Role of the C2 Domain of Factor VIIIa in the Assembly of Factor-X Activating Complex on the Platelet Membrane†

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ABSTRACT: Optimal rates of factor X (FX) activation require binding of factor IXa (FIXa), factor VIII(a) [FVIII(a)], and FX to activated platelet receptors. To define the FVIIIa domains that mediate platelet interactions, albumin density gradient washed, gel-filtered platelets (3.5×10^8/mL) activated by the thrombin receptor peptide, SFLLRN (25 μM), were incubated with ^{125}I-labeled FVIII C2 domain, or ^{125}I-FVIIIa, or ^{125}I-FVIII$_{(LC)}$, or peptides from the C2 domain region, with or without anti-C2 domain monoclonal antibodies (MoAb), ESH4 or ESH8. FVIIIa ($K_d \sim 1.7$ nM), FVIII$_{(LC)}$ ($K_d \sim 3$ nM), and the C2 domain ($K_d \sim 16$ nM) all interacted with ∼700−800 binding sites/platelet. Unlike FVIIIa, the C2 domain did not respond to the presence of excess EGR−FIXa (45 nM) and FX (1.5 μM) with enhanced binding stoichiometry and affinity. Both the MoAb ESH4 and a synthetic peptide corresponding to FVIII residues 2303−2332 (epitope for FVIII MoAb, ESH4) inhibited FVIIIa binding to platelets, whereas MoAb ESH8 and a C2 domain peptide corresponding to residues 2248−2285 (epitope for the FVIII MoAb, ESH8) failed to inhibit FVIIIa binding. Thus, a major platelet-binding site resides within residues 2303−2332 in the C2 domain of FVIIIa, and an additional site within residues 2248−2285 increases the stoichiometry and affinity of FVIIIa binding to activated platelets only in the presence of FIXa and FX but does not directly mediate FVIIIa binding to the platelet surface.

Factor VIII (FVIII)[1] is synthesized as a single polypeptide chain (∼300 kDa) containing 2332 amino acids (*1*, *2*) comprising a discrete domain structure, A1−a1−A2−a2−B−a3−A3−C1−C2, where a1, a2, and a3 indicate short spacers (∼30 aa) composed of acidic regions containing clusters of Asp and Glu residues (*3*). FVIII contains three "A" domains, homologous to regions in factor V (FV) and ceruloplasmin, a large heavily glycosylated "B" domain and two "C" domains that are homologous to those within FV and discoidin 1, a lectin (*4*, *5*). During the proteolytic activation of FVIII, the B domain is excised, leading to a noncovalent association of three polypeptide chains. Thrombin activation of FVIII is initiated by cleavage in the A3 domain at residue 1689 (*6*), which causes the release of FVIII from the vonWillebrand factor and is completed by cleavages in the heavy chain at residue 372 (A1 and A2 junction) and at residue 740 (A2 and B junction). FVIIIa is a trimer consisting of A1 and A2 domains of the heavy chain and the A3−C1−C2 subunits of the light chain (*5*, *7*, *8*). The activity of FVIIIa is transient due to rapid dissociation of the A2 domain from the remainder of the molecule (*5*−*8*).

In an attempt to define the molecular domains within the cofactor, FVIIIa, that bind to platelets and promote assembly of the F−X activating complex, we have recently demonstrated that both procofactor (FVIII) and active cofactor (FVIIIa) bind to platelets with enhanced affinity in the presence of the enzyme (EGR−FIXa) and the substrate (FX), thus, emphasizing the validity of a three-receptor complex on the platelet surface (*9*−*12*). The presence of the A2 domain increases both the affinity and the stoichiometry of FVIIIa binding to activated platelets (*9*). Earlier studies by Fay et al. (*8*, *13*, *14*) indicated that the A2 subunit of FVIIIa markedly increases the catalytic activity (i.e., k_{cat} of FIXa-catalyzed FX activation) by enhancing the reaction rate ∼100-fold. Furthermore, reconstitution of heterotrimeric FVIIIa from the isolated A2 subunit and A1/A3−C1−C2 dimer is also enhanced severalfold in the presence of FIXa and phospholipid (*15*). The addition of both A1 and A2 domains further enhances the affinity of FVIIIa binding to activated platelets in the presence of EGR−FIXa and FX (*9*), emphasizing that FX activation is a platelet-receptor-mediated process tightly coupled to receptor occupancy by FIXa, FVIIIa, and FX, and is dependent upon multiple protein−protein contacts. More recent studies using functional assays (*16*) further indicated that A1 and A2 subunits of FVIIIa synergistically stimulate FIXa catalytic activity yielding an overall increase in k_{cat} of over 1000-fold compared to FIXa alone. Taken together, our recent observa-

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[1] Abbreviations: Factor VIII (FVIII); factor IX (FIX); factor X (FX); light chain (LC); recombinant factor VIII (rFVIII); monoclonal antibody (MoAb); thrombin receptor agonists peptide (TRAP); washed gel-filtered platelets (wGFP).

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tions (*9*) from equilibrium binding studies and those of Fay et al. (*14*, *16*) from FX activation studies are consistent with the hypothesis that the primary mechanism for decay of the F−X activating complex under physiological conditions is the dissociation of the A2 subunit (*17*−*19*).

In this study, we aim to define the FVIII domains that mediate platelet interactions with FVIIIa and to determine the locus within the platelet membrane of the FVIIIa binding site. Recent studies indicate that the FVIIIa C2 domain interacts directly with negatively charged phospholipid membranes (*20*−*25*), whereas the binding of FV to phospholipid membranes is mediated by both the A3 domain (*26*) and the C2 domain (*27*) and is influenced by glycosylation within the C2 domain (*28*). In contrast, FVIII binding to phospholipid membranes requires more phosphatidylserine per binding site than does FV (*29*) and is independent of glycosylation. However, FVIII and FV compete with one another for phospholipid binding (*30*), whereas FV does not displace FVIII from platelet receptors, nor does it inhibit FX activation by FIXa in the presence of platelets and FVIIIa (*9*, *31*), implying a highly specific, receptor-mediated interaction of FVIIIa with activated platelets. Therefore, since no published information is currently available, we have defined the structural domains within FVIIIa that mediate its interaction with platelet membrane receptors. In this paper, we have characterized the role of the C2 domain of FVIII in the assembly of the F−X activating complex on human platelets.

EXPERIMENTAL PROCEDURES

Reagents. Carrier-free Na ^{125}I was obtained from Amersham Corp. (Arlington Heights, IL). The thrombin receptor agonist peptide (TRAP), SFLLRN-amide, was synthesized as previously described (*32*) using 9-fluorenyl-methyloxycarbonyl (FMOC) chemistry on an Applied Biosystem 430A Synthesizer and purified by reverse-phase high performance lipid chromatography (HPLC) to greater than 99% homogeneity by the Protein Chemistry Laboratory (Dr. John Lambris, Director) of the University of Pennsylvania (Philadelphia, PA). Methyl silicon oil (DC-200) and Hi phenyl silicon oil (125 DC-550) were purchased from William F. Nye, Inc. (Fairhaven, MA). All other reagents and chemicals used were the same as previously reported (*9*) and were obtained from Sigma Chemical Co., Aldrich Chemical Co. (Milwaukee, WI), or Calbiochem-Behring Corp. (San Diego, CA) and were the highest grade commercially available.

Proteins. Human FIX, FIXa, EGR−FIXa, FX, and α-thrombin (2800 NIH U/mg) were purchased from both Enzyme Research Laboratories Inc. (South Bend, IN) and Hematologic Technologies, Inc. (Essex Junction, VT). Highly purified recombinant FVIII (rFVIII, >4000 U/mg) was the generous gift of Baxter Health Care Corp. (Duarte, CA). The intact recombinant C2 (rC2) domain of FVIII expressed in *Pichia pastoris* cells was kindly provided by Drs. Kazuo Fujikawa and Kathleen P. Pratt, Department of Biochemistry, University of Washington (Seattle, WA). Anti-C2 monoclonal antibody (MoAb), ESH4, which is directed against the light chain, recognizes residues 2303−2332, and inhibits phospholipid binding, was obtained from American Diagnostica Inc. (Greenwich, CT). Similarly, anti-C2 MoAb ESH8, which is directed against the C2 domain, recognizes residues 2248−2285, and does not compete with ESH4 for FVIII binding, was also obtained from American Diagnostica Inc. The FVIII C2 domain peptides corresponding to residues 2303−2332 and 2248−2285, detailed in Table 1, were designed and synthesized according to conventional solid-phase procedures on an Applied Biosystems 430A Peptide synthesizer as previously described (*33*). A reverse-D analogue or "retro-inverso peptide" (*34*−*36*) corresponding to the reverse sequence of the C2 domain peptide, 2303−2332, was synthesized in a similar fashion utilizing D-amino acids instead of L-amino acids as used for the native peptide. Subunits of FVIII were prepared by the method described by Fay et al. (*8*) with slight modifications as described previously by us (*9*).

Radiolabeling of Proteins. FVIII was radiolabeled using the Bolton-Hunter reagent (*37*) to a specific radioactivity of ~2 × 10^6 cpm/μg while retaining virtually 100% of its specific biological activity as described previously (*9*). The ^{125}I FVIII C2 domain was prepared by the IODO-GEN

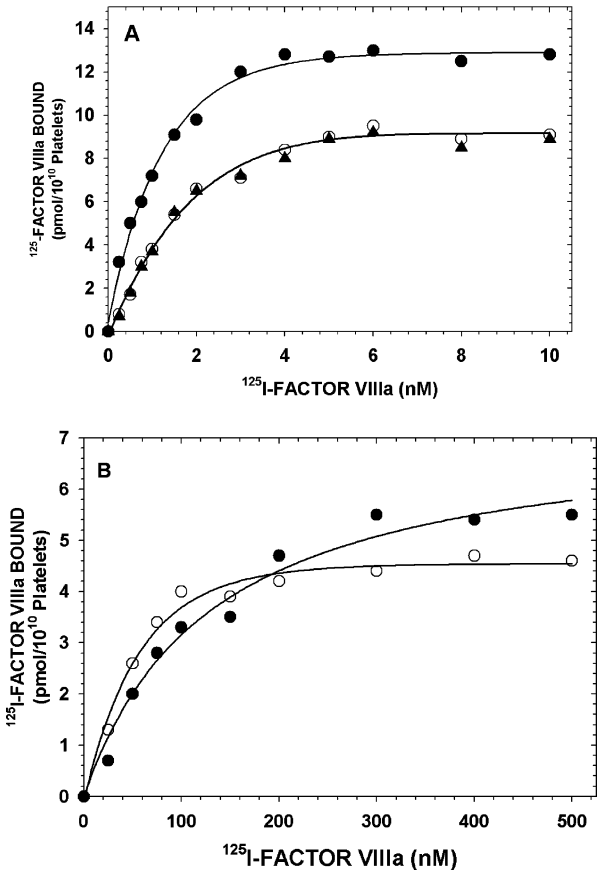

FIGURE 1: Effect of C2 domain antibodies on the specific binding of ^{125}I-factor VIIIa to activated human platelets in the presence of EGR−factor IXa and factor X. (A) Washed gradient gel-filtered platelets (wGFP) (3.5 × 10^8/mL) were incubated with thrombin receptor peptide, SFLLRN (25 μM), $CaCl_2$ (5 mM), and ^{125}I-FVIIIa in the absence (○) and in the presence (●) of EGR−FIXa (45 nM) and FX (1.5 μM). Closed triangles (▲) represent the binding of FVIIIa in the presence of C2 domain MoAb ESH8 (200 nM), in the presence of EGR−FIXa and FX. (B) Specific binding of ^{125}I-FVIIIa to activated human platelets in the presence of C2 MoAb ESH4 (200 nM) in the absence (○) and in the presence (●) of EGR−FIXa and FX. The plotted results are the mean of triplicate observations from four separate experiments, using platelets from four different donors. The SEM values are omitted for clarity but were generally <5% of the mean values and in no instance >10%.

Table 1: Synthetic Peptides Derived from the C2 Domain of Factor VIII

synthetic peptides	factor VIII C2 domain residues
residues 2303−2332	TRYLRIHPQSWVHQIALRMEVLGCEAQDLY
reverse-D analogue (2303−2332)	YLDQAECGLVEMRLAIQHVWSQPHIRLYRT
scrambled peptide (2303−2332)	YIGPCRALMTDVAHLIRLSWEVQHLEQYRQ
C2 peptide (2248−2285)	VKSLLTSMYVYEFLISSSQDGHQWTLFFQNGKVKVFQG

method (*38*) and had a specific radioactivity of ~2.5 × 10^6 cpm/μg.

Platelet Isolation and Binding Studies. Albumin density gradient washed gel-filtered platelets (wGFP) were isolated from human venous blood by a modification (*9*, *32*) of the method of Walsh et al. (*39*). In a typical binding experiment, wGFP (3.5 × 10^8/mL) in Ca^{2+}-free HEPES-Tyrode's buffer, pH 7.4, was incubated at 37 °C in a 1.5 mL Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled proteins, $CaCl_2$, platelet agonists, a thrombin receptor (PAR-1) activation peptide, SFLLRN-amide, or with 1.0 nM human α-thrombin, and other proteins. After incubation, aliquots (100 μL) were removed and centrifuged through a mixture of silicon oils as described previously (*40*). The data were analyzed, and the number of binding sites and K_d values were calculated from the means of six independent determinations, each done in duplicate using a Macintosh G4 Apple computer (Cupertino, CA) and the ligand program as modified by G. A. McPherson (Elsevier Science Publishers BV, The Netherlands).

RESULTS

Effect of FVIII C2 Domain Antibodies on FVIIIa Binding to Activated Platelets. To define the specific domains of FVIIIa that are responsible for platelet-receptor-mediated interaction, we studied the effects of C2 domain antibodies on the specific binding of ^{125}I-FVIIIa to activated human platelets in the presence of the enzyme (EGR−FIXa) and the substrate (FX). For this purpose, albumin density gradient washed and gel-filtered platelets (wGFP) were incubated with ^{125}I-FVIIIa in the presence or in the absence of anti-C2 domain monoclonal antibodies and $CaCl_2$ (5 mM), in the absence and in the presence of EGR−FIXa (45 nM) and FX (1.5 μM). Figure 1A represents the specific binding of ^{125}I-FVIIIa to activated platelets in the absence (○) and in the presence (●) of EGR−FIXa and FX. The presence of both the enzyme (EGR−FIXa) and the substrate (FX) increases both the number of binding sites (from 770 ± 133 to 1225 ± 200 sites per platelet) and the affinity of binding sites (K_d from 1.7 ± 0.15 nM to 0.8 ± 0.04 nM), Table 2. The anti-C2 MoAb, ESH4 (200 nM), which recognizes residues 2303−2332 and inhibits FVIII binding to phospholipids, partially inhibited ^{125}I-FVIIIa interaction with activated platelets (Figure 1B) to yield a stoichiometry and affinity of ~450 ± 75 sites per platelets; K_d ~90 ± 31 nM in the presence of both EGR−FIXa and FX or in the absence of the enzyme and the substrate (~350 ± 24 sites per platelet; K_d ~ 66 ± 15 nM). In contrast, ESH8 (200 nM), which recognizes residues 2248−2285 in the C2 domain of FVIII, does not inhibit FVIIIa−platelet interaction but interestingly abolished both the enhanced stoichiometry and the affinity of FVIIIa binding to platelets observed in the presence of EGR−FIXa and FX (compare lines 5 and 6 with lines 1 and 2 in Table 2).

Table 2: Effects of C2-Domain Antibodies on the Specific Binding of ^{125}I-Factor VIIIa to Activated Human Platelets

ligand	EGR−FIXa + FX	no. of sites/ platelet	apparent K_d (nM)
^{125}I-factor VIIIa	absent	770 ± 133	1.7 ± 0.15
	present	1225 ± 200	0.8 ± 0.04
^{125}I-FVIIIa + ESH4[a]	absent	350 ± 24	66 ± 15
	present	450 ± 75	90 ± 31
^{125}I-FVIIIa + ESH8[b]	absent	800 ± 72	1.5 ± 0.13
	present	850 ± 110	1.5 ± 0.12
^{125}I-FVIIIa$_{(LC)}$	absent	760 ± 70	3.0 ± 0.21
	present	1190 ± 160	2.0 ± 0.17

[a] ESH4 = recognizes aa 2303−2332; inhibits phospholipid binding. [b] ESH8 = recognizes aa 2248−2285; does not inhibit phospholipid binding.

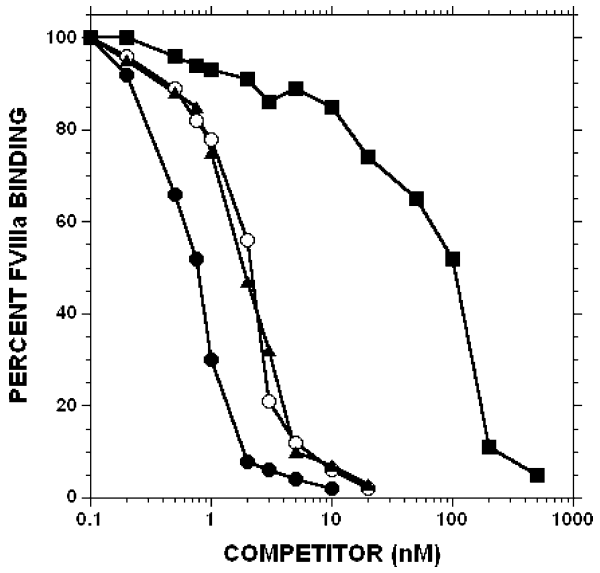

FIGURE 2: Inhibitory effects of C2 domain antibodies on ^{125}I-FVIIIa binding on activated human platelets in the absence and in the presence of EGR−FIXa and FX. Washed gradient gel-filtered platelets (wGFP) (3.5 × 10^8/mL) were incubated with thrombin receptor peptide, SFLLRN (25 μM), $CaCl_2$ (5 mM), and ^{125}I-FVIIIa (2 nM) and increasing concentrations of unlabeled FVIIIa in the absence (○) and in the presence (●) of EGR−FIXa (45 nM) and FX (1.5 μM). The inhibitory effects of C2 domain antibodies [ESH4 (■) or ESH8 (▲) at 200 nM] in the presence of EGR−FIXa and FX are represented by closed symbols. The plotted results are means of triplicate observations from four separate experiments, using platelets from four different donors. The SEM values are omitted for clarity but were generally <5% of the mean values and in no instance >10%.

To confirm and extend these studies, we carried out competition studies with unlabeled FVIIIa in the presence or in the absence of the same two anti-C2 domain monoclonal antibodies (Figure 2). As observed in the direct binding studies (Figure 1A,B), the presence of EGR−FIXa (45 nM) and FX (1.5 μM) decreased the concentration (IC_{50}) of unlabeled FVIIIa required to displace ^{125}I-FVIIIa ~5-fold. The anti-C2 domain MoAb, ESH4, was a potent inhibitor of FVIIIa binding to activated platelets, increasing the IC_{50}

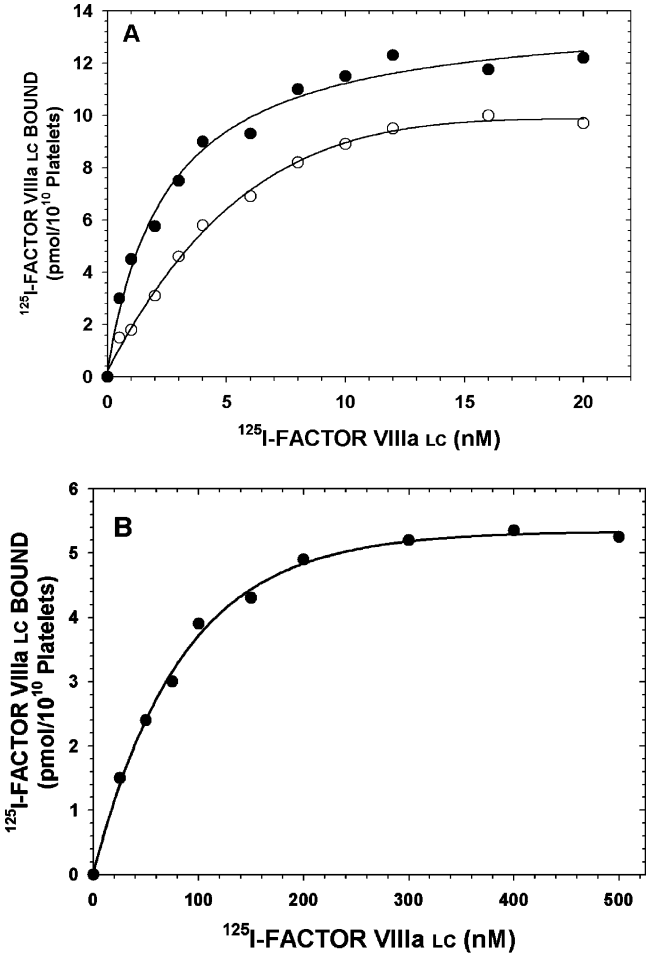

FIGURE 3: (A) Specific binding of ^{125}I-labeled FVIIIa light chain (LC) to activated human platelets in the absence and in the presence of EGR−FIXa and FX. Washed gel-filtered platelets (wGFP) (3.5×10^8/mL) were incubated for 20 min at 37 °C with SFLLRN (25 μM), $CaCl_2$ (5 mM), and ^{125}I-labeled FVIIIa$_{(LC)}$ in the absence (○) and in the presence (●) of EGR−FIXa (45 nM) and FX (1.5 μM). (B) Specific binding of ^{125}I-labeled FVIIIa$_{(LC)}$ to activated platelets in the presence (●) of C2 MoAb ESH4 (200 nM) in the presence of EGR−FIXa and FX. The plotted results are the means of triplicate observations from four separate experiments using platelets from four different donors. The SEM values are omitted for clarity but were generally <5% of the mean values and in no instance >10%.

of unlabeled FVIIIa to ∼110 nM. In contrast, and in agreement with the results of the direct binding studies, the MoAb ESH8 abolished the enhanced affinity of FVIIIa binding to activated platelets observed in the presence of EGR−FIXa and FX. As shown in Figure 3 and Table 2, by using ^{125}I-FVIIIa$_{(LC)}$ (i.e., 72 kDa A3−C1−C2 domain obtained by protease digestion), we were able to show saturable and reversible equilibrium binding of the FVIII LC to platelets ($K_d \sim 3.0 \pm 0.21$ nM; ∼760 ± 70 sites per platelets) in the absence and in the presence of EGR−FIXa and FX ($K_d \sim 2.0 \pm 0.17$; ∼1,190 ± 60 sites per platelet). The presence of ESH4 (200 nM) in binding assays partially inhibited (∼60%) FVIII$_{(LC)}$ binding ($K_d \sim 65 \pm 21$ nM; ∼500 ± 65 sites per platelets) (Figure 3B). These data provide strong evidence for the presence of (1) a major platelet-receptor-mediated binding site (within residues 2303−2332) in the C2 domain; and (2) an additional binding site (within residues 2248−2285) in the C2 domain that increases the stoichiometry and affinity of FVIIIa binding to activated

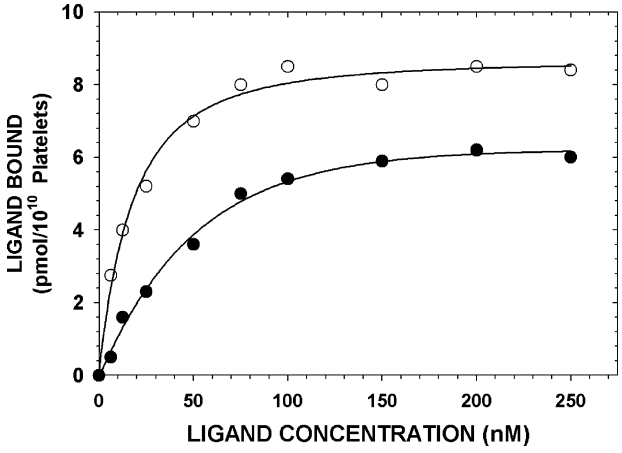

FIGURE 4: Specific binding of ^{125}I-FVIIIa rC2 domain and ^{125}I-FVIIIa C2 peptide to activated human platelets in the presence of EGR−FIXa and FX. Washed gel-filtered platelets (wGFP) (3.5×10^8/mL) were incubated for 20 min at 37 °C with SFLLRN (25 μM), $CaCl_2$ (5 mM), and ^{125}I-FVIIIa rC2 domain (○) or ^{125}I-FVIIIa C2 peptide (●) in the presence of EGR−FIXa (45 nM) and FX (1.5 μM). The plotted results are the means of triplicate experiments using platelets from four different donors. The SEM values are omitted for clarity but were generally <5% of the mean values and in no instance >10%.

platelets observed in the presence of FIXa and FX. Moreover, the isolated FVIII light chain (A3−C1−C2) binds to the same number of platelet sites in the presence and in the absence of EGR−FIXa and FX as does FVIIIa, albeit with slightly diminished affinity.

The Effects of the C2 Domain of FVIIIa and Synthetic Peptides on FVIIIa Binding to Activated Platelets. To further define the role of the C2 domain and various regions within the C2 domain of FVIII that are responsible for the assembly of the F−X activating complex on platelet membranes, we used the rC2 domain of FVIII expressed in *Pichia pastoris* cells and several peptides from the C2 domain of FVIII corresponding to residues 2303−2332 and 2248−2285. For this purpose, we labeled the rC2 domain (18 kDa) and FVIII C2 domain peptides corresponding to residues 2303−2332 and 2248−2285 with ^{125}I and then carried out equilibrium binding and competition studies to determine the stoichiometry, affinity, and inhibition constants of C2 domain interaction with platelet receptors. The isolated rC2 domain interacted with ∼700−800 binding sites per platelet with a $K_d \sim 16.0 \pm 2.5$ nM in the absence of EGR−FIXa and FX and failed to respond to the presence of excess EGR−FIXa (45 nM) and FX (1.5 μM) with enhanced binding stoichiometry and affinity ($K_d \sim 14.0 \pm 1.8$ nM) (Figure 4 (○) and Table 3). In contrast, both the affinity and the stoichiometry of ^{125}I-FVIIIa binding to activated platelets were enhanced in the presence of EGR−FIXa and FX (Table 3).

Competition studies with synthetic peptides corresponding to FVIII C2 domain residues 2303−2332 (comprising the epitope of FVIII MoAb ESH4) in the presence of EGR−FIXa and FX (Figure 5 and Table 4) showed inhibition of FVIIIa binding to activated platelets with a K_i value of 25 nM compared with native FVIIIa ($K_i \sim 1.5$ nM). In contrast, a C2 domain peptide corresponding to residues 2248−2285 (comprising the epitope for the FVIII MoAb ESH8) failed to inhibit FVIIIa binding significantly (Figure 5 and Table 4). Addition of these two peptides together at equimolar concentrations still resulted in inhibition of FVIIIa binding

Table 3: Binding Constants for Normal Factor VIIIa, Recombinant C2 Domain, and C2 Peptide

ligand	EGR−FIXa +FX	no. of sites/ platelet	apparent K_d (nM)
^{125}I-factor VIIIa	absent	750 ± 80	2.0 ± 0.18
	present	1280 ± 145	0.8 ± 0.05
^{125}I-rC2 domain	absent	800 + 66	16.0 + 2.5
	present	700 ± 65	14.0 ± 1.8
^{125}I-C2 peptide[a]	absent	ND[b]	ND
	present	666 ± 70	45 ± 8.0

[a] Corresponding to FVIII residues 2303−2332 (comprising the epitope for the FVIII MoAb, ESH4) (see Table 1). [b] ND = not determined.

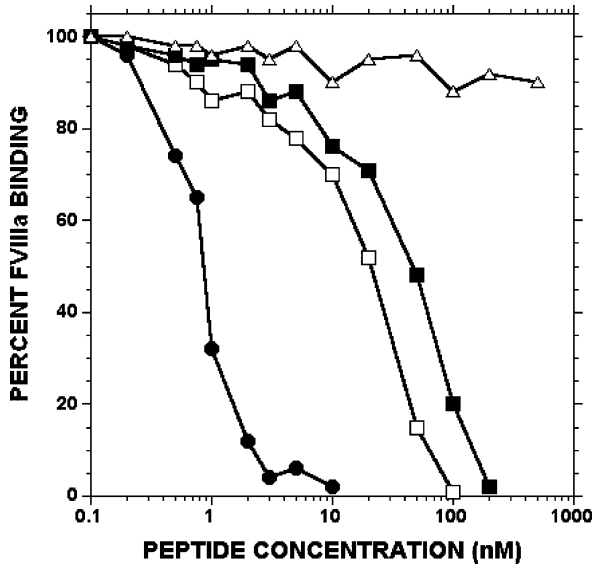

FIGURE 5: Analogue competition of radiolabeled FVIIIa binding to activated human platelets in the presence of EGR−FIXa and FX. Washed gel-filtered platelets (wGFP) (3.5×10^8/mL) were incubated for 20 min at 37 °C with SFLLRN (25 μM), $CaCl_2$ (5 mM), and ^{125}I-FVIIIa (4 nM) along with the increasing concentrations of synthetic peptides derived from the C2 domain of FVIIIa (Table 1). As shown in the figure, C2 peptide (2303−2332) (□) and reverse-D analogue (2303−2332) (■) inhibited FVIIIa binding to platelets as compared to the competition of unlabeled FVIIIa (●). Scrambled peptide (2303−2332) (△) showed no competition. The plotted results are means of triplicate experiments using platelets from four different donors. The SEM values are omitted for clarity but were generally <5% of the mean values and in no instance >10%.

Table 4: Effects of Factor VIIIa and Synthetic Peptides Corresponding to Factor VIII Residues in C2-Domain

competing factor VIIIa or C2-domain peptide	K_i (M) EGR−factor IXa + factor X	
	present	absent
factor VIIIa	1.5×10^{-9}	2.9×10^{-9}
C2-peptide[a] (2303−2332)	25×10^{-9}	35×10^{-9}
reverse-D analogue[a] (2303−2332)	49×10^{-9}	60×10^{-9}
scrambled peptide[a] (2303−2332)	2.5×10^{-3}	not determined
C2-peptide[b] (2248−2285)	NE[c]	NE

[a] Comprising the epitope for the FVIII MoAb ESH4 (see Table 1). [b] Comprising the epitope for the FVIII MoAb ESH8 (see Table 1). [c] NE = no effect at concentrations up to 10^{-2} M.

to platelets ($K_i \sim 45$ nM) (data not shown). These equilibrium binding and peptide competition studies are entirely consistent with the results of our studies with monoclonal antibodies as described above (Table 2, Figures 1A,B and 2), suggesting the presence of a major platelet-receptor-mediated binding site (within residues 2303−2332), comprising the epitope for MoAb ESH4 in the C2 domain of FVIIIa. An additional site (within residues 2248−2285, comprising the epitope for MoAb ESH8 in the C2 domain of FVIIIa) increases the stoichiometry and affinity of FVIIIa binding to activated platelets, only in the presence of FIXa and FX but does not mediate FVIIIa binding to the platelet surface. In control experiments, a reverse-D peptide analogue displayed an inhibitory effect similar to that observed for the C2 peptide (2303−2332), i.e., K_i 49 $\times$ 10^{-9} M (Table 4) and a "scrambled peptide" containing the same amino acids as the C2 peptide (2303−2332) had no effect (at concentrations > 2.5×10^{-3} M) on the binding of FVIIIa to activated platelets. Moreover, ^{125}I-C2 peptide (corresponding to FVIII residues 2303−2332; comprising the epitope for the FVIII MoAb ESH4) interacted with platelets with significantly reduced affinity ($K_d \sim 45$ nM; ~666 ± 70 sites/platelet) as compared to FVIIIa (Figure 4 (●) and Table 3). These results support the conclusion that residues 2303−2332 within the C2 domain of FVIIIa comprises the site utilized for binding to ~750 sites per platelet.

DISCUSSION

Unactivated platelets have no significant effect on the kinetics of FX activation by FIXa either in the presence or in the absence of thrombin-activated FVIII; however, thrombin-activated platelets decrease the K_m by more than 200-fold and permit FVIIIa to increase the k_{cat} by more than 50000-fold (*41*), yielding an overall increase in catalytic efficiency (k_{cat}/K_m) of more than 2×10^7-fold (*10*). The assembly of the FX activation complex involves the coordinated interactions of each of the three components, the enzyme (FIXa), the cofactor (FVIIIa), and the substrate (FX), with binding sites exposed on the surface of activated platelets (*11*, *12*). In the present study, we have defined the molecular domains within FVIII/FVIIIa that interact with platelets. As shown in Table 2, Figure 1A,B, and Figure 2, the anti-C2 MoAb, ESH4, which recognizes residues 2303−2332 and inhibits FVIII binding to phospholipids, inhibited ^{125}I-FVIIIa interaction with activated platelets ($IC_{50} \sim 110$ nM) in equilibrium binding assays, both in the presence and in the absence of EGR−FIXa and FX. In contrast, the MoAb, ESH8, which recognizes residues 2248−2285 in the C2 domain of FVIII, did not inhibit FVIII−platelet interaction but interestingly abolished both the enhanced stoichiometry and the affinity of FVIIIa binding to platelets observed in the presence of EGR−FIXa and FX ($K_d \sim 1.5 \pm 0.12$; ~850 ± 110 sites/platelet both in the absence and in the presence of EGR−FIXa and FX). Binding of ESH8 to FVIIIa results in significantly lower affinity of phospholipid binding, suggesting that ESH8 may block either a protein interaction or a conformational change within the C2 domain, which is responsible for the increase in FVIIIa affinity (compared with FVIII) for phospholipid (*24*). In the same study, it has been shown that ESH8 does not prevent FVIII binding to either the vonWillebrand factor or the platelets (*24*). By using ^{125}I-$FVIII_{(LC)}$ (i.e., 72 kDa; A3−C1−C2 domain) and the radiolabeled LC fragments (obtained by protease digestion), we were able to show saturable and reversible equilibrium binding of LC to platelets ($K_d = \sim 2.0 \pm 0.19$ nM; 1130 ± 180 sites/platelet). Similar affinities of $FVIII_{(LC)}$ interactions

with the platelet membrane have been reported by other investigators (*24*) and $FVIII_{(LC)}$ has been shown to be entirely responsible for the high-affinity binding to phospholipid membranes (*24*). These data provide strong evidence for the presence of (1) a major platelet-receptor-mediated binding site (within residues 2303−2332) in the C2 domain; and (2) an additional binding site (within residues 2248−2285) in the C2 domain that increases the stoichiometry and affinity of FVIIIa binding to activated platelets observed in the presence of EGR−FIXa and FX.

Earlier studies with synthetic peptides from the C2 domain region (residues 2303−2332) inhibited FVIII/PL binding, indicating that the C2 region of FVIII is involved in FVIII/PL interaction (*42*, *43*). Other recent studies (for reviews see refs *44* and *45*) also implicate the C2 domain in FVIII/PL interaction. Studies by Healey et al. (*46*) show that MoAb NMC−VIII/5 and other patient-derived inhibitory antibodies, also prevent FVIII/PL interaction, thus suggesting that the FVIII C2 domain is critical for interaction with PL. Therefore, to further define the role of the C2 domain of FVIII in the assembly of the F−X activating complex on the platelet membrane, we determined the affinities and stoichiometries of the interaction of both the C2 domain and the peptides from the C2 domain with activated human platelets. As shown in Table 3, the isolated rC2 domain interacted with the same number (∼700−800 sites/platelet) of platelet binding sites with which FVIIIa interacts with impaired affinity ($K_d \sim 16 \pm 2.5$ nM) compared with FVIIIa ($K_d \sim 2.0 \pm 0.18$ nM) in the absence of EGR−FIXa and FX. Moreover, our competition studies with synthetic peptides corresponding to the FVIII C2 domain (residues 2303−2332; comprising the epitope of FVIII MoAb ESH4) showed a marked inhibition of FVIIIa binding, with K_i of ∼25 nM compared with native FVIIIa or $FVIIIa_{(LC)}$ with K_i ∼1.5−2.0 nM (Table 4). These studies support the hypothesis that the C2 domain alone does not provide the totality of the binding energy for FVIIIa interaction with the platelet membrane and suggests that the A3 and/or C1 domain of $FVIII_{(LC)}$ may also contribute to the interaction with activated platelets. Our results are in agreement with the observations of Takeshima et al. (*21*) who showed that binding of the rC2 domain to synthetic vesicles was significantly weaker than that of the LC of FVIIIa, thus suggesting that other regions of FVIIIa may be required for an optimal membrane interaction. In contrast, Saenko et al. (*24*) using biosensor chip measurements of the binding of C2 domain, FVIIIa, and $FVIII_{(LC)}$ to synthetic membranes, concluded that C2 domain is entirely responsible for the binding of FVIIIa to phospholipids. In the present studies with synthetic peptides, we have utilized the strategy of preparing a "reverse-D peptide" or "retro-inverso peptide" (in which D-amino acids instead of L-amino acids were used to synthesize a peptide with a sequence opposite to that of the C2 domain peptide 2303−2332). This strategy "produces a structural isomer that maintains the original sterochemical conformation at all chiral centers but reverses the sequence" (*34*−*36*). Such a retro-inverso or reverse-D analogue assumes a conformation identical to the native peptide prepared from L-amino acids and therefore strongly confirms the conclusions drawn from the C2 peptide as well as those obtained from the scrambled peptide and the control C2 peptide, 2248−2285.

On the basis of the work done on phospholipid membranes, we know that specific membrane binding sites of FVIIIa involve stereoselective interactions of FVIIIa with PS headgroups (*47*). Gilbert and Arena (*48*, *49*) have shown that the FVIIIa binding affinities are affected by the mole fractions of specific phospholipid headgroups and by the nature of the acyl chain. These authors have shown that the effect of PS-containing membranes on the FVIIIa/FIXa complex is due primarily to a 1500-fold acceleration of k_{cat}, relative to the rate measured for the FVIIIa/FIXa complex in solution (*50*). Two high-resolution crystal structures of rC2 domain further implicated the FVIII C2 domain region in PL binding (*23*, *51*). These studies revealed the presence of three hydrophobic "feet" formed by side chains of Met 2199/Phe 2200, Val 2223, and Leu 2251/Leu 2252, which have been suggested by the authors to penetrate the membrane bilayer. More recently, using electron crystallographic studies, Stoilova-McPhie et al. (*25*) have identified four basic residues, Arg 2215, Arg 2220, Lys 2227, and Lys 2249, which lie close to the "feet" that were postulated to stabilize FVIII/PL binding by electrostatic interaction with negatively charged PL. Studies by these authors strongly support the contribution of these residues, together with a fourth loop structure, Trp 2313−His 2315, in forming the phospholipid-interacting domain of FVIIIa. Interestingly, this fourth loop lies within the sequence 2303−2332, which we have shown in this study to be partially responsible for its interaction with the platelet membrane and, according to Saenko et al. (*24*), solely responsible for FVIII binding to PS-containing membranes. Using Ala mutations of the residues comprising the hydrophobic "feet" Met 2199/Phe 2200 and Leu 2251/Leu 2252, Gilbert et al. (*22*) have shown that these mutations dramatically reduce the affinity of FVIII for PL.

In conclusion, this study clearly shows that the major binding site that mediates FVIIIa interaction with activated platelets resides within residues 2303−2332 in the C2 domain of FVIII. An additional site within residues 2248−2285 increases the stoichiometry and affinity of FVIIIa binding to activated platelets only in the presence of FIXa and FX but does not directly mediate FVIIIa binding to the platelet surface. Recent studies have shown that the binding of FVa to phospholipid membranes regulates the assembly of the prothrombinase complex and have provided experimental support for the joint participation of the C1 and C2 domains in the binding of FVa to phospholipid membranes (*52*). Whether similar molecular mechanisms exist for the coordinated involvement of the C1 and C2 domains of FVIIIa in the assembly of the F−X activating complex on the platelet surface and/or phospholipid membranes remains to be elucidated. Similarly, although the direct interaction of FVIIIa with the platelet surface appears to be mediated exclusively by the C2 domain (residues 2302−2332), protein interactive sites within the light chain (A3−C1−C2) and the heavy chain (A1−A2) clearly affect both the stoichiometry and the affinity of FVIIIa binding to the F−X activating complex on the platelet surface.

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REFERENCES

1. Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., and et al. (1984) Molecular cloning of a cDNA encoding human antihaemophilic factor, *Nature 312*, 342−347.
2. Wood, W. I., Capon, D. J., Simonsen, C. C., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hollingshead, P., Wion, K. L., et al. (1984) Expression of active human factor VIII from recombinant DNA clones, *Nature 312*, 330−337.
3. Lenting, P. J., van Mourik, J. A., and Mertens, K. (1998) The life cycle of coagulation factor VIII in view of its structure and function, *Blood 92*, 3983−3996.
4. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes, *Annu. Rev. Biochem. 57*, 915−956.
5. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes, *Blood 76*, 1−16.
6. Eaton, D., Rodriguez, H., and Vehar, G. A. (1986) Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity, *Biochemistry 25*, 505−512.
7. Lollar, P., and Parker, C. G. (1989) Subunit structure of thrombin-activated porcine factor VIII, *Biochemistry 28*, 666−674.
8. Fay, P. J., Haidaris, P. J., and Smudzin, T. M. (1991) Human factor VIIIa subunit structure. Reconstruction of factor VIIIa from the isolated A1/A3-C1-C2 dimer and A2 subunit. *J. Biol. Chem. 266*, 8957−8962.
9. Ahmad, S. S., Scandura, J. M., and Walsh, P. N. (2000) Structural and functional characterization of platelet receptor-mediated factor VIII binding, *J. Biol. Chem. 275*, 13071−13081.
10. Ahmad, S. S., London, F. S., and Walsh, P. N. (2003) The assembly of the factor X-activating complex on activated human platelets, *J. Thromb. Haemostasis 1*, 48−59.
11. Ahmad, S. S., and Walsh, P. N. (2002) Coordinate binding studies of the substrate (factor X) with the cofactor (factor VIII) in the assembly of the factor X activating complex on the activated platelet surface, *Biochemistry 41*, 11269−11276.
12. Ahmad, S. S., London, F. S., and Walsh, P. N. (2003) Binding studies of the enzyme (factor IXa) with the cofactor (factor VIII) in the assembly of factor-X activating complex on the activated platelet surface, *J. Thromb. Haemostasis 1*, 2348−2355.
13. Fay, P. J., Coumans, J. V., and Walker, F. J. (1991) von Willebrand factor mediates protection of factor VIII from activated protein C-catalyzed inactivation, *J. Biol. Chem. 266*, 2172−2177.
14. Fay, P., and Koshibu, K. (1998) The A2 subunit of factor VIIIa modulates the active site of factor IXa, *J. Biol. Chem. 273*, 19049−19054.
15. Fay, P. J., and Smudzin, T. M. (1992) Characterization of the interaction between the A2 subunit and A1/A3-C1-C2 dimer in human factor VIIIa, *J. Biol. Chem. 267*, 13246−13250.
16. Fay, P. J., Koshibu, K., and Mastri, M. (1999) The A1 and A2 subunits of factor VIIIa synergistically stimulate factor IXa catalytic activity, *J. Biol. Chem. 274*, 15401−15406.
17. Lollar, P., Parker, E. T., and Fay, P. J. (1992) Coagulant properties of hybrid human/porcine factor VIII molecules, *J. Biol. Chem. 267*, 23652−23657.
18. Lollar, P., Knutson, G. J., and Fass, D. N. (1984) Stabilization of thrombin-activated porcine factor VIII:C by factor IXa phospholipid, *Blood 63*, 1303−1308.
19. Fay, P. J., Beattie, T. L., Regan, L. M., O'Brien, L. M., and Kaufman, R. J. (1996) Model for the factor VIIIa-dependent decay of the intrinsic factor Xase. Role of subunit dissociation and factor IXa-catalyzed proteolysis, *J. Biol. Chem. 271*, 6027−6032.
20. Saenko, E. L., and Scandella, D. (1995) A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor, *J. Biol. Chem. 270*, 13826−13833.
21. Takeshima, K., Smith, C., Tait, J., and Fujikawa, K. (2003) The preparation and phospholipid binding property of the C2 domain of human factor VIII, *Thromb. Haemostasis 89*, 788−794.
22. Gilbert, G. E., Kaufman, R. J., Arena, A. A., Miao, H., and Pipe, S. W. (2002) Four hydrophobic amino acids of the factor VIII C2 domain are constituents of both the membrane-binding and von Willebrand factor-binding motifs, *J. Biol. Chem. 277*, 6374−6381.
23. Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K., and Stoddard, B. L. (1999) Structure of the C2 domain of human factor VIII at 1.5 Å resolution, *Nature 402*, 439−442.
24. Saenko, E. L., Scandella, D., Yakhyaev, A. V., and Greco, N. J. (1998) Activation of factor VIII by thrombin increases its affinity for binding to synthetic phospholipid membranes and activated platelets, *J. Biol. Chem. 273*, 27918−27926.
25. Stoilova-McPhie, S., Villoutreix, B. O., Mertens, K., Kemball-Cook, G., and Holzenburg, A. (2002) 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography, *Blood 99*, 1215−1223.
26. Kalafatis, M., Jenny, R. J., and Mann, K. G. (1990) Identification and characterization of a phospholipid-binding site of bovine factor Va, *J. Biol. Chem. 265*, 21580−21589.
27. Ortel, T. L., Devore-Carter, D., Quinn-Allen, M., and Kane, W. H. (1992) Deletion analysis of recombinant human factor V. Evidence for a phosphatidylserine binding site in the second C-type domain, *J. Biol. Chem. 267*, 4189−4198.
28. Kim, S. W., Ortel, T. L., Quinn-Allen, M. A., Yoo, L., Worfolk, L., Zhai, X., Lentz, B. R., and Kane, W. H. (1999) Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V modulates assembly of the prothrombinase complex, *Biochemistry 38*, 11448−11454.
29. Gilbert, G. E., Furie, B. C., and Furie, B. (1990) Binding of human factor VIII to phospholipid vesicles, *J. Biol. Chem. 265*, 815−822.
30. Gilbert, G. E., Drinkwater, D., Barter, S., and Clouse, S. B. (1992) Specificity of phosphatidylserine-containing membrane binding sites for factor VIII. Studies with model membranes supported by glass microspheres (lipospheres), *J. Biol. Chem. 267*, 15861−15868.
31. Nesheim, M. E., Pittman, D. D., Wang, J. H., Slonosky, D., Giles, A. R., and Kaufman, R. J. (1988) The binding of 35S-labeled recombinant factor VIII to activated and unactivated human platelets, *J. Biol. Chem. 263*, 16467−16470.
32. Scandura, J. M., Ahmad, S. S., and Walsh, P. N. (1996) A binding site expressed on the surface of activated human platelets is shared by factor X and prothrombin, *Biochemistry 35*, 8890−8902.
33. Ahmad, S. S., Wong, M. Y., Rawala, R., Jameson, B. A., and Walsh, P. N. (1998) Coagulation factor IX residues G4-Q11 mediate its interaction with a shared factor IX/IXa binding site on activated platelets but not the assembly of the functional factor X activating complex, *Biochemistry 37*, 1671−1679.
34. Degrado, W. F. (1988) Design of peptides and proteins, *Adv. Protein Chem. 39*, 51−124.
35. Goodman, M., and Chorev, M. (1979) On the concept of linear modified retro-peptide structures, *Acc. Chem. Res. 12*, 1−7.
36. Nair, D. T., Kaur, K. J., Singh, K., Mukherjee, P., Rajagopal, D., George, A., Bal, V., Rath, S., Rao, K. V., and Salunke, D. M. (2003) Mimicry of native peptide antigens by the corresponding retro-inverso analogs is dependent on their intrinsic structure and interaction propensities, *J. Immunol. 170*, 1362−1373.
37. Bolton, A. E., and Hunter, W. M. (1973) The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent, *Biochem. J. 133*, 529−539.
38. Tuszynski, G. P., Knight, L., Piperno, J. R., and Walsh, P. N. (1980) A rapid method for removal of [^{125}I]iodide following iodination of protein solutions, *Anal. Biochem. 106*, 118−122.
39. Walsh, P. N., Mills, D. C., and White, J. G. (1977) Metabolism and function of human platelets washed by albumin density gradient separation, *Brit. J. Haematol. 36*, 287−296.
40. Ahmad, S. S., Rawala-Sheikh, R., and Walsh, P. N. (1989) Comparative interactions of factor IX and factor IXa with human platelets, *J. Biol. Chem. 264*, 3244−3251.
41. Rawala-Sheikh, R., Ahmad, S. S., Ashby, B., and Walsh, P. N. (1990) Kinetics of coagulation factor X activation by platelet-bound factor IXa, *Biochemistry 29*, 2606−2611.
42. Foster, P. A., Fulcher, C. A., Houghten, R. A., and Zimmerman, T. S. (1990) Synthetic factor VIII peptides with amino acid sequences contained within the C2 domain of factor VIII inhibit factor VIII binding to phosphatidylserine, *Blood 75*, 1999−2004.
43. Gilbert, G. E., and Baleja, J. D. (1995) Membrane-binding peptide from the C2 domain of factor VIII forms an amphipathic structure as determined by NMR spectroscopy, *Biochemistry 34*, 3022−3031.
44. Pratt, K. P. (2000) in *Current Opinion in Drug Discovery & Development*, pp 516−526, PharmaPress Ltd., London.

45. Saenko, E. L., Ananyeva, N. M., Tuddenham, E. G., and Kemball-Cook, G. (2002) Factor VIII − novel insights into form and function, *Br. J. Haematol. 119*, 323−331.
46. Healey, J. F., Barrow, R. T., Tamim, H. M., Lubin, I. M., Shima, M., Scandella, D., and Lollar, P. (1998) Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII, *Blood 92*, 3701−3709.
47. Gilbert, G. E., and Drinkwater, D. (1993) Specific membrane binding of factor VIII is mediated by O-phospho-L-serine, a moiety of phosphatidylserine, *Biochemistry 32*, 9577−9585.
48. Gilbert, G. E., and Arena, A. A. (1995) Phosphatidylethanolamine induces high affinity binding sites for factor VIII on membranes containing phosphatidyl-L-serine, *J. Biol. Chem. 270*, 18500−18505.
49. Gilbert, G. E., and Arena, A. A. (1998) Unsaturated phospholipid acyl chains are required to constitute membrane binding sites for factor VIII, *Biochemistry 37*, 13526−13535.
50. Gilbert, G. E., and Arena, A. A. (1996) Activation of the factor VIIIa-factor IXa enzyme complex of blood coagulation by membranes containing phosphatidyl-L-serine, *J. Biol. Chem. 271*, 11120−11125.
51. Barrow, R. T., Healey, J. F., Jacquemin, M. G., Saint-Remy, J. M., and Lollar, P. (2001) Antigenicity of putative phospholipid membrane-binding residues in factor VIII, *Blood 97*, 169−174.
52. Peng, W., Quinn-Allen, M. A., and Kane, W. H. (2005) Mutation of hydrophobic residues in the factor Va C1 and C2 domains blocks membrane-dependent prothrombin activation, *J. Thromb. Haemostasis 3*, 351−354.

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