Coagulation Factor IX Residues G_4-Q_{11} 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†

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ABSTRACT: High-affinity, specific factor IX/IXa binding to platelets is mediated at least in part by amino acids (G_4-Q_{11}) exposed on the surface of the γ-carboxyglutamic acid (Gla) domain. Rationally designed, conformationally constrained synthetic peptides were screened for their capacity to inhibit factor IXa binding to platelets. Each of these peptides (G_4-Q_{11}, S_3-L_6, and F_9-Q_{11}) acted alone to inhibit factor IXa binding to ∼50% of the 500−600 sites/platelet with K_i values of 2.9 nM (G_4-Q_{11}), 24 nM (S_3-L_6), and 240 nM (F_9-Q_{11}), compared with native factor IXa (K_i ∼2.5 nM). The two peptides S_3-L_6 and F_9-Q_{11} added together at equimolar concentration demonstrated ∼50-fold synergism (K_i = 2.4 nM). Although both factor IX and the Gla peptide (G_4-Q_{11}) displaced 100% of bound factor IX and ∼50% of bound factor IXa, factor IX was ineffective (at >1000-fold molar excess) and the Gla domain peptide (G_4-Q_{11}) was relatively ineffective (K_i = 165 μM) in inhibiting platelet receptor-mediated factor X activation by factor IXa. We conclude that the Gla domain (G_4-Q_{11}) of factor IXa contains two conformationally constrained loop structures that mediate binding of factor IX/IXa to a shared site on activated human platelets which is separate and distinct from the site used by the enzyme, factor IXa, for assembly of the factor X activating complex.

The interaction between blood platelets and coagulation factors is essential for normal coagulation and hemostasis. In our previous studies, we have examined the mechanism by which platelets can promote factor X activation by factor IXa (*1*−*3*). We have demonstrated that when platelets are activated by physiologically relevant agonists in the presence of calcium ions, they expose high-affinity, saturable binding sites that are specific for factor IX and for factor IXa (*1*, *2*). In the absence of the cofactor (factor VIII) and the substrate (factor X) that are required for the assembly of the factor X activating complex, the enzyme (factor IXa) can occupy ∼500−600 sites per platelet, of which ∼250−300 can also be occupied by the zymogen (factor IX). Cross-competition studies (*1*) showed that whereas factor IXa can displace all the factor IX molecules, factor IX is capable of displacing only half the factor IXa. Furthermore, the affinity of these two binding sites is similar [dissociation constant (K_d)[1] ∼2.5−3.0 nM]. Therefore, it can be concluded that there are two classes of binding sites on the surface of activated platelets that do not require the presence of factor VIII or factor X: one class of sites that can occupy either the zymogen or the enzyme (herein referred to as the shared factor IX/IXa binding site), and another class of sites that is specific for the enzyme (herein referred to as the specific factor IXa binding site). In the presence of both the cofactor (factor VIII) and the substrate (factor X), the number of binding sites for both factor IXa and factor IX remains unchanged. When both factor VIII and factor X are present, the affinity of factor IXa binding is increased ∼5-fold (K_d ∼0.5 nM), but the affinity of factor IX binding is unaffected (*1*). This confirms the view that factor IX and factor IXa contain binding sites that interact with a discrete number of platelet receptors, which are independent of the presence of factor VIII and factor X, although the affinity of factor IXa binding is increased in the presence of both the cofactor and the substrate. Since platelet receptor occupancy by factor IXa is a major determinant of the catalytic efficiency of factor X activation (*1*, *3*), the objective of the present work was to identify the molecular domains within factor IX and factor IXa that mediate their interaction with platelet receptors and the assembly of the factor X activating complex.

Recently, we began an analysis of the structural features of the factor IX molecule that are important for the assembly of the factor X activating complex on the platelet surface. These studies have shown that a major determinant of factor

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[1] Abbreviations: K_d, dissociation constant; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; PPACK, D-phenylalanylprolylarginyl chloromethyl ketone.

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IX/IXa binding to human platelet receptors resides within its γ-carboxyglutamic acid (Gla) domain (*4*) whereas the first epidermal growth factor (EGF-1) domain of factor IXa appears not to be involved in factor IXa binding to platelets (*5*). In contrast, studies with chimeric factor IXa molecules in which the second epidermal growth factor domain (EGF-2) was replaced by that of factor X suggest that the EGF-2 domain may also be important for specific, high-affinity factor IXa binding to platelets in the presence of factor VIIIa and factor X (*6*). Our studies with both Gla-modified and Gla-domainless factor IXa molecules have shown that factor IXa binding to platelets is mediated in part, but not exclusively, by residues in the Gla domain of factor IX (*4*). Recently we have used nine different recombinant factor IX molecules to show that the high-affinity, specific binding of factor IXa to activated platelets in the presence or absence of factor VIIIa and factor X is mediated at least in part by amino acids exposed on the surface of the Gla domain within positions 3−11, possibly by residues 4, 5, 9, 10, and 11 (*7*).

We have constructed a computer-generated molecular model of the Gla domain of factor IX based upon the known structure of bovine prothrombin fragment 1 in order to identify amino acid residues exposed on the surface of the protein that might comprise a binding site required for interaction with a platelet receptor (*7*). Based upon this model, we have prepared rationally designed, conformationally constrained synthetic peptides which have been used to probe the functional role of the Gla domain in the interaction of factor IX/IXa with platelets. These studies lead to the surprising conclusion that this Gla domain "omega loop" structure (G_4−Q_{11}) mediates the binding of factor IX and factor IXa to a shared site on the activated platelet surface that is *not* the specific site utilized by factor IXa for the assembly of the functional factor X activating complex.

EXPERIMENTAL PROCEDURES

Materials. The chromogenic substrate S2337 [Bz-Ile-Glu-(γ-piperidyl)-Gly-Arg-*p*-nitroanilide] was purchased from AB Kabi Diagnostica (Stockholm, Sweden). *p*-Aminobenzamidine was obtained from Sigma Chemical Co. (St. Louis, MO). D-Phenylalanylprolylarginyl chloromethyl ketone (PPACK) was purchased from Calbiochem-Behring Corp. (San Diego, CA). Carrier-free $Na^{125}I$ was obtained from Amersham Corp. (Arlington Heights, IL). The thrombin receptor agonist peptide, SFLLRN-amide (*8*, *9*), was synthesized using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry on an Applied Biosystems 430A synthesizer and reverse-phase HPLC purified to greater than 99% homogeneity. All other reagents and chemicals used were the same as previously reported (*1*) and were obtained from Sigma Chemical Co., Aldrich Chemical Co. (Milwaukee, WI), or Calbiochem-Behring Corp. and were the highest grade commercially available.

Proteins. Human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin, were purified, assayed, and characterized as previously published (*1*). The conditions used for activation of factor VIII with human α-thrombin were identical with those previously published (*1*). All proteins were >98% pure as determined by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate (*10*). Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer (Bio-Rad, Richmond, CA). Normal, plasma-derived factor IX and a tyrosinated peptide comprising a sequence of amino acids (YAGA-CPGLDEFVQPC-AGAY) representing residues (G_4−Q_{11}) from the Gla domain were radiolabeled with ^{125}I by a minor modification of the iodogen method, as previously described (*1*), to a specific radioactivity in the range of $(2.0-2.5) \times 10^6$ cpm/μg for factor IX and $(0.5-1) \times 10^6$ cpm/μg for the tyrosinated (G_4−Q_{11}) peptide. Radiolabeling of the Gla peptide was carried out both in the absence and in the presence of excess sodium iodide (0.5 mg/mL). The calculated molar incorporation of ^{125}I was 1 atom of iodine per 230 peptide molecules in the absence of cold carrier iodide and 1−2 mol of iodine per mole of peptide in the presence of excess iodide. Activation of normal factor IX by purified factor XIa was carried out as previously described (*1*). Following gel electrophoresis, autoradiography of factor IXa was carried out to provide structural characterization of ^{125}I-labeled protein. ^{125}I-Labeled factor IXa migrated under reducing conditions as two polypeptide chains of M_r 27 000 and 17 000 representing the heavy and light chains and was indistinguishable from unlabeled plasma-derived factor IXa_N (not shown), thus confirming the purity and chain composition of both labeled and unlabeled factor IXa molecules.

Molecular Modeling. A model (*7*) of the factor IX Gla domain was constructed from residues 1−47 of the coordinates of the bovine prothrombin fragment 1 crystal structure (*11*). Amino acid replacements of factor IX within the prothrombin structure were made according to sequence alignment and were performed using the biopolymer module provided within the SYBYL computational chemistry package (Tripos Associates Inc., St. Louis, MO). The Amber force field, as implemented in SYBYL, was utilized in all subsequent calculations (*12*). Atomic parameters describing calcium and the Gla residues were added to the force-field tables. Because the atomic properties of calcium (a transition state metal) are inadequately described within the force field in order to account for the coordination complexes formed with the negative charges of the γ-carboxylated glutamic acids, the distance geometries measured in the prothrombin Gla domain coordinated complexes were held as restraints during the modeling process of creating the factor IX structure. On comparing our model with the actual coordinates of the factor IX Gla domain in the presence of calcium ions, our model was essentially superimposable on the backbone NMR structure reported recently by Freedman et al. (*13*).

After all amino acid replacements were completed, the structure was energy minimized to convergence using a conjugate-gradient approach. The newly minimized structure was then solvated with water (two solvent shells were added to ensure that all portions of the surface were adequately solvated) using the Silverware algorithm as implemented in SYBYL. The water−protein complex was again minimized prior to an energy-dependent simulation of molecular motion ($t = 100$ ps). Reviews of the trajectory files obtained after this dynamic run indicated that a stable low energy structure was obtained after ∼12 ps.

Peptide Synthesis. Using the techniques for molecular modeling described above, synthetic peptide analogues were designed comprising amino acids corresponding to the

Table 1: Synthetic Cyclic Peptides Derived from the Factor IXa Gla Domain Sequence Residues 3−11[a]

Synthetic Peptides	RESIDUE NUMBER IN FACTOR IX 3 4 5 6 7 8 9 10 11
Amino Acid in Factor IX	S G K L γ γ F V Q
(G_4-Q_{11})	C^a P G K L D E F V Q P C^a
(S_3-L_8)	C^a S G K L C^a
(F_9-Q_{11})	C^a F V Q C^a
YAGA (G_4-Q_{11}) AGAY	Y^bAGA-C^a P G K L D E F V Q P C^a - AGAY^b
Reverse-D Analog	C^a P Q V F E D L K G P C^a
Scrambled Peptide	C^a P E F G L Q K V D P C^a

[a] γ is γ-carboxyglutamic acid (Gla). [a]Designates a residue where cysteine was substituted for an amino acid in the native factor IXa sequence; [b]indicates tyrosine residues substituted for an amino acid in the native factor IXa Gla domain sequence.

segment of factor IXa Gla domain sequence spanning residues 1−14. Thus, each rationally designed peptide was separately modeled using energy minimization calculations that confirmed that the modeled peptides assumed a conformation similar to that of the putative native factor IX Gla domain. The peptides were synthesized according to conventional solid-phase procedures on an Applied Biosystems 430A Peptide Synthesizer by a modification of the procedure by Kent and Clark-Lewis (*14*). Each of the peptides was conformationally constrained using a disulfide bond created between Cys residues at the C- and N-terminus of each peptide. The sequences of these peptides are shown in Table 1.

Reduction and Alkylation and Refolding of Peptides. Peptides were dissolved in deionized water as a 100 mg/mL solution in a flask containing a stir bar in order to refold peptides containing cysteine residues. After the pH was adjusted to 8.5 with NH_4OH, the solution was allowed to stir at 5 °C for at least 3 days. The solution was then lyophilized. Alternatively, peptides were reduced with dithiothreitol (1 mM) and alkylated with iodoacetamide (5 mM) as described previously (*15*).

Characterization of Synthetic Peptides. All peptides used in this study were examined by high-performance liquid chromatography (HPLC) (both reverse-phase and gel filtration), and all demonstrated a single homogeneous peak (data not shown). This demonstrates the presence of a single homogeneous mixture of refolded peptides and not a mixed population of diverse polymers. The results were the same after reduction and alkylation of these same peptides. In addition, all peptides were examined for free SH groups (*16*) using the Ellman reagent, 5,5′-dithiobis(2 nitrobenzoic acid). It was determined that there was less than 0.02 mol of free SH/mol of peptide, which further verifies that these refolded peptides were homogeneous preparations consisting of intramolecular disulfide-bonded peptides.

Mass Spectrometry. Fast atom bombardment mass spectrometry was used to assess the homogeneity and molecular mass of each synthesized peptide with M_r <2000. Fast atom bombardment was performed as described previously (*15*). The spectra of these peptides revealed masses almost identical with the calculated values[2] (i.e., within 0.5%) with a single ion species observed in each case.

Binding Experiments. In a typical binding experiment, gel-filtered platelets [(3−4) × 10^8 platelets/mL], in calcium-free HEPES Tyrode's buffer, pH 7.4, were incubated at 37 °C in a 1.5 mL Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa (0.1−20 nM), or factor IXa derived peptides or ^{125}I-labeled peptide G_4−Q_{11} (at various concentrations), $CaCl_2$ (5 mM), human α-thrombin (0.1 unit/mL) in the presence or absence of factor X (1.5 μM), and thrombin-activated factor VIII (2 units/mL) as detailed previously (*1*). Platelets were separated from unbound proteins as previously described (*1*). The data were analyzed, and the number of binding sites and K_d were calculated from the mean of three independent determinations, each done in duplicate, as previously described (*1*) using a Macintosh Quadra 900 computer (Apple Computer, Inc., Cupertino, CA) and the LIGAND program as modified by G. A. McPherson (Elsevier Science Publishers BV, The Netherlands, 1985).

Measurement of Rates of Factor Xa Formation. Activation of factor X by factor IXa in the absence or presence of synthetic peptides was assayed at 37 °C in the presence of thrombin-stimulated, gel-filtered platelets, factor VIIIa, and $CaCl_2$ as described previously (*3*). The details of experimental conditions and concentrations of reactants are given under Results and in the figure legends. Inhibition of factor X activation by the synthetic peptides was analyzed using Dixon plots as previously described (*2*).

Calculations of Kinetic Constants, Inhibition Constants, and Free Energy of Binding. The derivation of kinetic constants for factor X activation by factor IXa was based on a one-enzyme, one-substrate model. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated from the mean ± SEM of five independent determinations each done in duplicate of factor X activation rates at variable factor X concentrations as described previously (*3*). Values of K_d were determined at variable factor IXa concentrations as previously described (*2*). The values of the turnover numbers (k_{cat}) were calculated by dividing V_{max} values either by the total factor IXa concentration or by the amount of enzyme (factor IXa) bound under the conditions of the experiment. This later value was obtained from the equation:

$$\text{amount bound} = \frac{B_{max}E}{K_d + E}$$

where B_{max} is the maximum amount of factor IXa bound or the total receptor concentration; E, total factor IXa concentration; and K_d, dissociation constant. The details of this calculation are provided in previous papers (*2*). The IC_{50} method of Cha (*17*) was used to determine the inhibition constants, as previously described (*15*). In the case of classical competitive inhibition, the IC_{50} (total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) is related to the substrate concentration as follows: $IC_{50} = {}^1/_2E_t + K_i + K_iS/K_m$, where E_t is the total enzyme concentration and S is the substrate

[2] Masses were obtained by addition of the average masses of amino acids which were determined using the atomic weights of the elements (C = 12.011; H = 1.0079; N = 14.0067; S = 32.06).

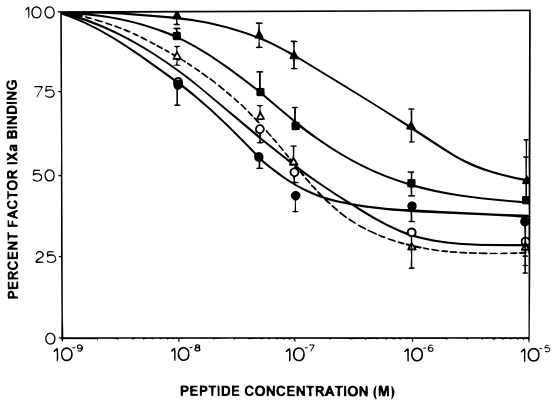

FIGURE 1: Analogue competition of radiolabeled factor IXa binding to platelets in the presence of factor VIIIa (2 units/mL) and factor X (1.5 μM). To confirm our hypothesis that residues 3−11 in the Gla domain of factor IX mediate factor IXa binding to activated platelets, we prepared synthetic peptides rationally designed and conformationally constrained with artificially introduced disulfide bonds (Table 1) on the basis of a computer-generated model. As shown in Figure 1, three peptides [G_4−Q_{11} (●); S_3−L_6 (■); and F_9−Q_{11} (▲)] inhibited factor IXa binding to platelets (for K_i see Table 2). The two shorter peptides (S_3−L_6 and F_9−Q_{11}) added together at equimolar concentrations (○) demonstrated ∼50-fold synergism when tested in the platelet binding assay. The "reverse-D peptide"(△) displayed inhibitory effects similar to those observed from peptide G_4−Q_{11}. The plotted results are the mean ± SEM of the duplicate observations from six separate experiments.

concentration. Thus, from the plot of IC_{50} versus S, K_i can be determined. The inhibition of factor X activation by synthetic peptide (G_4−Q_{11}) was analyzed by Dixon plots as previously described (*2*). Computation of Gibbs free energy ($\Delta G°$) differences was calculated from the following formula (*18*): $\Delta G° = -RT \ln K$ where R is the gas constant (1.987 cal K^{-1} mol^{-1}) and T is the absolute temperature [$T = 310$ K (37 °C)], and K_d is the dissociation constant. Using the above equation, the Gibbs free energy of binding was calculated from the equilibrium binding constant.

RESULTS

Molecular Modeling of the Gla Domain of Factor IX and Design of Conformationally Constrained Gla Peptides. A plausible three-dimensional structure of factor IX Gla domain was calculated using coordinates of the bovine prothrombin fragment 1 crystal structure (*7*). Based on this model, rationally designed synthetic peptides were prepared in which cysteine residues were introduced so that the resulting disulfide bond might stabilize the loop structure in a conformation likely to resemble the native binding surface (Table 1).

Analogue Competition of Radiolabeled Factor IXa Binding to Platelets in the Presence of Factor VIIIa and Factor X. The peptides shown in Table 1 were examined for their capacity to inhibit the binding of ^{125}I-factor IXa to platelets. The results shown in Figure 1 demonstrate that all three peptides (G_4−Q_{11}, S_3−L_6, and F_9−Q_{11}) were potent inhibitors of factor IXa binding to platelets with IC_{50} values of 1.0×10^{-7} M, 1.0×10^{-6} M, and 1.0×10^{-5} M, respectively, and calculated K_i values of 2.4×10^{-9} M, 2.4×10^{-8} M, and 2.4×10^{-7} M, respectively (Table 2). However, it was consistently observed that whereas the enzyme factor IXa was able to displace 100% of the bound ^{125}I-labeled factor IXa molecules, both the zymogen, factor IX, and the conformationally constrained synthetic peptides were capable of displacing only 50−70% of the bound ^{125}I-labeled factor IXa, leaving the remainder (30−50%) bound. Therefore, in estimating IC_{50} and K_i values, this residual binding was subtracted from the total bound, so that the IC_{50} was the concentration of competitor able to displace half the pool of displaceable factor IXa molecules. The rationale of this will become clear in subsequent experiments. When the binding assays were carried out with a mixture comprising equimolar amounts of the two shorter peptides (S_3−L_6 plus F_9−Q_{11}), these peptides were shown to display a 50-fold synergistic effect with an IC_{50} for the combined peptide 3.5 nM of 1×10^{-7} M and a calculated K_i of 2.4×10^{-9} M. Thus, the concentrations of the combined peptides required to inhibit factor IXa binding to platelets 50% were 50-fold lower than expected on the basis of their inhibitory activities when used alone. When the G_4−Q_{11} peptide was reduced and alkylated, it lost all inhibitory capacity, demonstrating the importance of conformational constraints on the modeled peptide. The "reverse-D peptide" (in which D-amino acids instead of L-amino acids were used to synthesize a peptide with a sequence opposite to that of G_4−Q_{11}) displayed inhibitory effects similar to those observed for peptide G_4−Q_{11}, i.e., $K_i = 3.7 \times 10^{-9}$ M (Table 2). A "scrambled peptide" containing the same amino acids as the G_4−Q_{11} peptide in a scrambled order had no effect (either after refolding or after reduction and alkylation) on the binding of factor IXa to activated platelets (Table 2).

Table 2: Effects of Factor IXa and Synthetic Gla Domain Peptides on the Binding of ^{125}I-Factor IXa to Platelets

competing factor IXa or Gla domain peptide	K_i (M) factor VIII + factor X present	K_i (M) factor VIII + factor X absent
factor IXa	0.5×10^{-9}	2.5×10^{-9}
G_4−Q_{11}	2.4×10^{-9}	2.9×10^{-9}
S_3−L_6	2.4×10^{-8}	
F_9−Q_{11}	2.4×10^{-7}	
S_3−L_6 and F_9−Q_{11}[a]	2.4×10^{-9}	3.0×10^{-9}
G_4−Q_{11}: R + A[b]	NE[c]	NE[c]
YAGA(G_4−Q_{11})AGAY	2.4×10^{-9}	
YAGA(G_4−Q_{11})AGAY (mock-labeled)	1.0×10^{-7}	
reverse-D analog	3.7×10^{-9}	5.0×10^{-9}
scrambled peptide	NE[c]	NE[c]

[a] Two peptides added together at equimolar concentration. [b] R + A = reduced and alkylated peptide. All other peptides were studied after refolding. [c] NE, no effect at concentrations up to 10^{-2} M.

Specific, Reversible, and Saturable Binding of ^{125}I-Gla Peptide (G_4−Q_{11}) to Activated Platelets in the Presence of Factor VIIIa and Factor X. Since as described above, the Gla peptide (G_4−Q_{11}) competes with ^{125}I-factor IXa for ∼50% of its platelet binding sites, the direct interaction of this peptide with the platelet surface was examined. To label this peptide with ^{125}I, we first added tyrosine residues to each end of the synthesized peptide (see Table 1) utilizing a spacer of sequence AGA at either end of this peptide between the cysteine residues and the tyrosines. When the tyrosinated peptide YAGA(G_4−Q_{11})AGAY was used in competition binding experiments, it retained the capacity to compete with ^{125}I-factor IXa binding to ∼50% of the sites on activated platelets with a K_i of 2.4×10^{-9} M (Table 2). When this tyrosinated peptide was "mock labeled" with cold iodine, it retained the capacity to compete with ∼50% of the sites for

Table 3: Binding Constants for Normal Factor IXa and Gla Peptide (G_4-Q_{11})

ligand	factor VIII	no. of sites per platelet	app K_d (nM)
factor IXa	absent	625 ± 66	2.8 ± 0.5
	present	600 ± 58	0.7 ± 0.06
peptide (G_4-Q_{11})	absent	327 ± 40	220 ± 55
	present	289 ± 35	195 ± 45

[a] Results presented represent the mean (± SEM) of four separate determinations.

^{125}I-labeled factor IXa, albeit with reduced affinity (K_i ~200 nM) presumably as a consequence of the introduction of 1−2 bulky iodine atoms into the relatively small peptide molecule. To determine whether this tyrosinated and iodinated peptide ^{125}I-(G_4-Q_{11}) binds to platelets, gel-filtered platelets (3.5×10^8 platelets/mL) were incubated, unstirred at 37 °C with labeled peptides in the presence of calcium chloride (5 mM), human thrombin (0.1 unit/mL), thrombin-activated factor VIII (2 units/mL), and factor X (1.5 μM). The incubation mixture was sampled at varying time intervals, and aliquots were centrifuged through silicone oil barriers to separate platelets from unbound proteins. To evaluate the specificity and reversibility of ^{125}I-(G_4-Q_{11}) interaction with platelets, the ability of unlabeled factor IXa to compete for its binding sites was studied. Radiolabeled peptide was mixed with buffer or 100 μg/mL cold factor IXa in the presence of $CaCl_2$ (5 mM), thrombin (0.1 unit/mL), factor VIIIa (2 units/mL), and factor X (1.5 μM). Unlabeled factor IXa competed with the radiolabeled Gla domain peptide for platelet binding sites since only ~25% (equivalent to nonspecific binding) of the ^{125}I-(G_4-Q_{11}) remained platelet-bound in the presence of a 100-fold molar excess of unlabeled factor IXa (data not shown). Neither a factor XI-derived synthetic peptide (C-P-R-G-G-I-S-G-Y-C from the A4 domain of factor XI) nor a prekallikrein-derived synthetic peptide ($N_{235}-K_{266}$) (data not shown) was able to displace ^{125}I-labeled Gla peptide (G_4-Q_{11}), whereas the unlabeled G_4-Q_{11} peptide was able to displace virtually all the labeled tyrosinated peptide. These data confirm that the binding of labeled Gla peptide to activated platelets is specific and reversible.

Since the binding to platelets of the Gla peptide ^{125}I-(G_4-Q_{11}) is specific and reversible, saturation binding studies were carried out in order to estimate the number of sites on activated platelets to which it binds. Saturable binding of the Gla peptide ^{125}I-(G_4-Q_{11}) to thrombin-stimulated platelets was observed (data not shown). Nonspecific binding was measured in the presence of excess unlabeled factor IXa (25 μg/mL or 0.44 μM) and was subtracted from total binding to obtain specific binding. The affinity and stoichiometry of binding for factor IXa and peptide ligands in the absence and presence of factor VIIIa and factor X were determined in four separate experiments, the means (±SEM) of which are given in Table 3. In the absence of factor VIIIa and factor X, there were 625 ± 66 sites/platelet (K_d 2.8 ± 0.50 nM) for factor IXa, and the presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on the number of binding sites for factor IXa and resulted in a decrease in the K_d for factor IXa binding from 2.8 ± 0.50 nM to 0.7 ± 0.06 nM. In comparison, the Gla peptide (G_4-Q_{11}) interacted with about half the number of factor IXa binding sites on the surface of activated platelets (327 ± 40 sites/platelet) in the absence of factor VIIIa and factor X with a K_d = 220 ± 55 nM. The presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on either the number of binding sites (289 ± 35 sites/platelet) or the K_d (195 ± 45 nM) for binding of the Gla peptide (G_4-Q_{11}) to activated platelets.

Theoretical Calculation of the Free Energy of Binding to Activated Human Platelets Mediated by Residues G_4-Q_{11} of the Factor IXa Gla Domain. Based on the determination that the K_d of binding of factor IX/IXa to 250−300 sites per activated human platelet is ~3.0 nM and the conclusion that all the binding energy required for this interaction resides within amino acid residues G_4-Q_{11}, the calculated Gibbs free energy required for this interaction is ~12.1 kcal/mol. In the presence of factor VIII and factor X, the free energy of binding of factor IXa to activated platelets increased to 13.2 kcal/mol.

Effect of Added Gla Peptide (G_4-Q_{11}) or Unlabeled Factor IX on the Specific Binding of ^{125}I-Factor IXa or ^{125}I-Factor IX to Thrombin-Activated Platelets in the Presence and Absence of Factor VIIIa and Factor X. In our studies of the assembly of the factor X activating complex on the platelet membrane, we have shown that factor IX and factor IXa share a common, low-affinity binding site on thrombin-activated platelets (B_{max} ~300 sites/platelet; K_d ~2.5 nM), that factor IXa binds to an additional 200−250 sites/platelet, and that the affinity of factor IXa (but not that of factor IX) binding is increased 5-fold in the presence of factor VIII and factor X (*1*). To determine whether the conformationally constrained cyclic peptide (G_4-Q_{11}) binds to the shared factor IX/IXa binding site or to the site specific for factor IXa, we examined the specific binding of ^{125}I-labeled factor IXa to thrombin-activated platelets in the presence of factor VIIIa and factor X, with and without the Gla peptide (G_4-Q_{11}) at 2 μM, and compared it with the specific binding obtained in the presence of excess factor IX (0.45 μM; 25 μg/mL). The results (Figure 2A) are plotted as specific factor IXa binding after subtracting nonspecific binding from total binding. Both in the presence and in the absence of added Gla peptide (G_4-Q_{11}), the binding of ^{125}I-factor IXa approached saturation at factor IXa concentrations of <10 nM. However, the amount of factor IXa bound in the presence of Gla peptide (G_4-Q_{11}) was ~5.5 pmol/10^{10} platelets as compared with ~8.5 pmol/10^{10} platelets in the absence of added Gla peptide (G_4-Q_{11}) and was indistinguishable from the specific binding isotherm obtained in the presence of added factor IX. Scatchard analysis of the data indicated that both factor IX and the conformationally constrained cyclic peptide (G_4-Q_{11}) competed with only about half (~250 sites/platelets) the factor IXa molecules bound to activated platelets with K_i's ~3 nM. To further confirm that the Gla peptide (G_4-Q_{11}) binds to the shared factor IX/IXa platelet binding site, we also determined the specific binding of ^{125}I-labeled factor IX to thrombin-activated platelets in the presence of factor VIIIa and factor X with and without added 2 μM Gla peptide (G_4-Q_{11}). The binding of factor IX approached saturation at a factor IX concentration of ~10 nM, at which concentration the amount of factor IX bound was ~4.5 pmol/10^{10} platelets (Figure 2B). In the presence of added Gla peptide (G_4-Q_{11}), the binding of ^{125}I-labeled factor IX was virtually identical with that which occurred

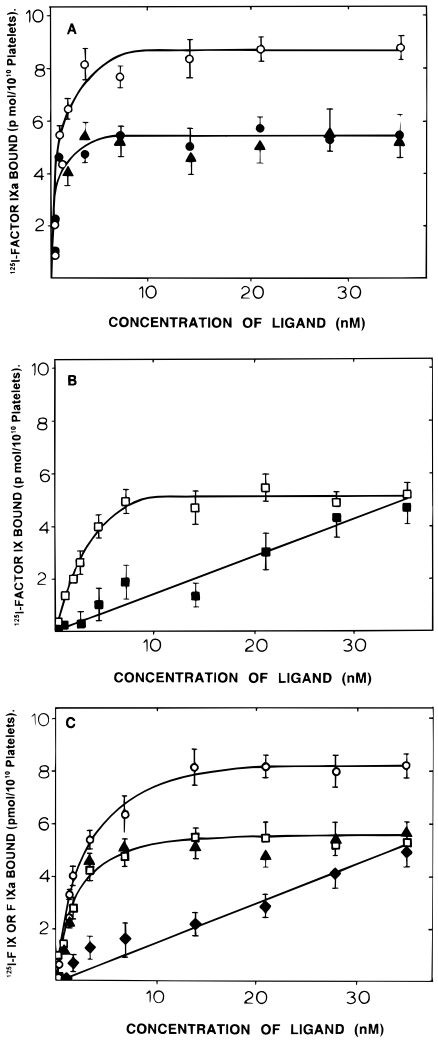

FIGURE 2: Effect of added Gla peptide (G_4-Q_{11}) or the unlabeled factor IX on the specific binding of ^{125}I-factor IXa to thrombin-activated platelets in the presence and absence of factor VIIIa and factor X. Gel-filtered platelets (3.5×10^8 platelets/mL) were incubated for 20 min at 37 °C with human α-thrombin (0.1 unit/mL), $CaCl_2$ (5 mM), and varying concentrations of ^{125}I-labeled factor IXa or factor IX in the presence or absence of thrombin-activated factor VIII (2 units/mL) and factor X (1.5 μM). Similar results were obtained with platelets activated with the thrombin receptor peptide (SFLLRN amide, 25 μM). Binding was determined as detailed under Experimental Procedures. Nonspecific binding was determined in the presence of excess unlabeled factor IXa (0.44 μM; 25 μg/mL) and was subtracted from total binding to obtain specific binding. The results (Figure 2A) represent the specific binding of factor IXa in the absence (○) or the presence of either 4.5 μM unlabeled factor IX (●) or 2 μM Gla peptide (G_4-Q_{11}) (▲), with factor VIII and factor X present. Figure 2B represents the specific binding of ^{125}I-factor IX in the absence (□) and presence (■) of 2 μM Gla peptide (G_4-Q_{11}) with factor VIII and factor X present. Figure 2C represents the effect of 2 μM Gla peptide (G_4-Q_{11}) on the specific binding of ^{125}I-labeled factor IXa (▲) and factor IX (◆) in the absence of factor VIIIa and factor X. The open circles and open squares represent the specific binding of ^{125}I-labeled factor IXa (○) and factor IX (□) in the absence of the Gla peptide and in the absence of factor VIIIa and factor X. The plotted results in Figure 2A−C are the mean ± SEM of duplicate observations from six separate experiments.

in the presence of excess unlabeled factor IX; i.e., it was entirely nonsaturable, suggesting that the Gla peptide (G_4-Q_{11}) interacts only with the shared factor IX/IXa binding site.

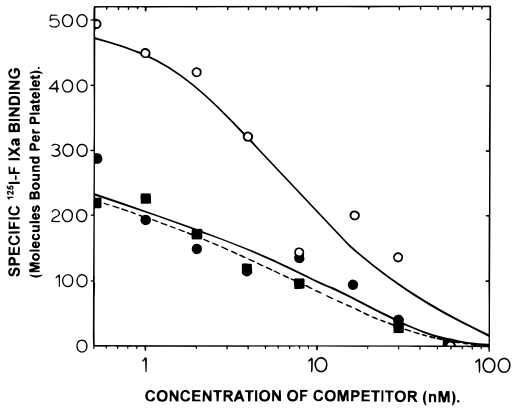

FIGURE 3: Effect of excess factor IX or Gla peptide on the competition binding of factor IXa with ^{125}I-labeled factor IXa to activated platelets. Competition binding experiments with 10 nM ^{125}I-factor IXa and 3.5×10^8 platelets/mL in the presence of either 300 nM factor IX (●) or 2 μM Gla peptide (■) were performed. The open circle (○) represents the binding of ^{125}I-factor IXa in the absence of excess factor IX or Gla peptide. The concentration of competitor shown on the abscissa is that of unlabeled factor IXa. The results represent the means of a single experiment carried out in triplicate.

Finally, to further strengthen our observation that the Gla peptide binds to the shared factor IX/IXa platelet binding site and not to the site specific to factor IXa only or to either the cofactor (factor VIIIa) and/or the substrate (factor X), we determined the effect of Gla peptide (G_4-Q_{11}) on the specific binding of ^{125}I-labeled factor IXa or factor IX to thrombin-activated platelets in the absence of factor VIIIa and factor X. The binding of both factor IXa and factor IX approached saturation at factor IXa or factor IX concentrations (~10 nM) at which the amount of factor IXa or factor IX bound was ~8.5 and ~5.0 pmol/10^{10} platelets, respectively (Figure 2C), or ~512 and ~301 sites per platelet, respectively. In the presence of added Gla peptide (G_4-Q_{11}), the binding isotherm of factor IXa was indistinguishable from the specific binding isotherm of factor IX binding in the absence of factor VIIIa and factor X. The presence of Gla peptide (G_4-Q_{11}) again resulted in entirely nonspecific and nonsaturable binding of ^{125}I-factor IX (Figure 2C). These data further confirm that the Gla peptide (G_4-Q_{11}) interacts only with the shared factor IX/IXa binding site, which does not require the presence of either the cofactor (factor VIIIa) or the substrate (factor X), or the assembly of the factor X activating complex (factor IXa/factor VIIIa/factor X).

Next, we carried out competition studies with unlabeled factor IX or the Gla peptide (G_4-Q_{11}) for ^{125}I-labeled factor IXa binding sites on thrombin-activated platelets in the presence of factor VIII and factor X (Figure 3). For this purpose, we incubated thrombin-stimulated platelets in the presence of $CaCl_2$ for 20 min at 37 °C with ^{125}I-labeled factor IXa and various concentrations of unlabeled factor IX or the Gla peptide (G_4-Q_{11}). When the residual binding of ^{125}I-labeled factor IXa was determined, it was apparent that the excess factor IXa, factor IX, and the Gla peptide (G_4-Q_{11}) all prevented >95% of ^{125}I-labeled factor IXa binding. From the results presented in Figure 3, it is clear that both factor IX and the Gla peptide (G_4-Q_{11}) molecules were able to compete with only half the binding sites (~250 sites/platelet) whereas cold factor IXa was able to compete with all of the

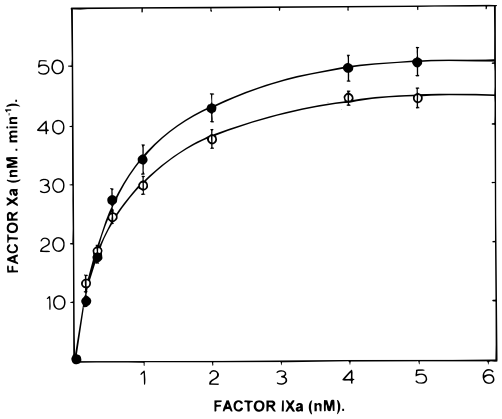

FIGURE 4: Effect of excess factor IX on the kinetics of factor X activation. Rates of factor Xa formation by the factor X activating complex were measured as a function of increasing factor IXa concentrations either in the presence (●) or in the absence (○) of 250 nM factor IX. The reactions were carried out in the presence of 250 nM factor X, 2 units/mL factor VIIIa, 5×10^7 platelets/mL (activated with the thrombin receptor peptide, SFLLRN amide, 25 μM), and 5 mM $CaCl_2$ in the presence or absence of excess factor IX. Similar results were obtained when platelets were activated with thrombin (0.1 unit/mL). The plotted results are the means of duplicate observations from four separate experiments.

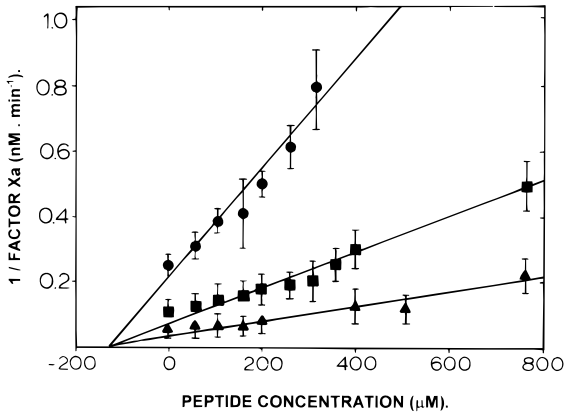

FIGURE 5: Rate of factor Xa formation by factor IXa in the presence of various concentrations of Gla peptide (G_4-Q_{11}). The rates of factor X activation were determined at three different concentrations of factor IXa [0.25 nM (●); 0.5 nM (■); and 2.5 nM (▲)] and in the presence of Gla peptide (G_4-Q_{11}). The reaction volume of 100 μL contained thrombin receptor peptide (SFLLRN amide, 25 μM) activated gel-filtered platelets (5×10^7 platelets/mL), 5 mM $CaCl_2$, 2 units/mL factor VIII, 1.5 μM factor X, and 0.5 mg/mL human serum albumin in HEPES buffer, pH 7.9. Platelets were preincubated with SFLLRN amide, factor IXa, $CaCl_2$, factor VIII, and the peptide (G_4-Q_{11}) for 1 h at 37 °C. Factor VIII was activated with 0.1 unit/mL thrombin before the performance of the assay. Similar results were obtained when platelets were activated with thrombin (0.1 unit/mL). The plotted results are the means of duplicate observations from six separate experiments.

~500 sites/platelet. These data indicate that there are in fact two separate and distinct classes of receptor sites on activated platelet membranes, one of which can interact with either factor IX or factor IXa via the Gla domain, whereas the other site is specific for the enzyme factor IXa and does not bind the zymogen, factor IX.

Effect of Excess Factor IX on Factor X Activation. To determine whether the factor IXa platelet binding site functionally important in the assembly of the factor X activating complex is the shared factor IX/IXa binding site or the specific factor IXa binding site, factor Xa generation rates were examined as a function of factor IXa concentration in the absence or presence of excess factor IX (Figure 4). Since the presence of a >50-fold molar excess of factor IX had insignificant effects on the kinetics of factor X activation, it would appear that the shared factor IXa/IXa binding site, from which factor IXa can be displaced by excess factor IX, is not essential for the assembly of the functional factor X activating complex.

Effect of Factor IX Gla Peptide (G_4-Q_{11}) on Rates of Factor Xa Formation by Factor IXa in the Presence of Factor VIIIa and Activated Human Platelets. To determine whether the factor IXa Gla peptide (G_4-Q_{11}) can inhibit the activation of factor X by factor IXa, we measured the rate of factor Xa formation at varying factor IXa concentrations in the presence of platelets, factor VIIIa, and increasing concentrations of the peptide. A Dixon plot of the resulting data (Figure 5) demonstrated that the Gla peptide (G_4-Q_{11}) is a competitive inhibitor of factor IXa binding to activated platelets with a $K_i = 165 \pm 35$ μM.

DISCUSSION

The contribution of platelets to factor X activation is receptor-mediated since platelets possess specific, high-affinity, saturable binding sites for factor IXa (*1*), factor VIII (*19*), and factor X (*20*), and receptor occupancy is correlated with rates of factor X activation (*2, 3*). Studies from our laboratory have demonstrated that activated human platelets, in the absence of factor VIII and factor X, expose 500−600 factor IXa binding sites per platelet with a K_d(app) of ~2.5 nM, of which 250−300 sites are shared by the zymogen, factor IX (*1*−*7*). The same number of factor IXa binding sites (500−600 per platelet) was demonstrated with enhanced affinity [K_d(app) ~0.5 nM] in the presence of factor VIII and factor X, which have no effect on the affinity or stoichiometry of factor IX binding (*1*−*7*). These observations led us to postulate that a single class of bifunctional receptors exists in the plasma membrane of activated platelets that can accommodate either one factor IX molecule (~250 sites/platelet, K_d ~2.5 nM) or two factor IXa molecules (~500 sites/platelet, K_d ~0.5 nM). The data presented in the present study now force us to revise this hypothesis.

The original purpose of this project was to investigate the role of the Gla domain of factor IX/IXa in its interaction with the plasma membrane of activated platelets. We have previously demonstrated that factor IXa molecules from which the Gla domain has been removed by enzymatic digestion or in which the Gla residues have been chemically modified exhibit a diminished capacity (i.e., both decreased affinity and decreased stoichiometry) to bind to activated platelets, although the k_{cat} for factor X activation, calculated on the basis of the number of bound factor IXa molecules, was normal (*4*). This observation led us to examine the role of specific residues within the Gla domain in this interaction. Studies with chimeric factor IXa molecules in which either factor X or factor VII sequences were inserted implicated amino acid residues (G_4-Q_{11}) within the so-called "omega loop" at the amino terminus of factor IX, possibly G_4, K_5, L_6, F_9, V_{10}, and Q_{11}, as important in the binding of factor IXa to activated platelets (*7*). Similar results were obtained in studies of factor IX binding to endothelial cells (*21*). These results are entirely consistent with recent elegant studies using

NMR spectroscopy to identify L_6 and F_9 as comprising portions of a hydrophobic patch at the amino terminus of factor IX that interacts with artificial phospholipid membranes (*22*).

An important and consistent observation made during our studies of chimeric, Gla-domainless, and Gla-modified factor IXa molecules with point mutations within the "omega loop" is that the number of sites on activated platelets with which these proteins interact is reduced by ~50% as compared with normal or wild-type factor IXa (*4, 7*). This observation suggests that there may in fact be two classes of binding sites within the factor IXa molecule, one of which mediates binding to ~250−300 sites/platelet through the Gla domain (G_4-Q_{11}), the other of which mediates binding to a separate and distinct set of sites (250−300/platelet) through some other domain within the factor IXa molecule. To confirm this supposition and to determine the functional role of these two putative sites in factor X activation, we have prepared conformationally constrained peptide analogues of the Gla domain binding site. These peptides were able to displace only ~50% of the bound factor IXa molecules (Figure 1) with K_i values nearly identical with the K_d for factor IXa binding to activated platelets in the absence of factors VIII and X (~2.5−3.0 nM) (Table 2). This confirms two important facts: first that only ~250−300 binding sites/platelet are mediated via the Gla domain, and second that all the binding energy required for this interaction (~12.1 kcal/mol) resides within amino acid residues G_4-Q_{11}. To determine more precisely the stoichiometry of binding of the Gla domain peptide to activated platelets, we carried out binding studies using a derivatized peptide with tyrosine residues to facilitate iodination. Unfortunately, "mock iodination" of the Gla peptide significantly decreased the affinity of this peptide for platelet binding sites since the K_i was increased from 2.4 nM to ~200 nM after iodination with cold carrier iodine (~1−2 atoms of iodine per peptide molecule). However, the "mock labeled" peptide was still able to displace ~50% of the bound factor IXa. Therefore, we assumed that the derivatized peptide would be a reasonable probe for determining the number of Gla peptide binding sites on activated platelets. The results of direct binding studies with the derivatized radiolabeled Gla peptide confirm the results with both derivatized and nonderivatized peptides and indicate that the stoichiometry of Gla peptide binding sites is almost exactly 50% of the total binding sites for factor IXa (Table 3). To address the question whether the sites to which the Gla peptide bind are those that are shared by both factor IX and factor IXa, we carried out direct (Figures 2A,B,C) and competition (Figure 3) factor IX and factor IXa binding studies in the presence and absence of the conformationally constrained Gla peptide G_4-Q_{11}. These experiments prove that the site to which the Gla peptide binds is the shared factor IX/IXa site and that factor IXa must also bind to a separate and distinct, high-affinity binding site on activated platelets that is specific for factor IXa and is mediated by a domain other than the "omega loop" (G_4-Q_{11}). When we determined the specific binding isotherms of ^{125}I-factor IX or factor IXa in the absence of factor VIIIa and factor X, the Gla peptide (G_4-Q_{11}) was able to prevent completely the binding of factor IX and to displace factor IXa from approximately half its platelet sites, leaving the remaining sites (approximately half) intact (Figure 2C). We conclude that the site to which the Gla peptide (G_4-Q_{11}) binds is the shared factor IX/IXa binding site on the surface of activated platelets and that the presence of neither the cofactor (factor VIIIa) nor the substrate (factor X) is required for the interaction of factor IX or factor IXa with this binding site.

Our conclusion that the "omega loop" peptide G_4-Q_{11} contains all the binding energy (~12 kcal/mol) required to mediate binding to the shared factor IX/IXa binding site is supported not only by the fact that the conformationally constrained peptide was able to displace factor IXa from the shared site with a K_i (2.9 nM) equivalent to the K_d for factor IXa binding, but also by the fact that an equimolar mixture of the peptides S_3-L_6 and F_9-Q_{11} had an equivalent capacity to G_4-Q_{11} in competition studies. Appropriate control peptides, including a "scrambled" peptide and the reduced and alkylated G_4-Q_{11} peptide, were without activity, indicating that the binding activity of G_4-Q_{11} is both sequence-specific and conformation-specific. This conclusion is supported by the observation that the conformationally constrained reverse-D analogue had potency equivalent to the G_4-Q_{11} peptide. The rationale for this experiment is that the reverse-D analogue, otherwise referred to as a retro-inverso peptide (*23, 24*), differs from the parent peptide in both the direction of the peptide bonds and the chirality of the amino acids, thereby maintaining the overall stereochemical relationships of the side chains. Therefore, such peptides retain the same surface characteristics as the native peptide but are resistant to proteolysis by proteases (*24*). The results with all these peptides (Table 2) support the conclusion that the sequence of amino acids G_4-Q_{11} comprising the "omega loop" at the amino terminus of the Gla domain contains a conformationally constrained, sequence-specific structure that mediates 100% of the binding of protein to the shared factor IX/IXa binding site.

Perhaps the most important question concerning the characterization of the Gla domain site shared by factors IX and IXa for binding to activated platelets is whether it is or is not the site utilized by factor IXa for the assembly of the functional factor X activating complex. To address this question, we initially examined the effect of a large molar excess of factor IX on the activation of factor X by factor IXa in the presence of activated platelets and factor VIII. Since factor IX displaces factor IXa from the shared site but not from the specific factor IXa binding site, the failure of factor IX to inhibit rates of factor Xa generation by various concentrations of factor IXa (Figure 4) suggests that the shared factor IX/IXa binding site is *not* the site involved in the assembly of the functional factor X activating complex. This conclusion is supported by the results of the experiment (Figure 5) in which the inhibitory effects of the Gla domain peptide (G_4-Q_{11}) on factor X activation by factor IXa were examined. This peptide, which displaces factor IX and factor IXa from their shared site on activated platelets with a K_i of ~3.0 nM, is required at ~50 000-fold higher concentration to inhibit factor IXa catalyzed factor X activation (K_i ~165 μM). We conclude that the "omega loop" (G_4-Q_{11}) of factor IX mediates the binding of factor IX and factor IXa to ~250−300 shared sites per activated platelet, and that the assembly of the functional factor X activating complex is mediated by the binding of factor IXa to an additional, separate and distinct site (~250−300 sites/platelet). We

present evidence elsewhere (manuscript in preparation) that the site mediating the binding of factor IXa to activated platelets and required for assembly of the functional factor X activating complex resides within the second epidermal growth factor domain.

The functional role of the shared factor IX/IXa binding site and the possible relationship between occupancy of this site and occupancy of the high-affinity, specific factor IXa binding site within the factor X activating complex is an important subject for future investigation. One possibility is that the shared factor IX/IXa binding site exists primarily to colocalize the substrate, factor IX, with the enzyme, factor XIa, on the surface of activated platelets, which contains binding sites for both factor XI (*25*) and factor XIa (*26*), and has been shown to promote the proteolytic activation of factor XI by factor XIIa (*27*) and by thrombin (Baglia and Walsh, unpublished experiments). This shared factor IX/IXa binding site could also serve as a source of platelet membrane-bound factor IXa molecules that can interact with the specific factor IXa binding site that is required for the assembly of the factor X activating complex.

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