The Distinct Roles That Gln-192 and Glu-217 of Factor IX Play in Selectivity for Macromolecular Substrates and Inhibitors†

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ABSTRACT: In this paper, we report functional characterization of positions 192 and 217 (chymotrypsinogen numbering system) in human factor IX and discuss the distinction and similarity of these two sites among the blood coagulation factors. Recombinant factor IXQ192E (residue glutamine at position 192 replaced by glutamic acid), IXQ192K, IXE217D, and IXE217R proteins exhibited 11%, 46%, 39%, and 2% of the wild-type factor IX's clotting activity, respectively. Binding of these variants to factor VIIIa (FVIIIa) was inefficient compared to that of wild-type factor IX, and the dissociation constants doubled for IXQ192E, 3-fold higher for IXQ192K and 4-fold higher for both IXE217D and IXE217R. In the presence of FVIIIa, all variant factor IX hydrolyzed factor X at the catalytic efficiencies correlating with respective clotting activities. However, FVIIIa greatly enhanced the catalytic efficiency of both IXE217 variants to a greater extent ($\sim 7 \times 10^4$-fold) as compared to its effect on the wild-type factor IXa and the other two IXQ192 variants [by a factor of $(1-2) \times 10^4$]. Moreover, while both IXQ192 variants demonstrated small substrate selectivity similar to that of wild-type factor IXa, the selectivity of both IXE217 variants was greatly altered. Mutations at position 192 disturbed the interaction of factor IXa with physiological inhibitors. Although all variants formed an SDS-stable complex with antithrombin III (ATIII) equally well in the presence of heparin and were readily inhibited by ATIII in the absence of heparin, activated IXQ192K exhibited a slower stable complex formation with ATIII without heparin. On the other hand, only IXQ192E showed decreased interaction with TFPI. Our results demonstrate that positions 192 and 217 play different roles unique to factor IX in specifying the interaction of factor IX with substrates and inhibitors.

Blood coagulation factors, i.e., prothrombin, factors VII, IX, and X, and protein C, belong to the serine protease super gene family, one of the most well-characterized groups of enzymes both structurally and functionally. They are vitamin K-dependent factors with a trypsin-like catalytic domain of histidine-aspartic acid-serine at comparable positions and functions (*1*). Unlike trypsin, the blood-clotting proteases interact rather specifically with their cognate cofactors, substrates, and inhibitors (*1*). Many studies of these interactions have suggested that positions 192 and 217 (chymotrypsinogen numbering system) are crucial for the selectivity of these enzymes, e.g., thrombin (*2*−*5*), factor X (*6*, *7*), and factor VII (*8*−*10*), and protein C of the anticoagulation system (*11*). Recombinant thrombin with the 192 position replaced by glutamine (thrombin E192Q) activated protein C approximately 20 times more rapidly than wild-type thrombin (*2*, *7*). In protein C, changing the glutamic acid at this position to glutamine increased the rate of inhibition of the activated protein C by α1-proteinase inhibitor by a factor of approximately 300 (*11*). Factor X contains glutamine at position 192, and replacing it by glutamic acid or methionine eliminates factor X's catalytic function toward prethrombin 2 (*6*, *7*). This is overcome by the presence of factor Va in the reaction, suggesting that factor Va could modify the conformation of the enzyme and/or the substrate (*6*). Factor VII exhibits a preference for lysine at position 192. Several studies, including ours, have shown that substitution of lysine with glutamine at this position in factor VII reduced its clotting activity to 44%, and substitution with glutamic acid generated a completely inactive molecule with greatly altered selectivity toward factor X, factor IX, and the synthetic substrate chromozyme tPA[1] (*8*, *10*). The glutamine substitution facilitated the interaction of factor VII with BPTI (bovine pancreatic trypsin inhibitor), whereas the glutamic acid substitution diminished its interaction. Furthermore, both factor VII variants (K192E and K192Q) were barely inhibited by TFPI (*8*). On the other hand, a recent study has indicated that, unlike with the coagulant proteins, position 192 does not contribute significantly to the substrate or inhibitor specificity of the tissue-type plasminogen activator in the fibrinolytic system (*12*).

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According to the crystal structure of the serine proteases, both positions 192 and 217 are within the second β-barrel of the catalytic domain. Position 192 is within loop 1 (residues 184−195) and is one of the extensive walls of residue 193 and 195−substrate hydrogen bindings (reviewed in ref *13*). Position 217 is in loop 2 (residues 213−228), close to the catalytic cleft of residues 214−216. Both positions 192 and 217 are thought to confer the recognition of either or both P2 and P3 residues (*14*, *15*). Position 217 plays an interesting role in the selectivity of thrombin. Like position 192, the substitution at 217 of thrombin affects thrombin's substrate selectivity. In particular, the substitution with alanine and lysine converted thrombin into an anticoagulant more than a procoagulant enzyme (*5*, *16*). The authors of this paper and others have reported that position 217 in factor VII is important for P3 recognition and inhibitor binding (*9*, *15*). In addition, factor VIIQ217A binds antithrombin III (ATIII) with twice the affinity of wild-type factor VII (*15*).

Among the vitamin K-dependent blood-clotting factors, factor IX has comparatively more stringent selectivity for substrates in these cognate enzyme−cofactor−substrate interactions. Factor VIIIa (FVIIIa), so far, is the only macromolecular protein cofactor identified for factor IXa. Upon complex formation with FVIIIa, the catalytic function of factor IXa toward its physiological substrate, factor X, is enhanced 200 000 times (*17*, *18*). By replacement and by point mutation studies, two regions at the catalytic domain of factor IX have been suggested to play some role in the FVIIIa-dependent stimulation (*19*, *20*). One is loop c34−40 (loop 199−204 of factor IX, ref *21*), located near the active site, and the other region is loop c132−134 (301−303 of factor IX, refs *20*, *22*) and a surface-exposed helix of residues 162−170 (330−338 of factor IX, refs *22*, *23*). The surface helix is in close proximity to the corresponding region in factor VII proposed for TF binding. However, the mutations at these regions had limited effects on the FVIIIa interaction. This leads to the question about whether any other regions of factor IX are also involved in FVIIIa interaction. In addition to that question, another question raised is whether the functions in which positions 192 and 217 are supposed to be involved will also confer the same aspects in factor IX. This study attempts to address the structural and functional contributions of positions 192 and 217 to the interaction of factor IX with substrates and inhibitors.

MATERIALS AND METHODS

Materials. All the restriction endonucleases and polymerases were obtained from New England Biolabs, Inc. (Beverly, MA). Geneticin (G418) was from GibcoBRL (Gaithersburg, MD). Factor IX deficient plasma, phosphatidylcholine (PC), phosphatidylserine (PS), and ATIII were purchased from Sigma (St. Louis, MO). The concentration of ATIII was calculated as described (*12*). Human factor XIa and factor X were from Enzyme Research Laboratory (South Bend, IN). Plasma-derived human factor VIII (Monoclate-P) was from Armore Pharmaceutical Co. Inc. (Aventis Behring, Marburg, Germany). Recombinant factor VIIa was prepared as described (*15*). Recombinant human TF was a gift from Dr. Wolfram Ruf (Department of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA). TFPI was a gift from Dr. Y. Kamikubo (Kaketsuken, Kyokushi Kikuchi, Kumamoto, Japan). Chromozyme tPA and ITS supplements used in serum-free media were from Boehringer Mannheim (Germany). Monoclonal antibodies A1, A5, and A7 against human factor IX were described previously (*24*). A *V*max microtiter plate reader (Molecular Devices Corp., Menlo Park, CA) equipped with a thermal controller was used for all spectrophotometric assays. QAE-Sephadex, Mono Q column, Akta FPLC purifier, and Enhanced Chemiluminescence (ECL) Western blotting detection reagents were from Amersham (Amersham Pharmacia BioTech, UK). Spectrozyme FXa and Spectrozyme FIXa were purchased from American Diagnostica (Greenwich, CT). Substrates S2222, S2288, S2238, S2366, and S2765 were from Chromogenix (Sweden), and CBS31.39 was from Diagnostica Stago (France).

In Vitro Mutagenesis, Expression of Factor IX, and Protein Purification. Mutagenesis was performed as described previously (*15*, *25*). The primer sequences were 5′-GAAGGAGGTAGAGACAGCTGT*GAA(or AAA)*GGAGATAGTGG-3′ and 5′-TTATTAGCTGGGGT*GAC(or CGC)*GAGTGCGCAATGAAAGGCAA-3′ for generating cDNA fragments with the Gln-362 and Glu-387 codons replaced by those of glutamic acid (or lysine) and aspartic acid (or arginine), respectively. The altered cDNA was fully sequenced before being expressed in the human 293 cells as described previously (*25*). To collect media with large quantities of factor IX, transfected cell clones were isolated and expanded in serum-free media as described (*15*, *25*). The factor IX concentration, estimated by ELISA, was approximately 0.5−2 mg/L.

Protein Purification, Immunoblotting, and Clotting Assay. Recombinant factor IX was purified from the cultured supernatant through a QAE Sephadex-A50 column eluted with 12−20 mM $CaCl_2$ in TBS (20 mM Tris-HCl, pH 7.4, 100 mM NaCl) and 0.5 mM benzamidine (*25*). Further purification was performed with a column coupled with the metal ion-dependent A7 antibody (*24*) or by a Mono Q column (*25*). Purified proteins were verified by SDS/PAGE and visualized by staining with silver nitrate (silver stain kit, Bio-Rad, Hercules, CA) or immunoblotting using antibody A1 followed by chemiluminescence (*24*, *25*). The protein concentration was measured by a protein assay kit (Bio-Rad) modified from the Bradford method (*26*). Factor IX clotting activity was measured by the one-stage clotting assay using factor IX deficient plasma as described previously (*27*).

Activation of Factor IX by Factor XIa and Factor VII−TF Complex. The effect of mutation on the activation of factor IX was analyzed at an enzyme-to-substrate ratio of 1:200 for factor XIa and 1:45 for VIIa−TF complex (*25*).

[1] Abbreviations: ATIII, antithrombin III or antithrombin; FVIIIa, factor VIIIa; IXQ192E and IXQ192K, IXE217D and IXE217R, the glutamine at residue 192 (amino acid residue 362 in factor IX) and the glutamic acid residue at 217 (387 in factor IX) replaced by glutamic acid and lysine and by aspartic acid and arginine, respectively; TFPI, tissue factor pathway inhibitor; PCPS, phosphatidylcholine and phosphatidylserine; ITS, insulin−transferrin−sodium selenite; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; PEG, polyethylene glycol; TF, tissue factor; tPA, tissue-type plasminogen activator; VIIaQ217A, VIIaK192Q, and VIIaK192E, activated factor VII with the glutamine at residue 217 (chymotrypsinogen numbering system) replaced by alanine and the lysine at residue 192 replaced by glutamine and by glutamic acid, respectively; BPTI, bovine pancreatic trypsin inhibitor; PPACK, D-Phe-Pro-Arg-chloromethyl ketone.

The final concentration of factor IX in reaction with factor XIa was 0.9 μM. Reaction was at 37 °C in TBS, 5 mM $CaCl_2$ (TBS/Ca), and 0.1% PEG. Activation of factor IX by VIIa−TF used factor VIIa in complex with relipidated recombinant TF (*15*). Factor IX (0.6 μM) was added to a 13 nM sample of the VIIa−TF complex in a total volume of 60 μL of TBS/Ca/0.1% PEG with 85 μM PCPS (from the relipidated TF). The reaction mixtures of both XIa and VIIa−TF analyses were withdrawn at timed intervals (0−60 min), placed in a gel loading buffer (1% SDS, 2 mM EDTA, and 8% glycerol), boiled for 5 min, and then revealed by SDS/PAGE and silver staining.

Enzyme Activities. Factor IXa and the amidolytic activity assays of the enzyme were prepared as described previously (*15*, *24*, *25*). The change of absorbance at 405 nm was recorded continuously at 37 °C on the plate reader, and the data were used to calculate K_m and k_{cat}'s (*15*). The amidolytic activities of factor IXa were also performed in the presence of freshly prepared FVIIIa (*25*). Five microliters of FVIIIa was mixed with 35 μL of factor IXa preincubated with PCPS in TBS/Ca/BSA. The mixture was allowed to stand at room temperature for 5 min to form the intrinsic tenase complex before the addition of 10 μL of a mixture of PCPS and different concentrations of peptide substrates to start the reaction. Final concentrations were as follows: wild-type or mutant factor IXa, 20 nM; FVIIIa, 6 nM; PCPS, 40 μM; and peptide substrates, 0.4−2 mM. Cleavage of factor X by wild-type and mutant factor IXa was assayed as described (*25*). In the absence of FVIIIa, factor IXa was incubated with PCPS at room temperature for 5 min. Spectrozyme FXa and factor X were added to the mixture to start the reaction at 37 °C in TBS/Ca/BSA/PCPS. Final concentrations were as follows: factor IXa, 10 nM; PCPS, 40 μM; Spectrozyme FXa, 0.5 mM; and factor X, 0.025−1 μM. When analysis was carried out in the presence of FVIII, 25 μL of FVIIIa was mixed with an equal volume of factor IXa. The mixture was allowed to stand for 5 min to form the intrinsic tenase complex before the addition of 50 μL of a mixture of PCPS, Spectrozyme FXa, and factor X to start the reaction. The intrinsic tenase activity was detected kinetically on the microtiter plate reader. Final concentrations were the following: wild-type or mutant factor IXa, 0.25 nM; FVIIIa, 0.4 nM; PCPS, 40 μM; factor X, 0−200 nM; and Spectrozyme FXa, 0.5 mM. The factor IXa activity in the presence and absence of FVIIIa was calculated by the following equation as described (*27*):

$$\text{absorbance } (A_{405}) = at^2 + bt + c$$

Binding of Factor IXa to Factor VIIIa. Binding experiments were performed by monitoring the intrinsic tenase activity at limited concentrations of FVIIIa, as described (*25*). The tenase complex was formed by incubating 25 μL of freshly prepared FVIIIa and 25 μL of different concentrations of wild-type or mutant factor IXa (0−20 nM), as described above. Activity of the intrinsic tenase complex was then measured by the addition of 50 μL of factor X and Spectrozyme FXa in TBS/Ca/BSA/PCPS. Final concentrations were as follows: FVIIIa, 0.4 nM; factor IXa, 0−5 nM; PCPS, 40 μM; factor X, 100 nM; and Spectrozyme FXa, 0.5 mM. Experiments were performed in duplicate for 3−5 independent reactions, and curves were fitted using all data points. The K_d values were derived by calculations described previously (*28*, *29*) using the equation:

$$[\text{IXa}-\text{VIIIa}] = \frac{[\text{IXa}]_t + [\text{VIIIa}]_t + K_d}{2} - \frac{\sqrt{([\text{IXa}]_t + [\text{VIIIa}]_t + K_d)^2 - 4[\text{IXa}]_t[\text{VIIIa}]_t}}{2}$$

Inhibition by ATIII and TFPI. The inhibition of factor IXa by ATIII was performed by gel analysis of the enzyme−inhibitor complex and by binding in solutions. When performed by gel analysis, incubation was at 37 °C in 100 μL of TBS/Ca containing 0.6 μM factor IXa and 2.9 μM ATIII with or without 0.6 unit/mL heparin. Aliquots (20 μL) of the reaction mixtures were withdrawn at timed intervals (0−30 min for presence of heparin and 0−24 h for absence of heparin), stopped by the gel loading buffer, and developed by SDS/PAGE and silver stain. When analysis of ATIII inhibition was assessed by slow-binding kinetics in the absence of heparin, the activated factor IXa (wild-type and mutants) was incubated at 37 °C with different concentrations of ATIII and CBS31.39 in 100 μL of TBS/Ca/BSA/PCPS. Absorbance at 405 nm was measured kinetically with a microtiter plate reader. Final concentrations were as follows: ATIII, 0−2.5 μM; FIXa, 10 nM; CBS31.39, 2.5 mM. Data of the slow-binding inhibition curves were calculated by the following two equations to derive the first-order rate constant, k', k_{ass}, and k_{dis}, as described by Morrison and Walsh (*30*):

$$A = v_s t + (v_0 - v_s)(1 - e^{-k't})/k' + A_0 \quad (1)$$

$$k' = k_{dis} + \frac{k_{ass}}{1 + [\text{S}]/K_m}[\text{I}] \quad (2)$$

where A is the absorbance at 405 nm at time t, v_0 and v_s are the initial and steady-state velocities, respectively, k' is the apparent first-order rate constant, and A_0 is the initial absorbance at 405 nm.

The reaction with TFPI was monitored by amidolytic assays in a microtiter plate format as described (*8*, *15*). Factor IXa (10 nM) was incubated with PCPS at room temperature for 5 min, and Spectrozyme FIXa (1 mM) and different concentrations (0−1 μM) of TFPI were then added to the mixture to start recording the change of absorbance for 120 min at 37 °C. The result was used to calculate the hydrolysis rate for each reaction. Residual activity of each factor IXa was determined relative to the activity in the absence of TFPI. The rates in the absence of TFPI were taken as 100%.

RESULTS

Effects of Mutations at Positions 192 and 217 on the Clotting Activity of Factor IX and Its Binding to Factor VIIIa. We have expressed four factor IX variants with single-point mutations at positions 192 and 217: two with Q192 replaced by glutamic acid (IXQ192E) and lysine (IXQ192K), and the other two with E217 replaced by aspartic acid (IXE217D) and arginine (IXE217R). All four variant proteins were secreted into the cultured supernatant at a level equivalent to that of the wild-type factor IX. The influence of the four substitutions on factor IX was first observed in the clotting

Table 1: Specific Activities[a] and FVIIIa Binding Parameters[b]

zymogen/enzyme	aPTT (%)	K_d (nM)
IXwt	100 ± 6.5	0.87 ± 0.02
IXQ192E	11.4 ± 1.1	1.69 ± 0.38
IXQ192K	46.0 ± 5.0	2.20 ± 0.09
IXE217D	39.4 ± 5.3	3.43 ± 0.29
IXE217R	1.9 ± 0.1	3.00 ± 0.37

[a] Shown are results from aPTT divided by protein concentrations determined by ELISA. Normal pooled plasma was used to construct a standard curve and defined as 100% for both aPTT and ELISA assays. [b] Mean ± standard deviation, $n = 3$.

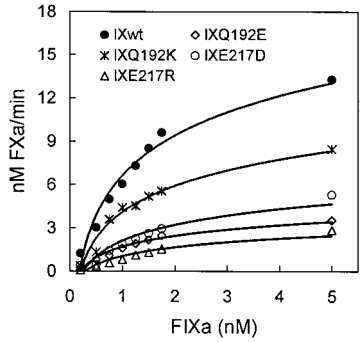

FIGURE 1: Binding of wild-type and mutant factor IXa to cofactor, FVIIIa. The total volume of the reaction mixtures was 100 μL, and the final concentrations were FVIIIa, 0.4 nM, factor X, 100 nM, Spectrozyme FXa, 0.5 mM, and factor IXa, 0−5 nM, in TBS/Ca/BSA/PCPS. The reaction was measured at 37 °C kinetically on a microtiter plate reader, and the results were calculated as described under Materials and Methods. Duplicates of 3 independent experiments were performed, and the mean of the rate of factor Xa (FXa) generation at each factor IXa concentration was plotted.

assay. As shown in Table 1, IXQ192K and IXE217D exhibited 40−50% of the normal clotting activity of factor IX. The other two variants, IXQ192E and IXE217R, showed only 11.4% and 1.9% of normal activity, respectively. None of the mutations prolonged the ox brain thromboplastin time or depicted the hemophila Bm phenotype (data not shown). All mutant factor IX's could be activated by factor XIa or by factor VIIa−TF complex, and the efficiency for product generation as observed in SDS/PAGE was indistinguishable from wild-type factor IX by either enzyme, suggesting that the lower clotting activities of the variants were not due to incomplete activation (data not shown). When the binding to FVIIIa was examined by evaluating the tenase activity of the factor IXa and FVIIIa complex, all the variants were defective in binding FVIIIa and displayed 2−4-fold increases in K_d as compared to wild-type factor IX (Table 1 and Figure 1). The results might partially explain the decreased levels of clotting activity of these mutants. However, the extent of the loss of the clotting activity observed with IXQ192E and IXE217R cannot be simply explained by the reduced affinity of the variants toward FVIIIa, since their counterparts, IXQ192K and IXE217D, had similar K_d's.

Activation of Factor X by Mutant Factor IXa's Was Enefficient. To assess the cause of different clotting activities, cleavage of factor X was analyzed with normal and mutant factor IXa's, and the results are shown in Figure 2. Table 2 summarizes the Michaelis−Menten parameters. In the absence of cofactor FVIIIa, all the mutants had more or less similar K_m values, with the exception of IXE217R, which displayed a significantly higher K_m (10-fold) relative to wild-type factor IX, and an overall 50-fold reduction of catalytic

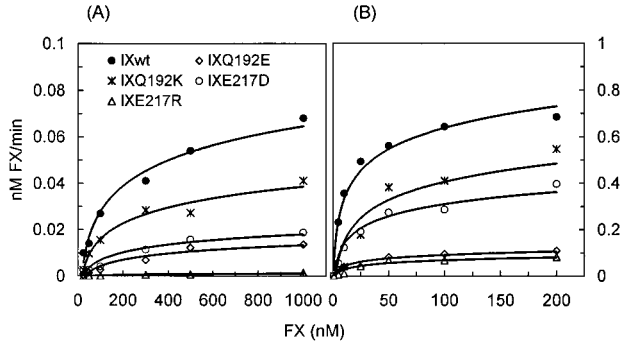

FIGURE 2: Kinetics of factor X activation by wild-type and mutant factor IXa. (Panel A) In the absence of FVIIIa. Total volumes were 100 μL. Final concentrations were factor IXa, 10 nM, Spectrozyme FXa, 0.5 mM, and factor X, 0.025−1 μM, in TBS/Ca/BSA/PCPS. Reaction conditions and calculations were as described in Figure 1. The K_m and V_{max} values were 274.4 ± 10.8 nM and 0.15 ± 0.05 nM FXa/min for wild-type factor IXa, 150 ± 25.8 nM and 0.014 ± 0.003 nM FXa/min for IXaQ192E, 151 ± 13.1 nM and 0.065 ± 0.007 nM FXa/min for IXaQ192K, 314.1 ± 25.8 nM and 0.018 ± 0.002 nM FXa/min for IXaE217D, and 3019.01 ± 37.2 nM and 0.045 ± 0.007 nM FXa/min for IXaE217R. (Panel B) In the presence of FVIIIa. Final concentrations were factor IXa, 0.25 nM, FVIIIa, 0.4 nM, factor X, 0−200 nM, and Spectrozyme FXa, 0.5 mM, in 100 μL of TBS/Ca/BSA/PCPS. The K_m and V_{max} values were 18.9 ± 1.4 nM and 2.48 ± 0.035 nM FXa/min for wild-type factor IXa, 15.9 ± 1.9 nM and 0.114 ± 0.009 nM FXa/min for IXaQ192E, 40.7 ± 5.5 nM and 0.61 ± 0.27 nM FXa/min for IXaQ192K, 51.5 ± 5.2 nM and 0.624 ± 0.009 nM FXa/min for IXaE217D, and 57.5 ± 8.6 nM and 0.16 ± 0.05 nM FXa/min for IXaE217R.

function that seems to correlate with its 1.9% clotting activity. The other variants were also defective in cleavage of factor X; factor IXQ192E revealed a 12-fold reduction in k_{cat}; IXQ192K, nearly 3-fold; and IXE217D, 8-fold, relative to wild-type factor IX. With the addition of FVIIIa, phospholipids, and calcium ions, the rate of catalysis (k_{cat}) increased dramatically for both wild-type and mutant factor IXa's (Figure 2 and Table 2). The effect of FVIIIa on the Q192 and E217 mutations is different. FVIIIa potentiated the rates (k_{cat}) of factor Xa generation by IXQ192E and IXQ192K up to as high as 2250 and 2830 times normal levels, respectively. It enhanced the k_{cat} of wild-type factor IXa by nearly the same figure (a factor of 2300). However, FVIIIa greatly enhanced the catalytic efficiency of IXE217D and caused a nearly 12 400-fold increase in the k_{cat}. FVIIIa also decreased the K_m values of the variants, especially that of IXE217R. Apparently, the decreased tenase activities of IXE217D and IXE217R were due to the combination of a 2.7- and 3-fold higher K_m (51.5 and 57.5 nM, respectively) as well as a 1.5- and 6-fold lower k_{cat} (0.371 and 0.096 s^{-1}, respectively) as compared to wild-type factor IXa.

Alteration of Substrate Selectivity of the Variant Factor IX's. We used a series of chromogenic substrates to investigate the selectivity of these variants. Table 3 and Table 4 summarize respective results analyzed in the absence and presence of FVIIIa. In the absence of FVIIIa, the K_m and k_{cat} were mostly unchanged or within a 1−2-fold difference (Table 3). Wild-type factor IXa preferentially hydrolyzed substrates with glycine at P2 (as in S2765), and basic or hydrophobic residues such as arginine, leucine, and isoleucine (as in S2765) but not glutamic acid (as in S2366) at P3. It appears that the activities of wild-type factor IXa, IXQ192E, and IXQ192K were very similar for many

Table 2: Kinetic Parameters for Factor IXa Activation of Factor X in the Presence and Absence of Factor VIIIa

	K_m (nM)	V_{max} (nM FXa min^{-1})	[enzyme] (nM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mol^{-1}\ s^{-1}$)
without FVIIIa					
IXwt	274.4 ± 10.8	0.150 ± 0.050	10	25×10^{-5}	1093
IXQ192E	150.0 ± 25.8	0.014 ± 0.003	10	2×10^{-5}	133
IXQ192K	151.0 ± 13.1	0.065 ± 0.007	10	10×10^{-5}	662
IXE217D	314.1 ± 25.8	0.018 ± 0.002	10	3×10^{-5}	96
IXE217R	3019.01 ± 37.2	0.045 ± 0.007	10	7.5×10^{-5}	24
with 0.4 nM FVIIIa[a]					
IXwt	18.9 ± 1.4	2.475 ± 0.035	0.071	0.577	30.5×10^6
IXQ192E	15.9 ± 1.9	0.114 ± 0.009	0.044	0.045	3×10^6
IXQ192K	40.7 ± 5.5	0.610 ± 0.269	0.036	0.283	6.9×10^6
IXE217D	51.5 ± 5.2	0.624 ± 0.009	0.028	0.371	7.2×10^6
IXE217R	57.5 ± 8.6	0.164 ± 0.049	0.028	0.096	1.7×10^6

[a] Concentrations of FIXa−FVIIIa complex were derived from experimental conditions and the K_d values of wild-type and mutant factor IXa for FVIIIa (see Table 1).

Table 3: Hydrolysis of Synthetic Peptide Substrates by Wild-Type and Mutant Factor IXa[a]

	enzymes				
substrates	IXwt	Q192E	Q192K	E217D	E217R
S2765	22.7	13	11.3	ND[c]	ND
(Z-D-R-G-R-pNA)	(3.9/0.172)	(3.3/0.251)	(3.3/0.291)		
Spectrozyme FIXa	13.5	23.1	9.2	20.8	20.4
(H-D-L-phG-R-pNA)	(3.5/0.26)	(4.1/0.177)	(3.3/0.359)	(12.5/0.6)	(2.5/0.122)
S2288	6.6	8.1	5.5	ND	ND
(D-I-P-R-pNA)	(2.4/0.363)	(2.6/0.32)	(2.7/0.5)		
Chromozyme tPA	2.6	2.9	2.9	ND	ND
($McSO_2$-D-F-G-R-pNA)	(2.58/0.995)	(3.3/1.15)	(2.8/0.93)		
CBS31.39	2.2	4	3.9	5.1	5.6
($McSO_2$-D-L-G-R-pNA)	(1.1/0.511)	(3.9/0.997)	(3.15/0.8)	(4.7/0.92)	(5.2/0.927)
Spectrozyme FXa	2.8	1.4	2.4	1.4	1.8
(MeO-CO-D-chG-G-R-pNA)	(8.3/3.8)	(2.3/1.6)	(3.6/1.5)	(3.3/2.4)	(2.8/1.6)
S2222[b]	0.21	0.18	0.19	0.23	0.17
(Bz-I-E-G-R-pNA)	(1/4.7)	(1.6/9.1)	(0.8/4.2)	(0.8/3.6)	(1.2/7.1)
S2366	ND	0.2	ND	4.3	0.75
(<E-P-R-pNA)		(0.2/0.997)		(0.83/0.192)	(1.84/2.45)
S2238	ND	1.5	ND	ND	ND
(D-F-Pip-R-pNA)		(2.1/1.432)			

[a] Shown are the k_{cat}/K_m ($mM^{-1}\ s^{-1}$) values. Individual k_{cat} (s^{-1}) and respective K_m (mM) are calculated from 3 or 4 experiments and shown in parentheses as the numerator (former) and the denominator (latter) without standard deviations. [b] Factor IXa concentration was 50 nM. [c] ND: not detectable.

substrates we tested. Exceptions were that IXQ192E hydrolyzed Spectrozyme FIXa better than S2765, and it displayed better levels of catalytic activity than wild-type factor IXa with CBS31.39, S2366, and S2238. For the E217 mutations, dramatic differences were found. Both IXE217D and IXE217R displayed barely detectable activity toward S2765, S2288, and Chromozyme tPA. Instead, both variants hydrolyzed Spectrozyme FIXa and CBS31.39 more efficiently than wild-type factor IXa, and they could hydrolyze S2366, toward which wild-type factor IXa displayed no detectable activity. In contrast to the effect of FVIIIa on factor IXa, in cleavage of factor X, FVIIIa had a minor effect in cleavage of small substrates. The amidolytic activities of wild-type factor IXa either were unchanged or only showed a 2−10-fold elevation in the presence of FVIIIa (Table 4). However, it had a profound effect on the E217 variants toward S2765, S2288, and Chromozyme tPA in that wild-type and mutant factor IXa demonstrated equivalent activities toward each individual substrate. The only exception was IXQ192E; with FVIIIa, it showed an 8-fold decrease in catalytic efficiency toward Spectrozyme FIXa.

Distinct Interaction of the Variant Factor IX's with Serpin- and Kunitz-Type Inhibitors. In the presence of heparin, the interaction of the factor IX variants with ATIII appeared similar to that of wild-type factor IXa (data not shown). However, different results were obtained when heparin was omitted in the reaction. Figure 3 depicts the formation of SDS-stable complexes of mutant and wild-type factor IXa with ATIII without heparin after 24 h incubation. While complex formation appeared normal with wild-type and mutant factor IXa, IXaQ192K did not form after it had been incubated for 10 h (Figure 3, panel A). The complex formation of IXaQ192K with ATIII occurred at much lower rates (Figure 3, panel B, lanes 5−8) compared to wild-type factor IXa (Figure 3, panel B, lanes 1−4). To determine whether ATIII is capable of inhibiting IXQ192K or not, the inhibition was assessed in soluble conditions containing small substrates, CBS31.39. The amidolytic activity of mutant and wild-type factor IXa in the presence of different concentrations of ATIII is depicted in Figure 3, panel C. It appears that ATIII inhibits IXQ192K as well as wild-type factor IXa and the other mutants since the measured association rate constant (k_{ass}) and dissociation rate constant (k_{dis}) were not much different for any of the factor IXa's. The effect of mutation at Q192 and E217 on the interaction with TFPI was assessed, and the results are shown in Figure 4. TFPI

Table 4: Substrate Catalysis by Factor Xase Complex[a]

substrates	enzymes				
	IXwt	Q192E	Q192K	E217D	E217R
S2765 (Z-D-R-G-R-pNA)	1.69 (2.9/1.72)	1.89 (2.08/1.10)	1.63 (2.17/1.33)	2.37 (2.42/1.02)	2.12 (2.25/1.06)
Spectrozyme FIXa (H-D-L-phG-R-pNA)	55.7 (19.2/0.344)	3.15 (16.7/5.3)	38.2 (22.9/0.6)	40.8 (20.4/0.5)	33.9 (20/0.58)
S2288 (D-I-P-R-pNA)	−[c]	−	−	−	−
Chromozyme tPA ($McSO_2$-D-F-G-R-pNA)	2.23 (4.58/2.05)	2.28 (6.67/2.93)	1.44 (4.17/2.9)	1.89 (5/2.64)	2.44 (5/2.05)
CBS31.39 ($McSO_2$-D-L-G-R-pNA)	29.1 (19.2/0.66)	22.6 (16.7/0.74)	33.2 (15.6/0.47)	31.2 (22.5/0.72)	27.3 (18.3/0.67)
S2222 (Bz-I-E-G-R-pNA)	ND[b]	ND	ND	ND	ND
Spectrozyme FXa (MeO-CO-D-chG-G-R-pNA)	8.2 (12.3/1.5)	5.1 (15.4/3)	3.9 (9.1/2.3)	5.8 (13.3/2.3)	6.7 (15.8/2.4)
S2366 (<E-P-R-pNA)	15.6 (4.7/0.3)	9.6 (5.4/0.56)	10.5 (4.4/0.42)	10.2 (6/0.59)	9.8 (6.3/0.64)
S2238 (D-F-Pip-R-pNA)	−	−	−	−	−

[a] Shown are the k_{cat}/K_m (mM^{-1} s^{-1}) values. Individual k_{cat} (s^{-1}) and respective K_m (mM) were determined as described in Table 3. [b] ND: not detectable. [c] (−): not determined.

inhibited the amidolytic activity of all the variants at a rate similar to that of wild-type factor IXa. In contrast, IXaQ192E was inhibited to a lesser extent, and a clear difference from the wild-type factor IXa persisted in the presence of high concentrations of TFPI. These data demonstrate that IXaQ192E is defective in forming inhibitor complex with TFPI.

DISCUSSION

The present study attempted to explore whether positions 192 and 217 of factor IX serve the same functions in factor IX as their corresponding sites in other coagulation factors. We found distinct influences that these two sites exert on factor IX, rather than the corresponding ones have on other clotting factors. In factor IX, replacing Q192 with either glutamic acid (as in thrombin and protein C) or lysine (as in factor VII) rendered factor IX less active toward factor X. In contrast, in both thrombin and protein C, better activity toward macromolecular substrates (fibrinogen and protein C in the case of thrombin, and factor Va in protein C) was observed with E192Q mutations (*2*, *7*, *11*). When mutated at Q192, factor IX also showed different levels of dependence on the cofactor, compared with similar mutational study in factor X. In factor X, decreased activity of the factor XQ192E mutant (*6*) in cleavage of prothrombin was only observed in the absence, but not the presence, of factor Va. In contrast, factor IXQ192E was only 10% as active as wild-type factor IX, regardless of the presence of FVIIIa. This indicates that binding of cofactors (FVIIIa and factor Va) may have different effects on position 192 of their cognate enzymes (factor IX and factor X, respectively) in terms of the activity toward their macromolecular substrates.

We found that the catalytic functions of the wild-type and the four mutant factor IX's toward factor X were consistent with their individual clotting activities (comparing Table 2 with Table 1). However, among the synthetic peptide substrates we have tested, none of them could predict the clotting activity or the catalytic efficiency of these enzymes in cleavage of factor X. In the presence and absence of FVIIIa, the K_m of wild-type and mutant factor IXa for factor X is very much smaller than those for chromogenic peptide substrates. All of these data suggest that the exosites (other than active site) for binding macromolecular substrates govern the assembly of the intrinsic factor Xase−substrate complex. Furthermore, in the presence of factor VIIIa, factor IXQ192K and those two E217 mutants displayed 2−3 times higher K_m than wild-type factor IXa in cleavage of factor X (Table 2), whereas their affinities for peptide substrates are indistinguishable from the wild-type (Table 4). The aforementioned fact combined with the fact that those mutants showed equivalence to wild-type factor IX amidolytic activities but reduced activities toward factor X also indicates that extended regions (exosites) are involved in the binding of macromolecular substrates, factor X (*31*, *32*). Mutations at Q192 and E217 may have affected the extended substrate/inhibitor binding sites. For example, position 192 is near K148, and it makes a salt bridge to R143 which is near the autolysis loop of factor IXa (*22*). The mutational effect of K148 on the interaction of factor IX with substrates and inhibitor has been described as the effect on the exosite alterations (*33*). Miyata et al. showed that exchange of G142 with E142 in factor IX would disrupt the specific conformational state in the active site environment of factor IXa, resulting in the malformation of the substrate binding site (*34*). In the case of position E217, we showed that the activities of the E217 mutants toward factor X were enhanced by factor VIIIa to a greater extent than that of wild-type (Table 2), and the binding of factor VIIIa *corrected* the substrate specificity for peptide substrates of these mutants. This indicates that the conformational change caused by the mutations at position 217 was somehow compromised by factor VIIIa binding. Besides, the fact that the E217 mutants had 4 times higher K_d for factor VIIIa suggests broader conformational effects caused by these mutations.

The lower catalytic function of the factor IXQ192 variants relative to wild-type factor IXa toward factor X in the presence and absence of FVIIIa was mainly due to the alterations in k_{cat}. In addition to the macromolecular substrate binding exosite(s) that might be affected in the IXQ192 variants, using chromogenic peptides as substrates, we found that the active site of factor IXa was also affected by

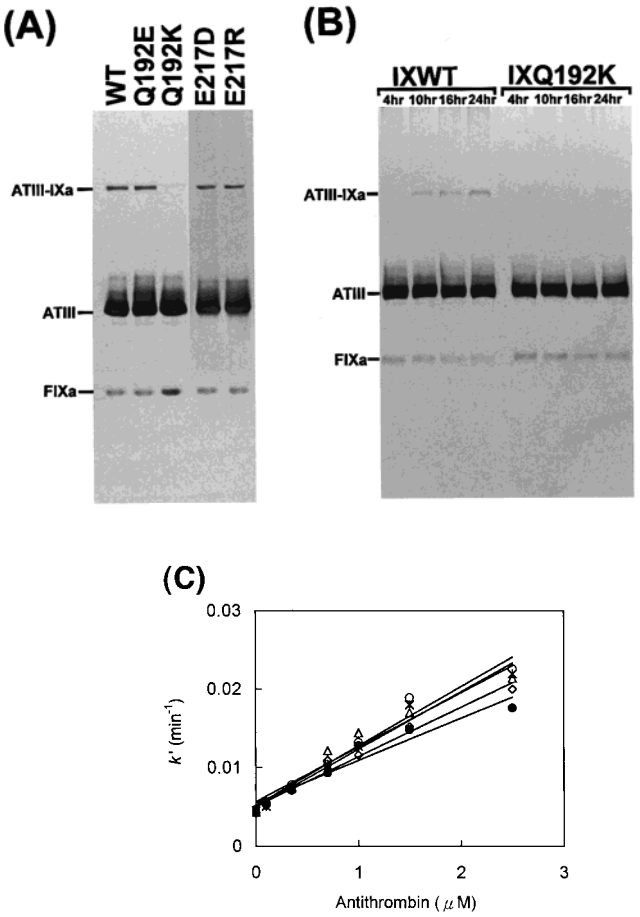

FIGURE 3: ATIII binding. (Panel A) Wild-type and mutant IXa (0.6 μM) were incubated overnight (12 h) with ATIII (2.9 μM) in the absence of heparin, followed by quenching with gel loading buffer, and subjected to SDS/PAGE under nonreducing conditions. The gel was stained with silver. The amount of protein loaded in each lane was 600 ng of factor IXa before reaction. (Panel B) The same conditions as in panel A. The reactions were withdrawn at the timed points shown above lanes. (Panel C) Binding of ATIII to normal and mutant factor IXa in the absence of heparin. Plot of k' at different inhibitor concentrations is shown. Calculation was as described under Materials and Methods. The k_{ass}, which is the slope of each line, was $(6.6 \pm 1.7) \times 10^{-3}$ M^{-1} min^{-1} for IXwt (●), $(6.8 \pm 0.7) \times 10^{-3}$ M^{-1} min^{-1} for IXQ192E (◇), $(7.3 \pm 0.1) \times 10^{-3}$ M^{-1} min^{-1} for IXQ192K (*), 7.6×10^{-3} M^{-1} min^{-1} for IXE217D (○), and $(7.2 \pm 0.4) \times 10^{-3}$ M^{-1} min^{-1} for IXE217R (△). The k_{dis} is the y intercept.

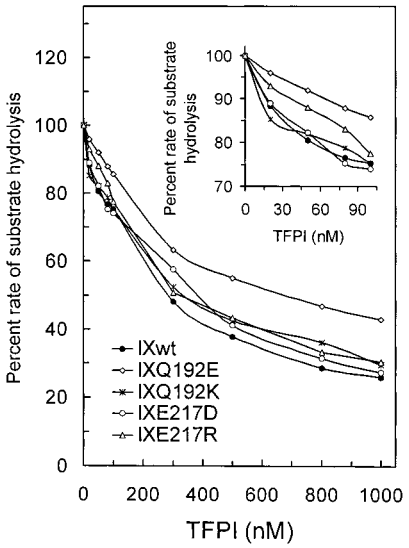

FIGURE 4: Interactions of wild-type and mutant factor IXa with TFPI. The final concentrations were as follows: factor IXa, 10 nM; TFPI, 0−1 μM; Spectrozyme FIXa, 1 mM; in TBS/Ca/BSA/PCPS. TFPI was added last to the reaction, and the rate of hydrolysis of Spectrozyme FIXa was determined immediately after the addition of TFPI and continued for 120 min at 37 °C on a microtiter plate reader. Only curves representing the linear phase of hydrolysis were used for calculation. Residual factor IXa activities relative to its activity in the absence of TFPI are shown. Means of duplicates of two independent experiments are shown. The inset depicts the inhibition by low concentrations of TFPI (0−100 nM).

mutations at Q192 (Tables 3 and 4). In general, both Q192 mutants were not effective in cleavage of the peptide substrates. In contrast to the selectivity order of wild-type factor IXa and IXaQ192K, factor IXaQ192E catalyzed putative thrombin's substrates, S2366 and S2238, more effectively than wild-type factor IXa. However, in the presence of FVIIIa, factor IXQ192E exhibited a dramatic decrease in catalytic efficiency toward Spectromzyme FIXa (Table 3) due to a 15-fold increase of K_m, which was not observed in the absence of FVIIIa. Since the binding of FVIIIa to factor IXa may cause a conformational change at the active site (*35*), and affect the positioning of the substrate at the active site, therefore, compared to the catalytic domain of wild-type factor IXa, the active site of the Q192 mutants might not be properly rearranged for substrates upon FVIIIa binding. Moreover, the phenol ring structure at the P2 position of Spectrozyme FIXa might repel the negatively charged glutamic acid residue in IXQ192E, which is more exposed in the factor IXa−VIIIa complex than in factor IXa alone.

Our analysis revealed that factor IXE217D is partially active and that IXE217R is almost inert in aPTT assays. Hemophilia B patients with E217 mutated to glycine, alanine, or lysine featured a CRM^+ phenotype with 1−2% factor IX clotting activity (*36*). Together with this study with IXE217R, it can be speculated that the negatively charged side chain of E217 has active roles in the physiological activity of factor IX. Our previous study has shown that factor VIIQ217E is as active as wild-type factor VII in clotting assays and in factor Xa generation experiments, and VIIQ217A is only 10% as active in clotting due to the lower catalytic activity (k_{cat}) toward factor X (*15*). It seems that in factor VII, the size of the side chain interferes with factor VII's activity, rather than its charge. In the case of factor IX, both the structure and the charge of position 217 are critically defined for the activity toward factor X. This is further demonstrated in the purified system that both the K_m and k_{cat} of the E217 mutants in cleavage of factor X were affected in the absence of FVIIIa (Table 2). This also suggests that the function of position 217 is related to the collision between the enzyme, factor IXa, and the substrate, factor X. In addition to the effect of E217 mutations on the exosites that may contribute to the altered interactions between factor IX and factor X, the synthetic peptide hydrolysis experiments revealed that residue 217 is involved in P3 recognition in factor IX as was observed in factor VII (*15*). When mutating E217 to aspartic acid and arginine, the new factor IX variants exhibited quite different selectivity for the peptide substrates from that of wild-type factor IXa (Table 4). The E217 mutants were not able to cleave S2765 with a basic P3 residue; however, they were effective in cleavage of S2222

and S2366 with acidic P3 residues. When factor VIIQ217 was mutated to glutamic acid (that is in wild-type factor IX), the activity of factor VIIa toward S2765 increased by a factor of 9. Apparently, a glutamic acid at position 217 significantly enhanced the hydrolysis of S2765 by both factor IX and factor VII. Moreover, Chromozyme tPA and CBS31.39 differ only at the P3 residue. While wild-type factor IX displays similar activity toward these two substrates, the two E217 variants cleaved only CBS31.39, also suggesting the involvement of position 217 in P3 recognition. The E217 in relation to P3 recognition may be implicated by the crystal structure. In the crystal structure of porcine factor IXa (*22*), the side chain of E217 makes a salt-bridge to lysine at position 224; however, there is no direct contact with the cocrystallized active site inhibitor. The distance between the backbone atoms of E217 and the backbone of P3 is about 5 Å (measured in the crystal structure from 1PFX.PDB). It is possible that the side chain of the E217 variants may be subjected to reorientation, replacing the salt-bridges to position 224 with direct interaction with the P3 residue. Alternatively, the change in the side chain of E217 may disturb the orientation of the adjacent residue, W215, which is highly conserved in P3 binding, and thus affect the P3 binding. Intriguingly, the defect in the E217 mutations was compensated for by FVIIIa, as revealed by the apparently lower K_m of factor IXE217R for factor X in the presence than in the absence of FVIIIa (Table 2). In the presence of FVIIIa, both IXE217 variants were effective toward Chromozyme tPA and S2765, suggesting both a profound effect of FVIIIa on the local structure of position 217 of factor IXa and also the importance of E217 on the selection for P3 side chains.

In trypsin and thrombin complexed with benzamidine, the inhibitor recognition pocket is formed by two segments consisting of residues 189−195 lining one side of the pocket and residues 214−220 on the other (*37*). In thrombin, replacing E217 with alanine decreased its interaction with ATIII both in the presence and in the absence of heparin (*38*). In factor VII, replacing Q217 with alanine facilitated the interaction of factor VII and ATIII while replacing it with glutamic acid decreased the interaction (*15*). These mutagenesis studies support the involvement of residue 217 in inhibitor binding. However, in factor IXa, mutations at 217 did not affect its binding to ATIII. The fact that in factor IX, the segment of residues 214−220 has greater mobility and flexibility than the counterparts in the other trypsin-like serine proteases may partly explain the result.

We found that factor IXQ192E showed normal ATIII binding but decreased the reactivity toward TFPI. In factor X, the mutant, factor XQ192E, has been reported to react with ATIII as effectively as wild-type factor X, whereas it reacted with BPTI and TFPI approximately 30 times more slowly than wild-type factor X (*6*). In contrast, thrombin and protein C with E192Q mutation exhibited better affinity for TFPI and BPTI (*3*, *11*). Therefore, it seems that among these serine proteases, Q192 and E192 are equally effective for these enzymes to bind ATIII, but, comparatively, Q192 seems to be advantageous for these proteins to bind BPTI and TFPI. In this study, we demonstrated that K192 is also effective for factor IXa to bind TFPI. Exactly how glutamine and lysine instead of glutamic acid would support the interaction of factor IXa with TFPI, and whether factor IXa interacts with the second kunitz domain of TFPI as factor Xa (*38*), thrombin E192Q (*3*), and protein C E192Q (*11*) still await further investigation. As observed in the superposition of factor Xa on the crystal structure of the second kunitz domain of TFPI complexed with trypsin, the carboxamide nitrogen atom of the Q192 of factor Xa makes a hydrogen bond with the cysteine (P2) of TFPI and also bridges to G216, G219, S147, and N143 of factor Xa through water molecules (*40*). This suggests that a hydrogen donor such as glutamine and lysine is preferred at position 192 for better binding with TFPI. In the case of interaction with ATIII, while the lysine at position 192 in factor IX did not affect TFPI binding, it caused slow interaction of factor IXQ192K with ATIII. As mentioned above, Q192 is near K148 (K316 of human factor IX), the mutation of which has affected exosites for factor X binding. The slow interaction of IXQ192K with ATIII was similar to that of the K148 mutants (IXK316E and IXK316A) with ATIII, which were readily inhibited by ATIII in soluble forms but were slow in making a stable 1:1 complex with ATIII in the absence of heparin (*34*). Thus, it is consistent with the idea that this region of factor IX affects the extended macromolecular recognition sites.

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