Binding of high-molecular-mass kininogen to the Apple 1 domain of factor XI is mediated in part by Val⁶⁴ and Ile⁷⁷

Frances S. SEAMAN,* Frank A. BAGLIA,* James A. GURR,† Bradford A. JAMESON*‡ and Peter N. WALSH*†§||

*Sol Sherry Thrombosis Research Center, †Department of Biochemistry, and §Department of Medicine, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A., and ‡Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

We have previously demonstrated the presence of a binding site for high-molecular-mass kininogen (HK), spanning residues Val⁵⁹–Lys⁸³, in the first Apple (A1) domain in the heavy-chain region of factor XI. We have now prepared conformationally constrained synthetic peptides and recombinant A1 domain (rA1) constructs to identify the specific amino acid residues that constitute the HK-binding site. Expression of the A1 domain (Glu¹–Ser⁹⁰) was achieved in a bacterial expression system following PCR amplification of the A1 domain from factor XI cDNA and ligation into an expression plasmid. The rA1 inhibited factor XI binding to HK [$K_i \sim (2-3) \times 10^{-7}$ M] in a manner indistinguishable from purified factor XI, indicating that all the information necessary for binding HK is contained within the A1 domain. To identify specific amino acid residues involved in binding HK, conformationally constrained peptides were synthe-

sized containing conservative amino acid substitutions at residues suspected to contain side chains involved in binding, including Val⁶⁴ → Ala, Glu⁶⁶ → Ala, Arg⁷³ → Ala and Ile⁷⁷ → Ala. Because normal results were obtained with all peptides with the exception of Val⁶⁴ → Ala and Ile⁷⁷ → Ala, which failed to compete normally with factor XI for binding to HK, we prepared two mutant rA1 domains (Val⁶⁴ → Ala and Ile⁷⁷ → Ala) by PCR-based site-directed mutagenesis, both of which exhibited diminished capacity to inhibit factor XI binding to HK. Competition studies with prekallikrein (PK) and a PK-dependent synthetic peptide suggested that PK and factor XI have a common surface in the A1 domain for binding HK of which Val⁶⁴ is a part. We conclude that the binding of factor XI to HK is mediated at least in part by Val⁶⁴ and Ile⁷⁷ in the A1 domain of factor XI.

INTRODUCTION

Human factor XI circulates in plasma as a complex with a non-enzymatic cofactor, high-molecular-mass kininogen (HK) (Thompson et al., 1977; Davie et al., 1979). This protein complex participates in the intrinsic phase of blood coagulation. Factor XI is proteolytically activated by factor XIIa after binding to a negatively charged surface through HK (Griffin and Cochrane, 1976; Bouma and Griffin, 1977; Kurachi et al., 1980). Factor XIa, the resulting serine protease, consists of two light chains and two heavy chains bridged by disulphide bonds, and catalyses the activation of factor IX (Bouma and Griffin, 1977; Kurachi and Davie, 1977; Kurachi et al., 1980; van der Graaf et al., 1983). The primary structure of factor XI has been elucidated from the sequence of a cDNA insert encoding factor XI (Fujikawa et al., 1986). This has demonstrated that four repeat sequences (domains A1, A2, A3 and A4) or Apple domains are present in the heavy chain, each encoded by two exons (Asakai et al., 1987). Early studies have demonstrated the presence of an HK-binding site in the heavy chain of factor XI (Baglia et al., 1989). Using immunological and proteolytic cleavage techniques we were able to identify a 47-residue cleavage product that is involved in the interaction of factor XI with HK in the A1 domain of the heavy chain of factor XI (Baglia et al., 1990). Further studies of the A1 domain utilized computer simulation to design constrained peptides whose folding patterns overlapped the model structure (Baglia et al., 1992). Our studies provided details of the fine mapping and chemical synthesis of the binding site on the A1 domain for HK, details which are consistent with the notion that the sequence of amino acids Val⁵⁹–Lys⁸³ of the heavy chain of factor XI contains two antiparallel β -strands connected by β -turns that together comprise a continuous surface

used in the binding of HK (Baglia et al., 1992). We have now examined the computer model of the A1 domain for amino acid residues whose side chains may participate in the HK-binding site, and have now prepared conformationally constrained synthetic peptides and recombinant A1 (rA1) constructs to identify the specific amino acid residues comprising the HK-binding site.

EXPERIMENTAL

Purification of proteins

Factor XI was purified from human plasma by immunoaffinity chromatography using a monoclonal antibody to factor XI (Sinha et al., 1985) and had a specific activity of 250 units/mg of protein where one unit is defined as the activity in 1 ml of pooled normal human plasma (Scott et al., 1984). HK (specific activity, 15 units/mg of protein) was purified by the method of Kerbirou and Griffin (1979). Prekallikrein (PK) was purified as described (Page and Colman, 1991). Factor XI and HK were assayed by minor modifications (Scott et al., 1984) of the kaolin-activated partial thromboplastin time (Proctor and Rapaport, 1961). All purified proteins appeared homogeneous by SDS/PAGE.

Radiolabelling of proteins

Purified factor XI was radiolabelled with ¹²⁵I by a minor modification (Sinha et al., 1985) of the iodogen method to a specific radioactivity of 5×10^6 c.p.m./ μ g of protein. The radio-labelled protein retained > 98% of its biological activity.

Protein analyses

Protein concentrations were determined by the Bio-Rad dye-

Abbreviations used: f.a.b., fast atom bombardment; HK, high-molecular-mass kininogen; PK, prekallikrein; rA1, recombinant A1.

|| To whom correspondence should be addressed, at: Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, U.S.A.

Table 1 Recombinant and synthetic peptides based on the known sequence of the Apple 1 domain of factor XI or prekallikrein (PK)

Key: ^a the numbers refer to the sequence of amino acids in factor XI or PK as reported by Fujikawa et al. (1986); ^b designates a residue where cysteine was substituted for amino acid in the native factor XI sequence; ^c designates a peptide in which cysteine replaced one or more amino acid(s) in the native factor XI sequence; ^d designates a peptide in which alanine replaced one amino acid in the native factor XI sequence; and ^r designates an expressed or recombinant peptide. Residues 1–55 are omitted for clarity and brevity.

Tandem repeat residue number ^a ...	60	70	80	90	Factor XI domain	Residue no. in factor XI
	F T C V L K D S V T E T L P R V N R T A A I S G Y S F K Q C S H Q I S				A1	Glu ¹ –Ser ^{90r}
	F T C V L K D S A ^d T E T L P R V N R T A A I S G Y S F K Q C S H Q I S				A1	Glu ¹ –Ser ^{90r} ^a
	F T C V L K D S V T E T L P R V N R T A A A ^d S G Y S F K Q C S H Q I S				A1	Glu ¹ –Ser ^{90r} ^a
	F T C V L K D S V T E T L P R V N R T A A I S G Y S F K Q C S				A1	Phe ⁵⁶ –Ser ⁸⁶
	V L K D S A ^d T E T L P R				A1	Val ⁵⁹ –Arg ^{70e}
	V L K D S V T A ^d T L P R				A1	Val ⁵⁹ –Arg ^{70e}
		N A ^d T C ^b A I S C ^b Y S F K			A1	Asn ⁷² –Lys ^{83ce}
		N R T C ^b A A ^d S C ^b Y S F K			A1	Asn ⁷² –Lys ^{83ce}
	V L K C ^b S V T E C ^b L P R				A1	Val ⁵⁹ –Arg ^{70c}
	C ^b G K D S V T E T G C ^b				A1	Lys ⁶¹ –Thr ^{67c}
					PK domain	Residue no. in PK
	F G C F L K D S V T G T L P K V H R T G A V S G H S L K Q C G				A1	Phe ⁵⁶ –Gly ⁸⁶

binding assay according to the instructions provided by the manufacturer (Bio-Rad, Richmond, CA, U.S.A.).

Binding of factor XI to HK

The binding of factor XI to HK was studied using poly(vinyl chloride) microtitre plates, the wells of which were coated with HK by incubation with 100 µl of the protein (100 µg/ml) for 2 h at room temperature. The binding assay and conditions used have been previously described (Baglia et al., 1989, 1990). Briefly, after residual binding sites in the wells were blocked, 100 µl of a mixture of ¹²⁵I-labelled factor XI and either buffer or peptide were added to the wells and incubated for 3–4 h at room temperature. The wells were thoroughly washed with PBS/BSA, dried, and counted in a γ-radiation counter.

Peptide synthesis

Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer by a modification of the procedure described by Kent and Clark-Lewis (1985) as previously described (Walsh et al., 1993). After synthesis, peptides were further purified by h.p.l.c. on a Vydac C18 column. Single peaks were further characterized by h.p.l.c. after refolding, or after reduction and alkylation (see below). The sequences of the synthetic peptides used in this study are given in Table 1.

Refolding and reduction and alkylation of peptides

In order to refold peptides containing cysteine residues, the peptide was dissolved in deionized water as a 0.1 mg/ml solution in a flask containing a stir bar. The pH was adjusted to 8.5 with NH₄OH and the solution was allowed to stir at 5 °C for at least 3 days. The resulting solution was lyophilized. Alternatively, peptides were reduced with dithiothreitol and alkylated with iodoacetamide as previously described (Sinha et al., 1985).

H.p.l.c.

The h.p.l.c. system employed was from Waters (Waters 600

Gradient Module, Model 740 Data Module, Model 46K Universal Injector and Lambda-Max Model 481 Detector). The reverse-phase chromatography described here was performed using a Waters C8 µBondapak Column, whereas gel filtration was carried out using a Waters Protein-Pak 60 Column as previously described (Baglia et al., 1991, 1992; Walsh et al., 1993).

Characterization of synthetic peptides

All the peptides utilized in this study were examined by h.p.l.c. (both reverse-phase and gel-filtration), and all demonstrated a single homogeneous peak (results not shown). When the refolded peptides were examined by h.p.l.c. (both reverse-phase and gel-filtration), single homogeneous peaks with identical retention times to the original mixtures (i.e. the unfolded h.p.l.c.-purified peptide) were observed. 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, i.e. single peaks with identical retention times to the original h.p.l.c.-purified peptide were observed by both reverse-phase and gel-filtration h.p.l.c. In addition, all reduced and alkylated or refolded peptides were examined for free SH groups using the Ellman reagent, 5,5'-dithiobis (2-nitrobenzoic acid). It was determined (Habeeb, 1972) that there was < 0.02 mol of free SH/mol of peptide, which further verifies that these refolded peptides were homogeneous preparations consisting of intramolecular disulphide-bonded peptide.

Fast atom bombardment (f.a.b.)-m.s.

F.a.b.-m.s. was used to assess the homogeneity and molecular mass of each synthesized peptide. F.a.b. was performed as previously described (Baglia et al., 1991; Walsh et al., 1993). The spectra of synthesized peptides revealed masses almost identical to calculated values (within 0.5%) with a single ion species detected in each case (results not shown).

Amplification of the A1 domain of human factor XI using the PCR

Primers corresponding to the A1 domain were used in the PCR with factor XI cDNA (a 2.1 kb *Eco*R1 fragment containing the complete factor XI cDNA coding sequence, a gift from Drs. Dominic W. Chung, Kazuo Fujikawa and Earl W. Davie of the University of Washington, Seattle) to create a 270 bp insert that is identical to the A1 domain of factor XI. The PCR was performed according to a method reported by the Cetus Corporation for their PCR reagent kit (Perkin Elmer Cetus Brochure).

Mutagenesis

Mutations were introduced using a PCR-based procedure (Picard et al., 1994) which employs *Pfu* DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.). The mutagenesis oligonucleotides used for this procedure were 5'GAAAGACAGTGGCTACAGAAACAC3' and 5'GACAGCAGCGGCTTCTGGGTATT3'. DNA products of the mutagenesis reactions were subcloned, then sequenced between *Pst*I and *Hind*III sites in order to verify the introduction of oligonucleotide-directed mutations and the absence of unplanned, polymerase-associated changes. The mutated fragments were finally transferred into *Escherichia coli* for expression.

QIAexpress pQE-9 vector

The pQE-9 construct (Qiagen Inc., Chatsworth, CA, U.S.A.) was used to express the A1 domain. This vector has an optimized, regulatable promoter/operator element, consisting of the *E. coli* T5 promoter and two *lac* operator sequences, a synthetic ribosome-binding site, designed for optimal mRNA recognition and binding, an optimized 6-His affinity tag coding sequence and a multicloning site. The QIAexpress high-level expression and protein purification system, a product of Qiagen Corporation (Chatsworth, CA, U.S.A.), uses the K-12-derived *E. coli* strain M15 [pREP 4] which allows a high level of expression. Expression from the pQE-9 vector is rapidly induced by the addition of isopropyl- β -D-thiogalactopyranoside which inactivates the repressor and clears the promoter. The full-length nucleotide sequence of each insert was determined by the dideoxy chain termination method (Sanger et al., 1977). The ligation of the A1 insert into the cloning site of pQE-9 and the expression of the A1 domain was performed according to protocols in the QIAexpress kit manual (Diagen GmbH, Qiagen Inc., 1992). Isolation of the rA1 was carried out using a (Ni-nitrilo-tri-acetic acid) resin which binds the 6-His tag on the expressed protein, according to the procedure outlined in the QIAexpress manual (Diagen GmbH, Qiagen Inc., 1992). The sequences of the recombinant peptides produced and used in this study are given in Table 1.

Computer modelling

A structural model (Figure 2) of the factor XI A1 domain was constructed as previously described (Baglia et al., 1992) using the computational chemistry package supplied by BioDesign Inc. (Pasedena, CA, U.S.A.) and a Silicon Graphics 4D280 parallel processing supercomputer.

RESULTS

Expression, isolation and characterization of A1 domain Glu¹-Ser⁹⁰ of factor XI

We used the QIAexpress system to express the A1 domain, Glu¹-Ser⁹⁰, of factor XI. Appropriate primers, the PCR and

Figure 1 Effects of native factor XI, rA1 factor XI and PK on the binding of ¹²⁵I-factor XI to HK

¹²⁵I-factor XI (0.04 mg/ml) was incubated with proteins at the concentrations indicated and the binding of ¹²⁵I-factor XI to HK was determined as described in the Experimental section. Data are shown for proteins described in Table 1 including native factor XI (○), rA1 factor XI (●) and PK (■). The concentrations of native factor XI was based on the subunit composition of the molecule having a molecular mass of 80 000 Da.

factor XI cDNA, were used to create an insert that is identical to the A1 domain of factor XI. This DNA was reamplified and integrated into the pQE-9 vector. The integrated DNA, containing sequences for the A1 domain, was expressed in this system. The level of expression of A1 protein was determined to be 20 mg/l of cell culture. Because the rA1 contains a 6 × His tag, the metal chelate adsorbent Ni-NTA was used, in a single-step isolation, to obtain the A1 domain according to the QIAgen manual (Diagen GmbH, Qiagen Inc., 1992). This peptide showed a single band upon SDS/PAGE (results not shown). The DNA insert containing the A1 domain was sequenced and was found to contain the identical nucleotide sequence found in the published nucleotide sequence of the A1 domain (results not shown). Finally, N-terminal analysis of the expressed A1 domain was identical to published human factor XI amino acid sequences (results not shown).

Effect of the recombinant A1 domain, Glu¹-Ser⁹⁰, on the binding of factor XI to HK

From our earlier studies it was apparent that the ability of the A1 domain of factor XI to bind HK was dependent on a unique contact surface (comprising specific amino acids) as well as conformational restraints inherent in the A1 domain (Baglia et al., 1990, 1992). This information was derived from studies with synthetic peptides. Therefore, we expressed the entire A1 domain in order to study in detail which specific amino acids are involved in the contact surface and to determine whether the intact A1 domain contributes to the binding of HK. Utilizing the factor XI-HK binding assay described in the Experimental section, we initially examined the effects of native factor XI, rA1 and PK on the binding of ¹²⁵I-labelled factor XI to HK (Figure 1). This experiment demonstrates that factor XI and the rA1 are indistinguishable in their capacities to inhibit ¹²⁵I-factor XI binding to HK [$K_i \sim (2-3) \times 10^{-7}$ M], a result which strongly suggests that all the information required for binding of factor XI to HK resides within the A1 domain. However, PK had a weak ability to inhibit factor XI binding to

Figure 2 Computer model of the A1 domain of the heavy chain of factor XI

A plausible three-dimensional structure was calculated from the primary structure of a truncated form of the A1 domain (Glu¹–Cys⁸⁵) and the known disulphide linkages, using Biograf Software (Molecular Simulations, Inc.) and a Silicon Graphics 4D 280 Parallel Processing Computer. A ribbon model of the protein backbone is shown with the loop structure Val⁵⁹–Val⁷¹ (shown as the loop containing Val⁶⁴ and Glu⁶⁶) and Asn⁷²–Cys⁸⁵ (shown as the loop containing Arg⁷³ and Ile⁷⁷). Amino acids are numbered according to the sequence in the mature protein. Four amino acids (Val⁶⁴, Glu⁶⁶, Arg⁷³ and Ile⁷⁷) are highlighted to reflect the suspicion that their side chains project into the surface that binds to HK.

HK ($K_i \sim 3 \times 10^{-3}$ M), suggesting that both PK and factor XI may contain a common site for binding HK.

Effect of A1-derived synthetic peptides on the binding of factor XI to HK

Using immunological and chemical cleavage techniques, we have previously identified a structure contained within residues Phe⁵⁶–Ser⁸⁶ in the first tandem repeat (A1) domain of the heavy chain of factor XI which binds HK (Baglia et al., 1990). Additional studies were performed using computer modelling for rational design of constrained peptides (Baglia et al., 1992). These studies have made clear the notion that the sequence of amino acids Val⁵⁹–Lys⁸³ of the heavy chain of factor XI may contain two antiparallel β -strands connected by β -turns that together might comprise a continuous surface used for binding to HK (Baglia et al., 1992). A computer model of the A1 domain (Figure 2) depicts the putative structure of this HK-binding site, which consists of two solvent-accessible loop structures. The binding energy appears to reside within these two peptide structures, Val⁵⁹–Arg⁷⁰ and Asn⁷²–Lys⁸³. Additional experiments were carried out with a synthetic peptide identical with Phe⁵⁶–Ser⁸⁶ in the A1 domain of PK. Because the amino acid sequence of factor XI is 58% identical (Fujikawa et al., 1986) with that of human plasma prekallikrein, we determined whether the corresponding region in PK competes with factor XI for binding to HK. We found that Phe⁵⁶–Gly⁸⁶ of the A1 domain of PK also inhibits ¹²⁵I-factor XI binding to HK with a K_i of $\sim 1 \times 10^{-3}$ M

Figure 3 Effect of A1 factor XI and PK-derived synthetic peptides on the binding of ¹²⁵I-factor XI to HK

Data are shown for peptide Phe⁵⁶–Ser⁸⁶ of factor XI (○) and Phe⁵⁶–Gly⁸⁶ of PK (△). The experimental design and results of control experiments were exactly as described in the Experimental section and in the legend to Figure 1.

(Figure 3). This is in contrast with Phe⁵⁶–Ser⁸⁶ of the A1 of factor XI, which inhibits factor XI binding to HK an order of magnitude better, with a K_i of $\sim 9 \times 10^{-5}$ M (Figure 3).

Effects of specific amino acid substitutions on inhibitory effects of synthetic peptides

In order to gain information about which specific amino acid residues comprise the HK-binding site, we prepared a panel of synthetic peptides with specific amino acid residues substituted (Table 1). Amino acid substitutions were made by examining the computer model (Figure 2) for residues that project their side chains into the predicted contact surface. The following amino acid residues of the A1 domain were chosen as possible candidates: Val⁶⁴, Glu⁶⁶, Arg⁷³ and Ile⁷⁷. In all the synthetic peptides prepared, the above amino acids were substituted with alanine which has a non-polar aliphatic side chain and represents a conservative amino acid substitution. Previously, we were unable to constrain Val⁵⁹–Arg⁷⁰ conformationally without a loss of activity after refolding (Baglia et al., 1992). Therefore, we chose not to introduce cysteines into synthetic peptides containing amino acid substitutions within the sequence Val⁵⁹–Arg⁷⁰. The concentration of peptide Val⁵⁹–Arg⁷⁰ required to inhibit by 50% factor XI binding to HK was 1×10^{-3} M, whereas the substitution of alanine for valine at position 64 greatly altered the activity of this peptide. Approx. 100-fold more concentrated peptide was required for 50% inhibition of factor XI binding to HK (K_i 1×10^{-1} M) (Figure 4a). When Glu was changed to Ala at position 66 of this peptide the activity (K_i 1×10^{-3} M) was the same as that of the 'wild-type' peptide (Figure 4a). These results indicate that Val⁶⁴ but not Glu⁶⁶ of the loop structure Val⁵⁹–Arg⁷⁰ may be an important component of the contact surface for binding HK.

We have previously reported that the loop structure Asn⁷²–Lys⁸³ can be conformationally constrained (by the introduction of cysteine residues) and folded. This conformationally constrained peptide inhibits the binding of factor XI to HK with a K_i of 1×10^{-4} M (Baglia et al., 1992). When Arg was changed to Ala at position 73 in this peptide there was essentially no effect

Figure 4 Effect of heavy chain factor-XI-derived synthetic peptides on the binding of ¹²⁵I-factor XI to HK

The experimental design and results of control experiments were exactly as described in the Experimental section and in the legend to Figure 1. (a) Data shown are for peptides Val⁵⁹-Arg⁷⁰ (Val⁶⁴ → Ala) (○), Val⁵⁹-Arg⁷⁰ (Glu⁶⁶ → Ala) (●) and Val⁵⁹-Arg⁷⁰ reduced and alkylated (■) (see Table 1). (b) Data shown are for peptides Asn⁷²-Lys⁸³ (●), Asn⁷²-Lys⁸³ (Arg⁷³ → Ala) (○) and Asn⁷²-Lys⁸³ (Ile⁷⁷ → Ala) (▲) (see Table 1).

on its ability to inhibit factor XI binding to HK (K_i 1×10^{-4} M, Figure 4b). However, when Ile was changed to Ala at position 77 in this peptide the ability of this peptide to inhibit the binding of factor XI to HK was altered, i.e. 10-fold more peptide was required for 50% inhibition (K_i 1×10^{-3} M, Figure 4b). Apparently, Ile⁷⁷ is an important contact site for factor XI interaction with HK, whereas Arg⁷³ is not.

Effect of site-directed mutants of the recombinant A1 domain, Glu¹-Ser⁹⁰, on the binding of factor XI to HK

In order to obtain additional evidence concerning the identity of specific amino acid residues of the A1 domain involved in binding HK, we used site-directed mutagenesis to prepare rA1 mutants containing conservative amino acid substitutions at residues suspected to contain side chains involved in binding, as predicted by computer modelling (Figure 2) and confirmed by the studies reported above. As shown in Table 1, we prepared two mutants of the rA1 domain by PCR-based site-directed mutagenesis: Val⁶⁴ → Ala and Ile⁷⁷ → Ala. Figure 5 demonstrates that the substitution of Val⁶⁴ with Ala in the A1 domain (the only

Figure 5 Effect of heavy chain factor-XI-derived recombinant wild-type and mutant Apple 1 domain peptides on the binding of ¹²⁵I-factor XI to HK

Data are shown for: peptides Glu¹-Ser⁹⁰r (see Table 1) either refolded (●) or reduced and alkylated (○); for recombinant peptide Glu¹-Ser⁹⁰r (Val⁶⁴ → Ala) refolded (■), and for recombinant peptide Glu¹-Ser⁹⁰r (Ile⁷⁷ → Ala) refolded (▲). The experimental design and results of control experiments were exactly as described in the Experimental section and in the legend to Figure 1.

amino acid change) results in a failure of the rA1 domain to inhibit factor XI binding to HK at concentrations greater than 5×10^{-5} M. The wild-type A1 domain inhibits by 50% factor XI binding to HK at a concentration of 10^{-7} M. Figure 5 also shows that the substitution of Ile⁷⁷ with Ala results in a diminished capacity of the rA1 domain to inhibit factor XI binding to HK. These results confirm the studies with synthetic peptides reported above and support the view that Val⁶⁴ and Ile⁷⁷ are essential for normal binding of factor XI to HK.

DISCUSSION

Earlier studies using immunological and chemical cleavage techniques, and more recent studies using synthetic peptides and computer modelling of protein structure, have investigated the structural characteristics of factor XI involved in binding HK (Baglia et al., 1990, 1992). Our studies support the notion that the sequence of amino acids encompassing Val⁵⁹-Lys⁸³ of factor XI heavy chain may be important for the binding of HK (Baglia et al., 1992). In order to gain information about the HK-binding site on factor XI we used computer modelling of the A1 domain to generate a testable three-dimensional structure (Figure 2), using the primary amino acid sequence and the disulphide linkages within the A1 domain (Baglia et al., 1992). This model may or may not accurately reflect the solution structure of the factor XI A1 domain; however, it does predict a possible contact surface for interaction with HK. We used the model as a hypothetical paradigm to design functionally active peptides in our previous experiments (Baglia et al., 1992). It has been shown that the calculated structure of the HK-binding site in the A1 domain consists of two antiparallel β -strands connected by β -turns defined by amino acid residues Val⁵⁹-Arg⁷⁰ and Asn⁷²-Lys⁸³ (Baglia et al., 1992). Our present study was undertaken to describe in detail which amino acids in this domain (A1) interact with HK. In the first part of our study we screened a panel of synthetic peptides that contain substitutions in amino acids that were predicted, on the basis of our computer model, to be possible contact sites for binding to HK. In the latter part of

our study we made rA1 peptides with amino acid substitutions in an attempt to corroborate our findings with synthetic peptides and to determine whether the entire domain might contribute to the binding of HK.

In order to perform site-directed mutagenesis experiments of the HK-binding site in the A1 domain, it was important to express the rA1 in its native, fully functional conformation. The rA1, expressed in a pQE-9 vector construct, had the same amino acid sequence as Glu¹-Ser⁹⁰ of plasma factor XI. The rA1 has inhibitory activity capable of competing with the binding of ¹²⁵I-factor XI to HK that is identical to that of purified plasma-derived factor XI. Furthermore, the two synthetic peptides comprising residues Val⁵⁹-Arg⁷⁰ and Asn⁷²-Lys⁸³ when added together at equimolar concentration had approximately the same K_i ($\sim 10^{-7}$ M) as intact factor XI and the rA1 domain (Baglia et al., 1992). These results strongly suggest that all the information required for binding of factor XI to HK resides within the A1 domain, specifically within residues Val⁵⁹-Arg⁷⁰ and Asn⁷²-Lys⁸³.

The results of our present experiments also support the hypothesis that the sequence of amino acids Val⁵⁹-Lys⁸³ in the A1 domain of the heavy chain of factor XI contains specific amino acids which comprise a contact site used for binding to HK. The evidence supporting this conclusion is as follows: (a) An A1-derived peptide, Val⁵⁹-Arg⁷⁰ with a Val⁶⁴ → Ala substitution, failed to compete with factor XI for binding to HK (Figure 4). (b) This same substitution in the expressed rA1 (Glu¹-Ser⁹⁰) also failed to compete with factor XI binding to HK (Figure 5). (c) An A1-derived peptide, Asn⁷²-Lys⁸³ with a Ile⁷⁷ → Ala substitution, failed to compete normally with factor XI for binding to HK (Figure 4). (d) This same substitution in the expressed rA1 (Glu¹-Ser⁹⁰) also failed to compete normally with factor XI binding to HK (Figure 5). (e) Normal results were obtained with substitutions of Glu⁶⁶ → Ala and Arg⁷³ → Ala (Figure 4). Thus, these experiments demonstrate that the presence of amino acid residues Val⁶⁴ and Ile⁷⁷, but not Glu⁶⁶ or Arg⁷³, is important for optimal interaction of the A1 domain of factor XI with HK.

We also examined the plasma protein PK, a blood coagulation factor with four tandem repeats that share high sequence identity with factor XI (Fujikawa et al., 1986), to determine whether it can compete for binding of factor XI to HK. Because factor XI and PK are highly homologous (58 %) and because both of these proteins bind to HK (Kerbiou et al., 1980; Schapira et al., 1982; Scott et al., 1982; Bouma et al., 1983; Bock and Shore, 1983; van der Graaf et al., 1984; Bock et al., 1985; Shimada et al., 1985; Tait and Fujikawa, 1986, 1987), an examination of the A1 domain in PK might give important clues about which amino acids present in factor XI and PK are involved in binding HK. Our experiments demonstrate that PK was able to inhibit factor XI binding to HK with a K_i of 1×10^{-3} M (Figure 1). However, the peptide Phe⁵⁶-Gly⁸⁶ of PK A1 domain (a homologue of the factor XI A1 domain peptide Phe⁵⁶-Ser⁸⁶) also inhibited ¹²⁵I-factor XI binding to HK with the identical K_i (1×10^{-3} M) as PK. This suggests that the only site that is common to PK and factor XI for binding HK is Phe⁵⁶-Ser⁸⁶ (factor XI) or Phe⁵⁶-Gly⁸⁶ (PK). This confirms experiments which mapped the HK-binding site of PK to the A1 domain (Herwald et al., 1993). As shown in Figure 6, there is a 68 % amino acid sequence identity between factor XI and PK in the region between residues 56 and 86. The amino acid comprising a putative contact surface (Val⁶⁴), as defined for factor XI in the present study, is present in the PK A1 domain in a region of six identical amino acids. Thus, the amino acids comprising the putative contact surface loop (residues 60–65) of factor XI that are also present in PK are the following: Leu-Lys-Asp-Ser-Val-Thr. This suggests that these amino acids

Figure 6 Comparison of amino acid sequences of human factor XI and plasma PK

The positions that have identical residues are boxed.

may comprise a common site for binding HK in both proteins. However, Ile⁷⁷, also indicated by the present studies to be an important component of the HK-binding surface within factor XI, is a valine in the PK A1 domain (Figure 6). Whether or not the sequence Ala-Val-Ser-Gly (residues 76–79) in PK also comprises a portion of this common binding site for HK, similar to Ala-Ile-Ser-Gly in factor XI, is unanswered by our present studies. However, it has been suggested that a sequence of amino acids spanning portions of the A3 and A4 domains of PK comprises a predominant binding site for HK (Page and Colman, 1991; Herwald et al., 1993). It is possible that this latter site confers specificity upon PK for binding to HK and accounts for the fact that PK competes very poorly with factor XI for binding to HK, as shown in Figure 1.

In conclusion, the present results imply that the side chains of valine at position 64 and isoleucine at position 77 are both important in defining the structure of the HK-binding site in factor XI. It is possible that these side chains are important for the formation or stabilization of the loop structures of which they are a part through ionic interactions, hydrogen bonding or van der Waals interactions. Alternatively, the side chains may comprise a portion of the solvent-accessible surface which interacts with HK. We favour the second explanation because our computer model predicts that Val⁶⁴ and Ile⁷⁷ are located on the very tips of the loop structures which could be easily envisioned as being in direct contact with HK. However, a definitive interpretation of our results will have to await the acquisition of structural information from X-ray crystallography or n.m.r.

This study was supported by research grants from the National Institutes of Health (HL46213, HL45486 and HL25661) and from the W. W. Smith Charitable Trust. We thank: Drs. Dominic W. Chung, Kazuo Fujikawa and Earl W. Davie (University of Washington, Seattle) for the generous gift of factor XI cDNA; Dr. Susan C. Bock (Temple University School of Medicine, Philadelphia) for advice with mutagenesis experiments; Drs. Jimmy Page and Robert Colman (The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia) for the generous gift of prekallikrein; and Patricia Pileggi for assistance in manuscript preparation.

REFERENCES

- Asakai, R., Davie, E. W. and Chung, D. W. (1987) *Biochemistry* **26**, 7221–7228
- Baglia, F. A., Sinha, D. and Walsh, P. N. (1989) *Blood* **74**, 244–251
- Baglia, F. A., Jameson, B. A. and Walsh, P. N. (1990) *J. Biol. Chem.* **265**, 4149–4154
- Baglia, F. A., Jameson, B. A. and Walsh, P. N. (1991) *J. Biol. Chem.* **266**, 24190–24197
- Baglia, F. A., Jameson, B. A. and Walsh, P. N. (1992) *J. Biol. Chem.* **267**, 4247–4252
- Bock, P. E. and Shore, J. D. (1983) *J. Biol. Chem.* **258**, 15079–15086
- Bock, P. E., Shore, J. D., Tans, G. and Griffin, J. H. (1985) *J. Biol. Chem.* **260**, 12434–12443
- Bouma, P. N. and Griffin, J. H. (1977) *J. Biol. Chem.* **252**, 6432–6437
- Bouma, P. N., Vlooswijk, R. A. A. and Griffin, J. H. (1983) *Blood* **62**, 1123–1131
- Davie, E. W., Fujikawa, K., Kurachi, K. and Kiesel, W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 277–318
- Diagen GmbH (1992) *Diagen Inc.* (J. Crowe, Diagen Inc., K. Henco, Diagen GmbH, Eds)
- Fujikawa, K., Chung, D. W., Hendrickson, L. and Davie, E. W. (1986) *Biochemistry* **25**, 2417–2424
- Griffin, J. H. and Cochrane, C. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2554–2558

- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Herwald, H., Johnen-Dockent, W., Abd Alla, S., Hock, J., Bouma, B. and Muller-Esterl, W. (1993) *J. Biol. Chem.* **269**, 14527–14535
- Kent, S. B. H. and Clark-Lewis, I. (1985) *Synthetic Peptides in Biology and Medicine* (Alitalo, K., Partanen, P. and Vakeri, A., eds.), pp. 29–58, Elsevier Science Publishers, Amsterdam
- Kerbiriou, D. M. and Griffin, J. H. (1979) *J. Biol. Chem.* **254**, 12020–12029
- Kerbiriou, D. M., Bouma, B. N. and Griffin, J. H. (1980) *J. Biol. Chem.* **255**, 3952–3958
- Kurachi, K. and Davie, E. W. (1977) *Biochemistry* **16**, 5831–5839
- Kurachi, K., Fujikawa, K. and Davie, E. W. (1980) *Biochemistry* **19**, 1330–1338
- Page, J. D. and Colman, R. W. (1991) *J. Biol. Chem.* **266**, 8143–8148
- Perkin Elmer Cetus Brochure for Gene Amp PCR Reagent Kit No. N801-0043, Copyright 1990 by Perkin-Elmer Cetus Instruments.
- Pickard, V., Ersdal-Badju, E., Lu, A. and Bock, S. C. (1994) *Nucleic Acids Res.* **22**, 2587–2591
- Proctor, R. R. and Rapaport, S. I. (1961) *Am. J. Clin. Pathol.* **36**, 212–219
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Schapira, M., Scott, C. F., James, A., Silver, L. D., Kueppers, F., James, H. L. and Colman, R. W. (1982) *Biochemistry* **21**, 567–572
- Scott, C. F., Schapira, M., James, H. L., Cohen, A. B. and Colman, R. W. (1982) *J. Clin. Invest.* **69**, 844–852
- Scott, C. F., Sinha, D., Seaman, F. S., Walsh, P. N. and Colman, R. W. (1984) *Blood* **63**, 42–50
- Shimada, T., Kato, H., Maeda, H. and Iwanaga, S. (1985) *J. Biochem. (Tokyo)* **97**, 1637–1644
- Sinha, D., Koshy, A., Seaman, F. S. and Walsh, P. N. (1985) *J. Biol. Chem.* **260**, 10714–10719
- Tait, J. F. and Fujikawa, K. (1986) *J. Biol. Chem.* **261**, 15396–15401
- Tait, J. F. and Fujikawa, K. (1987) *J. Biol. Chem.* **262**, 11651–11656
- Thompson, R. E., Mandle, R. and Kaplan, A. P. (1977) *J. Clin. Invest.* **60**, 1376–1380
- van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M. and Griffin, J. H. (1983) *J. Biol. Chem.* **258**, 9669–9675
- van der Graaf, F., Rietveld, A., Keus, F. J. A. and Bouma, B. N. (1984) *Biochemistry* **23**, 1760–1766
- Walsh, P. N., Baglia, F. A. and Jameson, B. A. (1993) *Methods Enzymol.* **222**, 65–96