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Identification of a Putative Binding Site for Negatively Charged Surfaces in the Fibronectin Type II Domain of Human Factor XII

An Immunochemical and Homology Modeling Approach

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Key words

Factor XII, binding site, fibronectin type II

Summary

Monoclonal antibodies directed against functional sites of proteins provide useful tools for structure-function studies. Here we describe a mAb, KOK5, directed against the heavy chain region of human coagulation factor XII (FXII), which inhibits kaolin-induced clotting activity by preventing the binding of FXII to kaolin. Furthermore, mAb KOK5 enhances FXII susceptibility for cleavage by kallikrein and supports FXII autoactivation. Hence, mAb KOK5 likely is directed against the binding site of FXII for negatively charged surfaces. Screening of two phage-displayed random peptide libraries with mAb KOK5 selected phages that could be grouped on the basis of two amino acid consensus sequences: A) FXFQTPXW and B) HQ/LCTHR/KKC. Sequence A contains two motifs: one shares homology with FXII amino acid residues 30-33 (FPFQ), the second one with residues 57-60 (TPNF); both amino acid stretches belonging to the fibronectin type II domain of FXII. Sequence B also reveals homology with part of the fibronectin type II domain, i.e. the stretch 40-47 (HKCTHKGR). A three-dimensional model of FXII residues 28-65, obtained by homology modeling, indicated that the three amino acid stretches 30-33, 40-47 and 57-60 are close to each other and accessible for the solvent, i.e. in a form available for interaction with the monoclonal antibody, suggesting that mAb KOK5 recognizes a discontinuous epitope on the fibronectin type II domain of FXII. Peptides corresponding to FXII sequences 29-37 ($FXII^{29-37}$) or 39-47 ($FXII^{39-47}$), were synthesized and tested for the capability to inhibit FXII binding to negatively charged surfaces. Peptide $FXII^{39-47}$ inhibited the binding of labeled FXII to kaolin and effectively prevented both dextran sulfate- and kaolin-induced activation of the contact system in plasma. Hence, we suggest that the fibronectin type II domain of FXII, in particular residues 39 to 47, contribute to the binding site of FXII for negatively charged surfaces.

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Introduction

Human factor XII (FXII), together with prekallikrein and high molecular weight kininogen, contributes to the plasma contact system which mediates the activation of several plasma cascades including fibrinolysis, bradykinin formation and complement system (1-8).

FXII, like other serine proteases of plasma cascade systems, consists of several structural domains, i.e. starting from the amino-terminus, a fibronectin domain type II, an epidermal growth factor-like domain, a fibronectin domain type I, a second epidermal growth factor-like domain, a kringle domain, a proline-rich region and the catalytic domain (9-11).

FXII is produced by the liver and circulates in plasma as a single-chain inactive zymogen. Upon activation the zymogen is converted to a two-chain active protease, activated FXII (FXIIa). Activation of FXII is elicited after its binding to a negatively charged activator, usually released after tissue injury, infections, and inflammatory conditions or present on artificial surfaces such as prosthetic heart valves or dialysis membranes. FXII has been demonstrated to bind also to endothelial cells and to neutrophils (12, 13), but it remains to be established if these cells may serve as activators of FXII *in vivo*. *In vitro*, interaction with negatively charged surfaces induces a conformational change (14-16) of single-chain FXII resulting in enhanced susceptibility to proteolytic cleavage at amino acid residues 353-354 which cleavage may occur via autoactivation or, more efficiently, by kallikrein (17-19). Although the pathophysiologic activating surface(s) is still unknown, artificial negatively charged surfaces such as kaolin, ellagic acid, sulfatide micelles and high molecular weight dextran sulfate, have been extensively used to study the activation of FXII and of the contact system *in vitro* (20-24).

A putative binding site for negatively charged surfaces has been mapped initially at amino acid residues 134-153 (25) by identifying the epitope(s) recognized by a mAb, B7C9, which inhibits kaolin-induced FXII clotting activity. Later studies located the epitope for mAb B7C9, and hence the surface binding region, at the amino-terminus of FXII between residues 1 and 28 (26, 27). However, recent studies with a recombinant FXII protein lacking the residues 3 to 19 indicate that these residues, although containing the epitope for mAb B7C9, do not contain the binding site for negatively charged surfaces, and that mAb B7C9 inhibits the clotting activity of FXII by interfering with factor XI activation (28). The latter observation implicates also that FXII binding site for negatively charged surfaces is located elsewhere in the molecule.

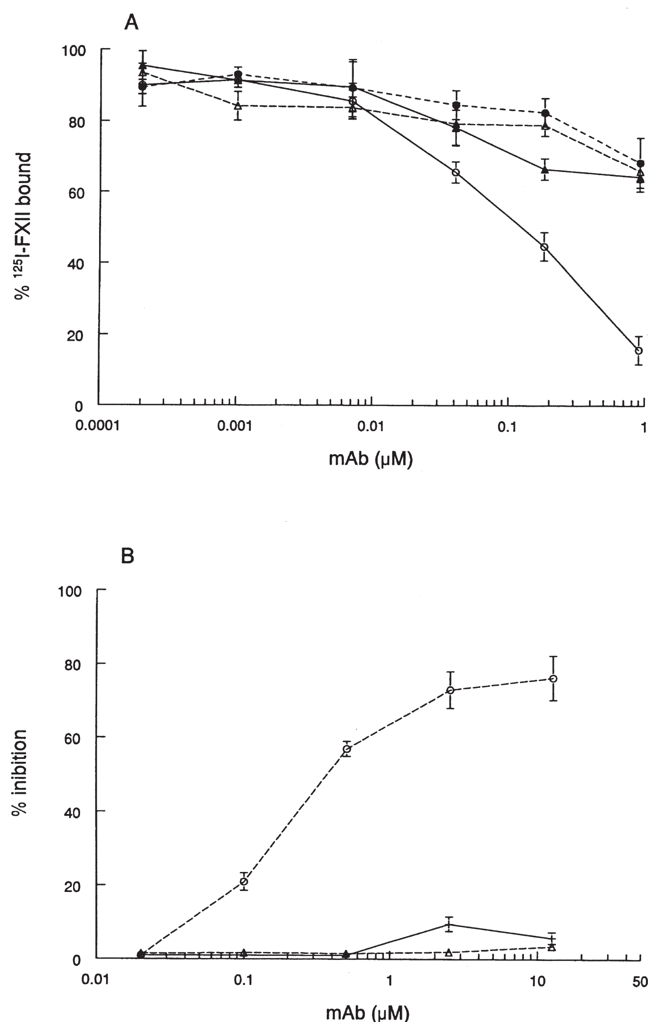

Fig. 1 A) Inhibition of FXII binding to kaolin by mAb KOK5. Fifty µl of ^{125}I-FXII (0.027 pmoles/ml) were added to 0.5 ml polypropylene tubes and incubated at room temperature for 1 h with 10 µl of PBS, 0.1% (wt/vol) Tween-20 (PT) containing increasing amounts of mAbs. Thereafter, 50 µl of kaolin (0.032 mg/ml in PT) was added. After 10 min at room temperature tubes were centrifuged for 2 min at 10,000 × g and the pellets were counted for radioactivity. Binding was expressed as the percentage of the total counts that were found in the pellet in the absence of mAbs. Results represent the means ± SD of three experiments. Symbols: mAb KOK5 (○), mAb F3 (▲), mAb F1 (△), mAb B7C9 (●). B) Inhibition of FXII clotting activity by mAb KOK5. Fifty µl of purified FXII (500 nM) were preincubated with 50 µl of serial dilutions of either mAb KOK5 (○) or mAb F1 (△) in PBS for 15 min and then tested in an FXII-clotting assay as described in Material and Methods. In addition 50 µl of purified FXII (500 nM) were first incubated for 2 min with kaolin and then with serial dilutions of mAb KOK5 for 15 min (+); whereafter the residual FXII clotting activity was measured. Results are expressed as percentage of inhibition compared to the clotting activity measured in the absence of mAbs and are the mean ± SD of three experiments

Here we describe a mAb, KOK5, which interferes with the binding of FXII to kaolin and, upon binding, induces contact system activation in plasma by increasing FXII susceptibility to activation. Thus the epitope for mAb KOK5 on FXII likely overlaps the binding site for negatively charged surfaces. By screening two phage-displayed libraries with mAb KOK5 we identified a nonapeptide corresponding to amino acid residues 39-47, belonging to the fibronectin type II domain of FXII, that competes the binding of FXII to kaolin and inhibits both dextran sulfate- and kaolin-induced contact system activation in plasma. Hence we postulate that residues 39-47 of FXII contribute to the binding site(s) of FXII for negatively charged surfaces.

Material and Methods

General. Dextran sulfate of MW 500,000 (DS500), protein G-Sepharose and soybean trypsin inhibitor (SBTI) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hexadimethrine bromide (Polybrene) was from Janssen Chimica (Beerse, Belgium), the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) was from Chromogenix AB (Mölndal, Sweden). FXII-deficient plasma was obtained from George King Biomedical Inc. (Overland Park, KS).

Proteins. FXII and prekallikrein were immunopurified from 150 ml of human citrated plasma (29, 30). Kallikrein was prepared as described (30). All protein preparations were more than 95% homogeneous as determined by SDS/polyacrylamide gel electrophoresis. Protein concentrations were determined by radioimmunoassays (31).

Purified FXII was labeled with ^{125}I by the chloramine-T method. Free label was separated by dialysis against phosphate buffered saline, pH 7.4 (PBS), containing 0.1% (wt/vol) Tween-20. The specific activity of the labeled preparation was 4.2×10^7 cpm/µg of protein. The labeled FXII was electrophoresed on SDS/polyacrylamide gel and visualized by autoradiography to assess the quality of preparation. The labeled factor XII retained greater than 95% of biological activity as determined by measuring the clotting activity and the amidolytic activity after activation with kallikrein in the presence of dextran sulfate.

Monoclonal antibodies. mAb KOK5, an IgG1 antibody, was obtained by fusing spleen cells from mice immunized with 27 µg of purified human FXII with Sp 2/0-Ag14 myeloma cells (29). mAb KOK5 was affinity purified on Protein G-Sepharose according to manufacturer's instructions. F(ab')2 and F(ab') fragments of mAb KOK5 were prepared according to standard procedures (29). Mabs F1 and F3 directed against the kringle domain and the light-chain region of FXII, respectively, have been described before (29, 30). mAb B7C9 was kindly provided by Dr. R. Pixley (Temple University, Philadelphia, PA).

FXII binding to kaolin. Binding of labeled FXII to kaolin was determined as described (28). Briefly, 50 µl of ^{125}I-FXII (0.027 pmoles/ml) were added to 0.5 ml polypropylene tubes and incubated at room temperature for 1 h with 10 µl of PBS, 0.1% (wt/vol) Tween-20 (PT) containing increasing amounts of mAbs (0.02-10 µM). Thereafter, 50 µl of kaolin (0.032 mg/ml in PT) were added and the mixtures were incubated for 10 min at room temperature. The tubes were centrifuged for 2 min at 10,000 g and the pellets were counted for radioactivity. Under these conditions, no significant binding of ^{125}I-FXII to the tubes (less than 0.5%) was observed.

To study the effect of synthetic peptides on the binding of ^{125}I-FXII to kaolin, 10 µl of serial dilutions (10-0.001 mM) of the peptides and 50 µl of ^{125}I-FXII (0.025 pmoles/ml) in PT were added to 50 µl of kaolin (0.032 mg/ml). After 10 min at room temperature tubes were centrifuged and pellets were counted for radioactivity. Binding was expressed as the percentage of the total counts that were found in the pellet in the absence of peptides.

Clotting assay for FXII. Fifty ml of purified FXII (500 nM) was preincubated for 15 min with 50 ml of serial dilutions (25-0.04 µM) of either mAb KOK5, its F(ab')2 or F(ab') fragments in PBS. Thereafter three different dilutions of the mixtures were assayed for remaining FXII coagulant activity using a modification of the activated partial thromboplastin time (32). The clotting activity of the samples was calculated on the basis of a calibration curve obtained with pooled normal human plasma.

FXII activation. Activation of FXII by kallikrein was carried out in a 96-well microtiter plate (Nunc, Roskilde, Denmark) as described (19). Briefly: 25 µl of prewarmed FXII (125 nM), 25 µl of five-fold dilutions of mAb KOK5 (25-0.2 µM), its F(ab')$_2$ or F(ab') fragments, 25 µl of prewarmed kallikrein (25 nM) and 25 µl of buffer to yield final concentrations of 50 mM Tris-HCl, 150 mM NaCl, 0.1% (wt/vol) Tween-20, pH 7.8, were incubated for 30 min at 37° C. Thereafter the amount of FXIIa formed was determined from the rate of

hydrolysis of the chromogenic substrate S-2302: 50 µl of assay mixture (S-2302 1 mM, SBTI 100 mg/ml in Tris 50 mM, NaCl 150 mM, pH 7.8) were added to the wells and the increase in absorbance at 405 nm was recorded at time intervals of 2 min by a Bio-Kinetics Reader (Bio-tek instruments Inc.) set at 37° C. Under these conditions, the rates of increase in absorbance were constant for at least 10 min and were used to calculate the amount of FXIIa generated in the wells on the basis of a calibration curve obtained with known amounts of fully activated FXII. In control experiments it was established that the amount of SBTI added was sufficient to prevent the conversion of the chromogenic substrate by kallikrein at concentrations of up to 100 mM.

Autoactivation of FXII was also studied in a 96-well microtiter plate: 25 µl of FXII (500 nM), 25 µl of five-fold serial dilutions of mAb KOK5 (25-0.2 µM) and 50 µl of buffer yielding final concentrations of 50 mM Tris-HCl, 50 mM NaCl, 0.1% (wt/vol) Tween-20, pH 7.8, were incubated for 60 min at 37° C. Thereafter, 50 µl of assay mixture (see above) were added to wells and the increase in absorbance at 405 nm was recorded.

Activation of the contact system in plasma. Plasma activation was carried out in 96-well microplates Dynatech (Plochingen, Germany): 40 µl of serial dilutions of mAb KOK5 (5-0.04 µM) in PBS were preincubated for 5 min at 37° C, thereafter 40 µl of prewarmed EDTA-plasma (31) were added. The microplate was shaken for 3 min at room temperature and then incubated for 20 min at 37° C, after which time the activation was stopped by adding 120 µl of stop buffer (PBS, 0.1 mg/ml SBTI, Polybrene 0.05%, wt/vol).

To study the effects of synthetic peptides on the activation of contact system in EDTA-plasma in the presence of DS500, 20 µl of DS500 (100 µg/ml in PBS) were preincubated for 10 min with 20 µl of serial dilutions of peptides (10-0.04 mM), thereafter 40 µl of prewarmed EDTA-plasma were added. The microplate was shaken for 3 min at room temperature and then incubated for 20 min at 37° C, after which time the activation was stopped by adding 120 µl of stop buffer (see above).

To study the effects of synthetic peptides on the activation of contact system in EDTA-plasma by kaolin, 20 µl of kaolin (5 mg/ml) were mixed with 20 µl of serial dilutions of peptides (10-0.04 mM) and 40 µl of EDTA-plasma. After a 20 min-incubation at 37° C the reaction was stopped by adding 120 µl of stop buffer, and sample were centrifuged for 1 min at 10,000 × g to discard kaolin pellets.

Contact system activation in plasma was assessed by measuring the generation of FXIIa-, kallikrein- and factor XIa-C1-inhibitor complexes. The amount of FXIIa-C1-inhibitor and kallikrein-C1-inhibitor complexes generated in EDTA-plasma was determined by radio-immunoassays, as described (31). Generation of factor XIa-C1-inhibitor complexes was determined by an enzyme-linked immunosorbent assay (ELISA) (33). Sample dilutions were tested in duplicate and results were calculated by comparison with an in-house standard that consisted of normal pooled human plasma fully activated by either DS500 (50 µg/ml) or kaolin (5 mg/ml).

Determination of the affinity of mAb KOK5 for different FXII species. Fifty µl of two-fold serial dilutions of FXII or FXIIa (125-1 nM) were preincubated overnight at 4° C with 50 µl of biotinylated mAb KOK5 (250 ng/ml) in PT, 0.1% (wt/vol) bovine serum albumin (BSA). Thereafter samples were added to maxisorp plates (96 wells) that had been coated overnight at 4° C with 100 µl of purified FXII (80 ng/ml) in 0.1 M $NaHCO_3$ pH 9.6, and blocked by incubation for 45 min at room temperature with washing buffer (PBS, 0.1% Tween 20 wt/vol). After 1 h incubation at room temperature plates were washed 5 times and then incubated for 30 min with streptavidin-horseradish peroxidase 1:1000 diluted in PBS, 0.1% (wt/vol) Tween-20, 0.2% (wt/vol) gelatin (PTG). After washes bound peroxidase was visualized by incubating the plates with 0.11 M sodium acetate, pH 5.5 containing 0.1 mg/ml 3,3',5'tetramethylbenzidin (Merck, Darmstadt, Germany) and 0.003% (v/v) H_2O_2. Reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm on a Multiscan plate reader (Labsystems, Helsinki, Finland).

Identification of the epitope recognized by mAb KOK5. Two random phage-displayed libraries were used in this study: A 15-mer peptide library described and obtained from Dr. J. J. Devlin (34), and a 9-mer peptide library fused to pVIII (35) kindly provided by Dr. F. Felici (Istituto di Ricerche di Biologia Molecolare P. Angeletti, Pomezia, Rome, Italy). The affinity purification of phages bearing peptides recognized by mAb KOK5 was performed as follows: 7.5 µg of mAb KOK5 were bound to 3 mg of Dynatech beads coated with anti-mouse IgG antibodies (Dynal A. S, Oslo, Norway), according to manufacturer's procedure. Thereafter, 1×10^{12} phages (50 µl) from the twice amplified 15-mer library were incubated with 6 µg of KOK5-beads in 200 µl of PBS, 0.1% (wt/vol) bovine serum albumin (BSA) at 4° C for 16 h by the head over head rotation. Unbound phages were discarded and beads were washed 10 times with 1 ml PBS, 0.1% (wt/vol) Tween-20, 0.1% (wt/vol) BSA. Bound phages were then eluted with 200 µl of 0.1 M HCl, adjusted to pH 2.2 with glycine, for 15 min at room temperature. The eluate was transferred to a microfuge tube, neutralized with 12 µl of 2 M Tris base, amplified as described (36), and used for a second round of panning. A control reaction and panning were carried out on beads coated with an irrelevant mAb. A third round of panning was carried out using ten times less immobilized mAb. Reactive clones were identified from the output of the third round of selection by an ELISA using mAb KOK5 as the capturing antibody and a polyclonal sheep-anti-M13 biotin conjugate (5 Prime-3 Prime, Boulder, CO, USA) as the detecting antibody (37). More than 80% of clones were found to give a positive signal and 18 were chosen to be sequenced (34). Phages binding to mAb KOK5 were also isolated from the 9-mer-peptide library by a similar procedure as described above for 15-mer peptides. After the third round of panning positive plaques were identified by immunoscreening on nitrocellulose filters (36). The isolated plaques were then grown to be tested in the ELISA and to be sequenced. Twenty-three clones were sequenced using the DYEnamic™ Direct cycle sequencing kit (-40 M13 DYEnamic ET primer) (Amersham, Buckinghamshire, UK). Amino acid sequences, deduced from nucleotide sequences, are given in the single letter code and were aligned to FXII sequence by using PC/Gene software release 6.60 July1,1991 (IntelliGenetics Inc.,Geneva, Switzerland).

FXII modeling. Homology modeling of the FXII residues 28-61 (corresponding to residues 47-80 in the swissprot database fa 12_human entry) was carried out on Silicon Graphics R500SC, using the program INSIGHT II (Dayringer et al., 1986). The model was subjected to limited energy refinement (program Discover, Steepest Descent Algorithm, Biosym Technologies, San Diego, CA).

Peptides. Synthetic peptides were synthesized at the synthetic peptide facility of the Amsterdam-Leiden Institute for Immunology by Dr. J. W. Drijfhout (Dept. of Immunohaematology and Blood Bank, University Hospital Leiden, Leiden). Synthetic peptides were made on Abimed 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany) as described (38). The peptides were isolated and purified by repeated ether precipitations, dissolved in 10% acetic acid and lyophilized. The amino acid content was checked by reverse phase high-performance liquid chromatography (HPLC). The integrity of the synthetic peptides was confirmed by mass spectrometry. The peptides were resuspended in water at an approximate concentration of 10 mM and stored in aliquots at -70° C. The peptide concentration was determined by BCA protein assay reagent (Pierce, Rockford, Illinois, USA). Peptides containing cysteine were also tested after reduction and alkylation (39).

Results

Effects of mAb KOK5 on the binding of FXII to negatively charged surfaces. The binding of radiolabeled FXII to kaolin in the presence of mAb KOK5 was investigated. As shown in Fig. 1 mAb KOK5 inhibited binding of ^{125}I-FXII to kaolin in a dose dependent manner whereas no inhibition was observed by other mAbs to FXII such as mAb F1, F3 and B7C9 (Fig. 1A).

Accordingly, mAb KOK5 was found to inhibit kaolin-induced clotting activity of purified FXII in a concentration dependent manner (Fig. 1B). Maximum inhibition was 73.5% of the total coagulant activity and was observed at a 10:1 molar ratio of intact mAb to FXII. $F(ab')_2$ and F(ab') fragments of mAb KOK5 also inhibited FXII clotting activity, to 63.1% and 60.5% respectively, at molar ratio of 20:1 $(Fab')_2$ and 40:1 (Fab') (data not shown). In contrast, mAb KOK5 was not able to inhibit the clotting activity of purified FXII preincubated with kaolin at any concentration tested (Fig. 1B) indicating that the epitope for

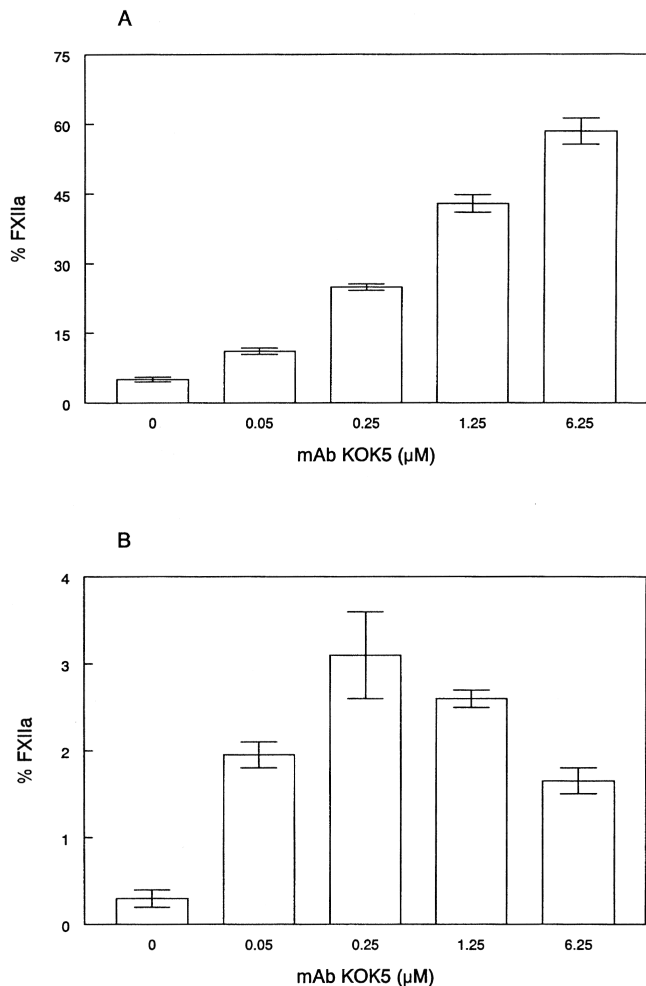

Fig. 2 Effects of mAb KOK5 on activation of purified FXII. A) Activation by kallikrein: Twenty-five μl of prewarmed FXII (125 nM), 25 μl of serial dilutions of mAb KOK5, 25 μl of prewarmed kallikrein (25 nM) and 25 μl of buffer yielding final concentrations of 50 mM Tris-HCl, 150 mM NaCl, 0.1% (wt/vol) Tween-20, pH 7.8, were incubated for 30 min at 37° C. Thereafter 50 μl of assay mixture containing S-2302 were added and the increase in absorbance at 405 nm at 37° C was recorded. B) Autoactivation: Twenty-five μl of prewarmed FXII (500 nM), 25 μl of serial dilutions of mAb KOK5 and 50 μl of buffer yielding final concentrations of 50 mM Tris-HCl, 150 mM NaCl, 0.1% (wt/vol) Tween-20, pH 7.8, were incubated for 60 min at 37° C. Thereafter 50 μl of assay mixture containing S-2302 were added and the increase in absorbance at 405 nm at 37° C was recorded. Results are expressed as the percentage of the maximum amount of FXIIa present after full activation and represent the mean ± SD of three experiments

mAb KOK5 is not accessible on kaolin-bound FXII. mAb F1, used as a control, did not have any effect on FXII clotting activity (Fig. 1B). These results together suggested that the epitope for mAb KOK5 overlaps the binding site of FXII for negatively charged surfaces.

Effects of mAb KOK5 on the activation of purified FXII. Since the binding of FXII to negatively charged surfaces increases the rate of FXII activation by kallikrein we asked whether the binding of FXII to mAb KOK5 will have effects on its activation. The rate of activation of purified FXII by kallikrein was enhanced in the presence of mAb KOK5 and increased with the concentration of mAb KOK5. At a mAb:FXII molar ratio of 40:1 the amount of FXIIa formed, after 30 min incubation, was about 50% of the total amount of FXII present whereas, in the absence of antibody, only 5% of total FXII was activated (Fig. 2A). If FXII activation by kallikrein was carried out in the presence of DS500 (50 μg/ml) after 30 min 90% of FXII was activated (data not shown). To rule out the possibility that the activation of factor XII was due to some proteases still present after mAb KOK5 purification two different preparations, purified from different batches of culture supernatant by affinity chromatography with either Protein G-sepharose or a rat mAb against kappa-light chain of mouse immunoglobulin, were used. The same results were obtained with both preparations. Moreover, mAb KOK5 did not have any effect on the amidolytic activity of FXIIa. These experiments showed that mAb KOK5 could substitute for the activating surface regarding activation of FXII by kallikrein.

The presence of mAb KOK5 also supported FXII autoactivation. Indeed the efficiency of autoactivation was dependent on the concentration of mAb KOK5, reaching an optimum at a mAb:FXII molar ratio 2:1 and decreasing at higher concentrations (Fig. 2B). At optimal conditions almost 3% of FXII was activated after 1 h incubation at 37° C in the presence of mAb KOK5, 61% in the presence of DS500 (2 μg/ml) (data not shown) and 0.3% in the absence of any activator (Fig. 2B).

Activation of the contact system in plasma by mAb KOK5. Since mAb KOK5 enhanced FXII activation by kallikrein and supported FXII autoactivation we investigated whether mAb KOK5 could also induce contact system activation in plasma. To this, the amounts of FXIIa-, kallikrein- and factor XIa-C1-inhibitor complexes generated in normal human EDTA-plasma incubated with increasing concentration of mAb KOK5 were assessed. C1-inhibitor is the major inhibitor in plasma of all three proteases, which constitute the contact system (33, 40, 41); therefore the generation of protease-C1-inhibitor complexes represents activation of the plasma contact system (22, 33, 42, 43). Results were expressed as the percentage of the maximum amount of each complex generated in normal human EDTA-plasma by DS500 (50 μg/ml), which was arbitrarily set at 100%. As shown in Fig. 3, mAb KOK5 induced generation of protease-C1-inhibitor complexes in a dose-dependent manner. The maximum amount of FXIIa- and kallikrein-C1-inhibitor complexes was generated at a mAb : FXII molar ratio of 10 : 1 and

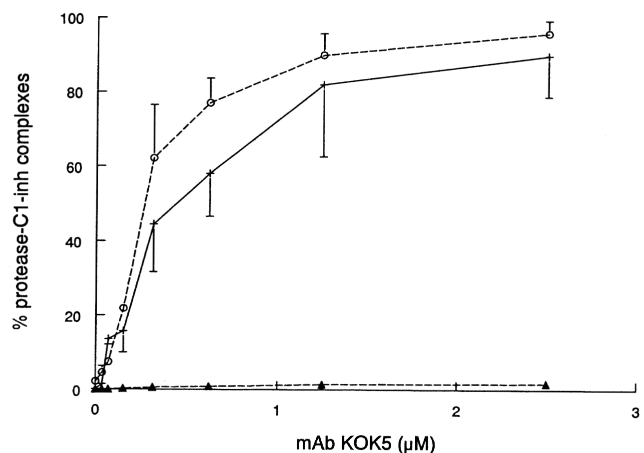

Fig. 3 Activation of the contact system in plasma by mAb KOK5. Forty μl of EDTA-plasma were incubated for 20 min at 37° C with 40 μl of two-fold dilutions of mAb KOK5 in PBS as described in Material and Methods. The reaction was stopped by adding 120 μl of stop buffer (see Materials and Methods) and the amount of FXIIa-C1-inhibitor (+), kallikrein-C1-inhibitor (○) and factor XIa-C1-inhibitor (▲) complexes generated was measured. Results, expressed as percentage of the maximum amount of complexes generated in pooled plasma by DS500 (50 μg/ml), represent the mean values ± SD from three experiments

Table 1 Contact system activation in plasma induced by mAb KOK5

Activator	FXIIa-C1-inhibitor	kallikrein-C1-inhibitor
mAb KOK5	81.9 ± 19.4	89.9 ± 5.8
F(ab')2 KOK5	77.2 ± 34.3	75.6 ± 6.6
F(ab') KOK5	20.3 ± 7.3	40.8 ± 6.5
mAb KOK5 + Polybrene	79.2 ± 13.4	54.1 ± 12.2
mAb F3	0.3 ± 0.02	0.5 ± 0.03

Forty µl of EDTA-plasma were incubated, for 20 min at 37° C, with 40 µl of mAb KOK5 (2.5 µM) with or without Polybrene (0.05% (wt/vol)), KOK5 $(Fab')_2$ (2.5 µM), KOK5 (Fab') (5 µM) and mAb F3 (2.5 µM) as control. The reaction was stopped by adding 120 µl of stop buffer (PBS, 0.1 mg/ml SBTI, Polybrene 0.05%, wt/vol) and the amount of FXIIa-Cl-inhibitor and kallikrein-Cl-inhibitor complexes generated was measured as described in Material and Methods. Results, expressed as percentage of the maximum amount of complexes generated in pooled plasma by DS500 (50 µg/ml), represent the mean values ± SD from three different experiments.

was comparable to that obtained by activating the contact system with DS500. On the contrary, the amount of factor XIa-C1-inhibitor complexes formed when plasma was incubated with mAb KOK5 was less than 2%, which is consistent with the observation that factor XI needs to bind to negatively charged surfaces to be efficiently activated by FXII (44). Activation of contact system was also observed when $F(ab')_2$ or F(ab') fragments of mAb KOK5 were added to EDTA-plasma, though somewhat less efficient (Table 1). Polybrene (0.1%) only slightly decreased the activation of the contact system by mAb KOK5. No activation was induced by the mAb F3 used as control (Table 1).

Affinity of mAb KOK5 for FXII different species. Since mAb KOK5, upon binding, increased FXII susceptibility to activation, possibly inducing a conformational change, we asked whether mAb KOK5 would bind with different affinity to different FXII species in solution. To this the dissociation constant of mAb KOK5 to native FXII and to activated FXII was determined according to the method of Friguet et al. (45). We found that the dissociation constant of mAb KOK5 for activated FXII ($Kd = 1.6 \pm 1 \times 10^{-9}$) was ten times lower than that for native FXII ($Kd = 12 \pm 4 \times 10^{-9}$) indicating a higher affinity of mAb KOK5 for FXII which had undergone a conformational change.

Identification of the epitope for mAb KOK5. To localize the epitope recognized by mAb KOK5 we screened two phage-displayed random peptide libraries, a 15-mer-peptide library and a 9-mer-peptide library, respectively. The amino acid sequences of the peptides displayed by the phages selected with mAb KOK5 were deduced from phage DNA sequences and consensus sequences, characterized by conserved amino acid residues, were derived by comparison of the selected oligopeptides (Table 2). Among the phages isolated from the 15-mer library thirteen clones shared the motif SLXP/Q (X being an amino acid residue with aromatic side chain). Three clones, harboring two different sequences, showed the consensus HQ/LCTHR/KKC homologous to FXII residues 40-47 (HKCTHKGR). From the 9-mer-peptide library 20 clones were isolated and sequenced. Fifteen clones shared the motif SLXP, similar to that found in the 15-mer library. Five independent clones shared with clone (F12.15p8), isolated from the 15-mer library, the consensus FXFQTPXW. The latter sequence contains two motifs: one shows homology with FXII residues 30-33 (FPFQ), the second one with FXII residues 57-60 (TPNF). The three FXII amino acid stretches 30-33, 40-47 and 57-60 all belong to the fibronectin type II domain of FXII.

We used molecular modeling techniques to build a three-dimensional model of the fibronectin type II domain of FXII. The model is based on the direct alignment of residues 28-65 of human FXII with the N-terminal sequence of the Bos primigenius taurus (cattle) seminal fluid protein pdc-109, the structure of which is known (46) (Table 3). In the three-dimensional model FXII amino acid residues 30-33, 40-45 and 57-60, appeared to be close to each other and accessible for the solvent in a form available for interaction with the monoclonal antibody (Fig. 4). Thus the molecular modeling results indicated that the three FXII amino acid stretches, identified by the homology with the phage

Table 2 Peptides displayed by the mAb KOK5 reactive phage clones

Clone	frequency	sequences																						
F12.15p6	2/18								W	V	V	S	S	H	Q	C	T	H	R	K	C	Y		
F12.15p10	1/18								X	T	P	X	T	H	L	C	T	H	K	K	C			
														H	**Q/L**	**C**	**T**	**H**	**R/K**	**K**	**C**			
F12.15p8	2/18		E	F	P	F	Q	T	P	P	W	E	M	I	G	E	X							
F12.9p13	2/23		E	F	L	F	Q	T	P	A	W	T	K											
F12.9p22	2/23		E	F	R	F	Q	T	P	L	W	N	T											
F12.9p25	1/23		E	F	R		Q	T	P	Q	W	H	G											
			E	**F**	**X**	**F**	**Q**	**T**	**P**	**X**	**W**													
FXII 28-50		C	H	**F**	**P**	**F**	**Q**	Y	H	R	Q	L	Y	**H**	**K**	**C**	**T**	**H**	**K**	**G**	**R**	P	G	P
FXII 51-69		Q	P	W	C	A	T	**T**	**P**	**N**	**F**	D	Q	D	Q	R	W	G	Y	C				

Deduced amino acid sequences of the 15-mer and 9-mer peptides displayed by phage clones isolated with mAb KOK5 as described in Material and Methods. Sequences are given in the single letter code. The sequences have been aligned according to the most conserved amino acid residues and two consensus have been derived (bold). W and F are both amino acid with aromatic side chains. The FXII region in which alignments with the selected phage-displayed sequences were found encompassed residues 28–69 and is reported in the last two rows, homologous stretches being in bold.

Table 3 Alignment of FXII residues 28-65 with the N-terminal sequence of the Bos primigenius taurus (cattle) seminal fluid protein

			30										40										50										60					
fa12_h	C	H	F	P	F	Q	Y	H	R	Q	L	Y	H	K	C	T	H	K	G	R	P	G	P	Q	P	W	C	A	T	T	P	N	F	D	Q	D	Q	R
	|		|	|	|		|			:		|	:	:	|	|	:		|		:				:	|	|	:		:	|	|	:	|		|		
pdc-109	C	V	F	P	F	I	Y	G	G	K	K	Y	E	T	C	T	K	I	G		S	M	W	M	S	W	C	S	L	S	P	N	Y	D	K	D	R	A

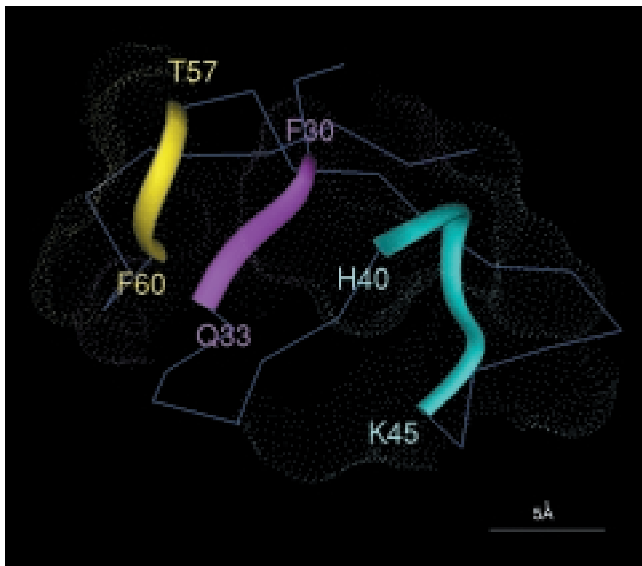

Fig. 4 Molecular model of the fibronectin type II domain of human FXII. The C-alpha trace of the N-terminal region of the FXII three-dimensional model is shown in blue; solvent accessible surface and ribbon representation corresponding to residues F30 to Q33, H40 to C45 and T57 to F60 are displayed in magenta, cyan and yellow, respectively

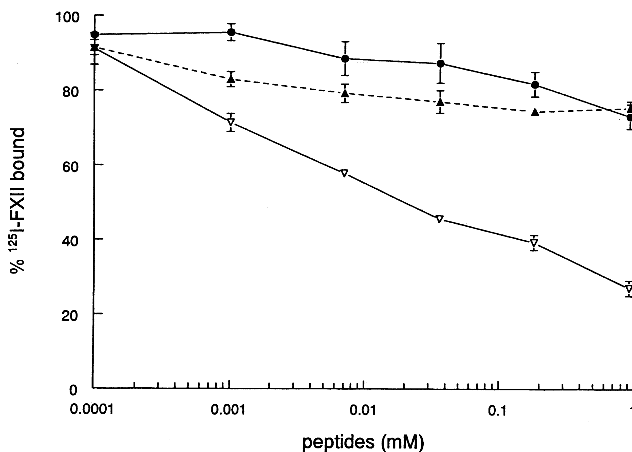

Fig. 5 Inhibition of FXII binding to kaolin by synthetic peptides. Ten µl of serial dilutions of the peptides and 50 µl of ^{125}I-FXII (0.025 pmoles/ml) in PT were added to 50 µl of kaolin (0.032 mg/ml). After 10 min at room temperature tubes were centrifuged and pellets were counted for radioactivity. Results are expressed as the percentage of the total counts that were found in the pellet in the absence of peptides and are the means ± SD of four determinations. Symbols: FXII$^{29\text{-}37}$ (●), FXII$^{39\text{-}47}$ (▽), FXII$^{3\text{-}19}$ (▲)

sequences selected with mAb KOK5, contained residues which may contribute to a discontinuous or conformational epitope.

Effects of synthetic peptides on binding of FXII to kaolin. Since mAb KOK5 inhibited the binding of FXII to kaolin we studied whether synthetic peptides corresponding to FXII regions which shared homologies with the phages selected with mAb KOK5 were able to interfere with the binding of FXII to negatively charged surfaces. Two peptides: HFPFQYHRQ (FXII$^{29\text{-}37}$) and YHKCTHKGR (FXII$^{39\text{-}47}$), corresponding to the FXII sequences indicated by the computer analysis as the sequences having maximal homology with the peptides displayed by the isolated phages, were chemically synthesized (Table 4) and tested for their capability to inhibit the binding of FXII to kaolin. As shown in Fig. 5 peptide FXII$^{39\text{-}47}$ inhibited the binding of ^{125}I-FXII to kaolin with an IC_{50} of 0.05 ± 0.006 mM. On the contrary, peptide FXII$^{29\text{-}37}$ did not show any effect on ^{125}I-FXII binding to kaolin (Fig. 5). As a control, the effects of two other synthetic peptides on the binding of FXII to kaolin were investigated: i) a scrambled peptide containing the same residues as FXII$^{39\text{-}47}$ but in a random sequence (Table 4), which inhibited the binding of ^{125}I-FXII to kaolin with an IC_{50} comparable to that of FXII$^{39\text{-}47}$ (0.06 ± 0.01 mM) and ii) peptide FXII$^{3\text{-}19}$ (Table 4), containing residues 3 to 19 of the amino terminus of FXII molecule (which have been indicated to contain a putative binding site for negatively charged surfaces) which did not inhibit the binding of ^{125}I-FXII to kaolin (Fig. 5). No effects of reduction and alkylation on the capability of the synthetic peptides to inhibit the binding of ^{125}I-FXII to kaolin were observed (data not shown).

Effects of synthetic peptides on contact system activation. The above results prompted us to study the effects of the peptide FXII$^{39\text{-}47}$ on contact activation induced in EDTA-plasma by either DS500 or kaolin. In EDTA-plasma incubated with either DS500 or kaolin the synthetic peptide FXII$^{39\text{-}47}$ inhibited, in a dose dependent manner, the activation of contact system as measured by the formation of FXIIa-C1-inhibitor complexes (Fig. 6). The concentration of FXII$^{39\text{-}47}$ required to inhibit contact system activation (as generation of FXIIa-C1-inhibitor complexes) to 50% was 0.25 ± 0.1 mM in the presence of DS500 and 0.53 ± 0.3 mM in the presence of kaolin (Table 5). The peptide was able to inhibit to less than 50% also the generation of kallikrein- and factor XIa-C1-inhibitor complexes (Table 5). As expected from the binding experiment results, peptide FXII$^{29\text{-}37}$ did not have any effect on contact system activation (Fig. 6).

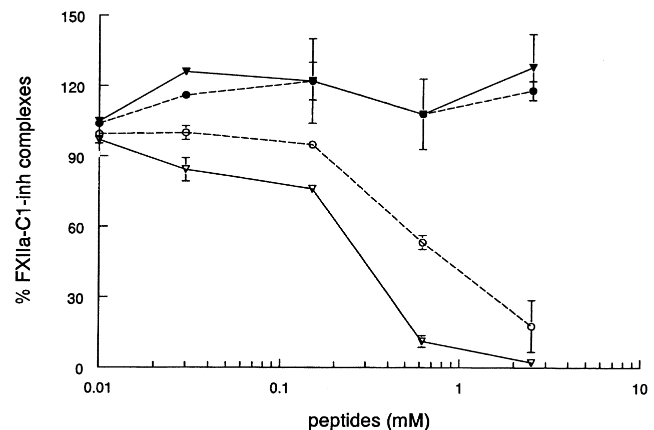

Fig. 6 Inhibition of contact system activation by synthetic peptides. Twenty µl of either DS500 (100 µg/ml) (triangles) or of kaolin (5 mg/ml) (circles) were preincubated for 10 min with 20 µl of serial dilutions of peptide FXII$^{29\text{-}37}$ (closed symbols) or FXII$^{39\text{-}47}$ (open symbols). Thereafter 40 µl of prewarmed EDTA-plasma were added. After 20 min incubation at 37° C the reaction was stopped adding 120 µl of stop buffer (see Materials and Methods). The amount of FXIIa-C1-inh complexes was determined. Results are expressed as the percentage of the maximum amount of complexes generated in the absence of peptide and are the means ± SD of four determinations

Discussion

A putative binding site of FXII for negatively charged surfaces has been mapped at the N-terminal part of the molecule, but a recent study with a FXII recombinant protein lacking this region, challenges this view. In the present paper we describe a mAb, KOK5, that fulfills all criteria for being directed against the binding site of FXII for negatively charged surfaces, i.e. it inhibits the binding of FXII to kaolin, whereas, upon binding to FXII, it supports autoactivation and enhances susceptibility of FXII for cleavage by kallikrein. By screening phage-displayed random peptide libraries with mAb KOK5 we have identified three amino acid sequences, located within the fibronectin type II domain of FXII, one of which, spanning residues 39-47, competes the binding of FXII to negatively charged surfaces, and hence possibly contributes to the binding site of FXII for negatively charged surfaces.

MAb KOK5, as well as its F(ab')2 and F(ab') fragments, enhanced FXII susceptibility for cleavage by kallikrein indicating that mAb KOK5, similar to mAb F1 that we previously described (29,30), can substitute for the activating surface to induce the conformational changes in FXII necessary for its activation (15). Notably, mAb KOK5 showed a ten-fold higher affinity for activated FXII compared to native FXII suggesting that recognition between mAb KOK5 and FXII invokes an induced fit, i.e., the binding reaction induces conformational changes (in the antibody and in the antigen) leading to a better complementarity between epitope and paratope (47). MAb KOK5 induced activation of FXII and prekallikrein, but not that of factor XI, in plasma. This activation, in contrast to that by DS500, was not influenced by the presence of Polybrene. These findings rule out the possibility that the mAb-induced FXII activation was merely due to traces of negatively charged surfaces; even though to date there is no explanation for the decreased amount of kallikrein-C1-inhibitor complexes generated in plasma in the presence of mAb KOK5 and Polybrene compared to mAb KOK5 alone. The finding that mAb KOK5-induced autoactivation was dose dependent reaching an optimum at a mAb:FXII molar ratio 2:1 is in agreement with previous studies demonstrating that the autoactivation of factor XII on negatively charged activating surfaces, as for example high molecular weight dextran sulfate, is dose-dependent (19). Autoactivation reaches an optimum when the molar ratio between dextran sulfate and factor XII is low (i.e. the factor XII molecules are bound close to each other on one dextran sulphate molecule) and decreases when the molar ratio between dextran sulfate and factor XII increases (i.e. the factor XII molecules are spread on the activating surface and can not activate each other). We postulate that also in the KOK5-induced activation an increased ratio between the activating surface and factor XII decreases the efficiency of contact between factor XII molecules and then autoactivation.

Finally, mAb KOK5, upon preincubation with FXII, prevented the binding of ^{125}I-FXII to kaolin and inhibited kaolin-mediated FXII clotting activity indicating that its epitope overlaps or is very close to the binding site for negatively charged surfaces on FXII.

This led us to map the epitope recognized by mAb KOK5. The oligopeptides selected by screening the phage-displayed random peptides libraries with mAb KOK5 could be grouped in three classes on the basis of consensus sequences that were found in several contexts which indicates that the sequences were derived from independent phages. The amino acid sequence most frequently represented (SLXP/Q) showed no discernible homology to the original FXII sequence suggesting that it represents a "mimotope" sequence, i.e. a peptide structurally unrelated to the immunizing epitope but functionally mimicking its antibody binding activity (48-51). Notably, "mimotope" sequences are often selected by mAbs which recognize discontinous epitopes (48 to 51). Analysis of the other selected oligopeptides sequences allowed us to derive a consensus sequence (FXFQTPXW) which contained two juxtaposed motifs: FXFQ resembling FXII residues 30-33 (FPFQ) and TPXW homologous to FXII residues 57-60 (TPNF) (W and F being both amino acids with aromatic side chains), implicating that mAb KOK5 may recognize a discontinuous epitope (48). We hypothesized that; while apart in the linear sequence of FXII, in the folded protein the two amino acids stretches 30-33 (FPFQ) and 57-60 (TPNF) are close to each other. The three-dimensional model of FXII fibronectin type II domain, obtained by homology molecular modeling techniques, showed that the two stretches consisting of amino acid residues 30 to 33 and 57 to 60 constitute two turns exposed to the solvent on the surface of the protein, the distance between their C-alpha being 4-6 Å. Therefore, the molecular modeling results supported the hypothesis that these two FXII amino acid stretches contained residues which may contribute to a discontinuous or conformational epitope (52-54).

By screening the phage-displayed libraries with mAb KOK5 we isolated also three phages bearing a sequence (HQ/LCTHR/KKC) homologous to FXII residues 40-47 (HKCTHKGR). The three-dimensional model of the fibronectin type II domain showed that FXII residues 40 to 45 contain another turn which is also exposed to the solvent close to the stretch 30-33 and therefore could contribute to the epitope for mAb KOK5. The localization of mAb KOK5 epitope on the fibronectin II domain of FXII is consistent with previous results obtained with FXII recombinant proteins deleted of specific domains, showing that mAb KOK5 recognizes an epitope located on one of the first three amino-terminal domains of FXII (32).

Table 4 Amino acid sequences of the synthetic peptides derived from FXII sequence

PEPTIDES	SEQUENCES
FXII$^{29\text{-}37}$	H F P F Q Y H R Q
FXII$^{39\text{-}47}$	Y H K C T H K G R
scrambled	K R C G K T H Y H
FXII$^{3\text{-}19}$	P W E A P K E H K Y K A E E H T V

Sequences are given in the single letter code.

Table 5 Inhibition of contact system activation by the synthetic peptide FXII$^{39\text{-}47}$ (IC_{50} values)

Activator	DS500	kaolin
FXIIa-C-inh	0.25 ± 0.1	0.53 ± 0.3
kal-C1-inh	0.34 ± 0.1	6.92 ± 0.7
FXIa-C1-inh	3.64 ± 1.0	1.87 ± 0.6

Twenty µl of either DS500 (100 µg/ml) or kaolin (5 mg/ml) were incubated with 20 µl of serial dilutions of synthetic peptide (0.04-10 mM) and 40 µl of EDTA-plasma as described in Material and Methods. Thereafter the amount of protease-C1-inh complexes formed was determined. The inhibition displayed by peptide FXII$^{39\text{-}47}$ is expressed as IC_{50} value (mM), i.e. the concentration of peptide that inhibited to 50% the generation of protease-C1-inh complexes. Results are the means ± SD of four experiments.

Mab KOK5 inhibited the binding of FXII to kaolin indicating that its epitope overlaps or is very close to the binding site of FXII for negatively charged surfaces. The finding that the synthetic peptide consisting of FXII residues 39-47 inhibited binding of labeled FXII to kaolin and prevented contact system activation induced in plasma by either DS500 or kaolin confirmed that mAb KOK5 binds to a region of FXII which directly contributes to the binding to negatively charged surfaces. The FXII region comprising residues 39 to 47 has a net positive charge (+ 3.13 at pH 7) distributed among nine amino acids of which three are positive charged amino acids and two are histidines. The latter have been shown to play a role in the binding of FXII to negatively charged surfaces (55). *In vitro,* the activation rate of FXII in the presence of negatively charged surfaces is dependent on the ionic strength (56) which indicates that ionic interactions are relevant in the binding of FXII to negatively charged surfaces. The finding that also the scrambled peptide was able to compete the binding of ^{125}I-FXII to kaolin, suggests that the spatial positions of the amino acids in the stretch 39 to 47 are not important. This is in line with data that many different negatively charged surfaces, possibly with different three-dimensional structures, can activate FXII (20-24). However, the overall amino acid composition of the stretch 39-47 appears to be important, since the synthetic peptide FXII$^{3-19}$, representing a FXII region which also contains three positively charged amino acids and two histidines, does not compete the binding of ^{125}I-FXII to kaolin (see Fig. 5).

In summary by screening phage-displayed random peptide libraries with mAb KOK5 in combination with molecular modeling, we have identified three short amino acid sequences (30-33, 39-47 and 57-60) belonging to the fibronectin type II domain of FXII, that are exposed on the surface of the molecule and are in a configuration that they can all contribute to a conformational, discontinuous epitope. Since one of the isolated sequences, corresponding to residues 39-47 of FXII, suppresses activation of the contact system in vitro by competing the binding of FXII to negatively charged surfaces, we suggest that amino acid residues 39 to 47 contribute to a putative binding site of FXII for negatively charged surfaces.

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