independent factor IXa binding. Studies done in the presence of annexin V indicated that synthetic phospholipid bilayers did not mimic activated platelets with regard to binding of factor IXa, as determined by equilibrium binding studies and kinetic studies of factor X activation. Their collected data are consistent with the existence of a binding site for factor IXa on the activated platelet surface comprising both phospholipid and protein moieties. These combined observations indicate that other membrane components, in addition to anionic phospholipids, regulate procoagulant protein binding to cellular membrane surfaces.

Model of Platelet Activation-dependent Prothrombinase Complex Assembly and Function—While it has been clearly shown that factor Va functions as at least part of the receptor for factor Xa on platelets (5, 17), our current functional binding studies indicate that prebound factor Va alone is not sufficient to mediate factor Xa binding to the activated platelet membrane. The current study also demonstrates that expression of EPR-1 or an EPR-1-like molecule by activated platelets is involved in the assembly of a functional prothrombinase complex at their membrane surface. We propose a hypothetical model for the expression, assembly, and function of the prothrombinase complex receptor, which is consistent with all known data (12–15, 17) (Fig. 6). In this model, neither factor Va nor factor Xa binds to anactivated human platelets. Subsequent to agonist-induced activation, binding of each ligand is distinct and can be correlated with the extent of platelet activation. A subthreshold level of activation results in release of functional factor Va from α-granules, which parallels expression of platelet membrane binding sites (i.e. a putative receptor) for either platelet-released or plasma forms of factor Va. Even when maximal factor Va binding is achieved, the bound factor Va is not competent to support factor Xa binding. Rather, factor Xa binding is only achieved with additional platelet activation such that EPR-1, in proximity to bound factor Va, is expressed in a conformation where it is able to support a factor Xa binding interaction. Furthermore, only one-half of the bound factor Va molecules interact with EPR-1 to form a competent factor Xa binding site. Factor Xa binding to the activated platelet is regulated further by, in addition to EPR-1 and bound factor Va, anionic phospholipid made available subsequent to platelet activation. Thus, the expression of a supramolecular membrane receptor for the prothrombinase complex by platelets regulates their ability to effectively generate thrombin and effect formation of a platelet-fibrin clot at the site of vascular injury.

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Effector Cell Protease Receptor-1, a Platelet Activation-dependent Membrane Protein, Regulates Prothrombinase-catalyzed Thrombin Generation

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