High Molecular Weight Kininogen-binding Site of Prekallikrein Probed by Monoclonal Antibodies*

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A panel of monoclonal antibodies against human prekallikrein was raised in mice and characterized with respect to the major antigenic epitopes. Of 19 antibodies tested, nine were directed against the light chain portion performing the proteolytic function of activated kallikrein, and nine recognized the heavy chain mediating the binding of prekallikrein to high molecular weight (H-)kininogen. Among the anti-heavy chain antibodies, one (PK6) interfered with the procoagulant activity of prekallikrein, and prolonged in a concentration-dependent manner the activated partial thromboplastin time of reconstituted prekallikrein-deficient plasma (Fletcher factor). Antibody PK6 was subtyped IgG1,k and had an apparent K₉₅ of 6.8 ± 0.44 · 10⁻⁹ M⁻¹ for prekallikrein. Functional analyses revealed that PK6 does not interfere with prekallikrein activation by activated Hageman factor (β-F XII), and has no effect on the kininogenase function of activated kallikrein. Monoclonal antibody PK6 but none of the other anti-heavy chain antibodies completely prevented complex formation of prekallikrein with H-kininogen, and readily dissociated preformed complexes of prekallikrein and H-kininogen. Likewise, Fab’ and F(ab’)₂ fragments of PK6 blocked H-kininogen binding to prekallikrein. A synthetic peptide of 31 amino acid residues encompassing the entire prekallikrein binding region of H-kininogen effectively competed with PK6 for prekallikrein binding indicating that the target epitope of PK6 is juxtaposed to, if not incorporated in, the H-kininogen-binding site of prekallikrein. Extensive cross-reactivity of PK6 with another H-kininogen-binding protein of human plasma, i.e. factor XI, suggested that the structure of the target epitope of PK6 is well conserved among prekallikrein and factor XI, as would be expected for the kininogen-binding site shared by the two proteins. It is anticipated that monoclonal antibody PK6 will be an important tool for the precise mapping of the hitherto unknown kininogen-binding site of prekallikrein.

Mammalian blood coagulation is a well-orchestrated process of cellular and molecular events designed for the rapid occlusion of leaky blood vessels by thrombus formation (1). Two major cascades, i.e. the endogenous (2) and the extrinsic system, serve in fibrin generation thereby involving a large number of proteins, including the non-enzymatic cofactor high molecular weight kininogen (H-kininogen) and the zymogen prekallikrein (Fletcher factor) (for reviews, see refs. 3, 4). In concert with two other proenzymes, i.e. factor XII (Hageman factor) and factor XI (plasma thromboplastin antecedent), they trigger the endogenous blood coagulation cascade and the kinin-forming pathway on subendothelial surfaces ("contact phase") (4). Local assembly of the various factors is facilitated by direct binding of factor XII and H-kininogen to the contact phase, and by non-covalent complex formation (5) between H-kininogen and prekallikrein (Kₛₑ₅ = 8.3·10⁻⁷ M⁻¹) or H-kininogen and factor XI (Kₛₑ₅ = 3.4·10⁻⁷ M⁻¹), respectively (6, 7).

The binding site for prekallikrein contained in H-kininogen has been mapped to the extreme COOH terminal portion of the kininogen light chain (8) corresponding to positions 565–595 of mature human H-kininogen (9, 10). This binding segment overlaps the corresponding binding region of factor XI covering positions 556–613 of the H-kininogen light chain sequence (11). Synthetic peptides encompassing the predicted prekallikrein-binding site of H-kininogen effectively mimic the binding of H-kininogen to prekallikrein (11). The corresponding binding site(s) for H-kininogen contained in prekallikrein and factor XI are known to be harbored by their heavy chains (7, 12); however, their precise localizations remain to be determined.

In the study of protein-protein interactions and protein functions, monoclonal antibodies which selectively interfere with functional role(s) expressed by their target proteins have proven to be useful tools (13–15). A monoclonal anti-human prekallikrein antibody was selected which inhibits activation of prekallikrein by factor XII, on a surface without competing for H-kininogen binding of prekallikrein or impairing the enzymatic activity of activated kallikrein (16). This antibody allowed identification of a previously unrecognized epitope of the prekallikrein heavy chain portion required for the interaction with factor XII on the contact surface (16). Furthermore, a monoclonal antibody against factor XII was raised which inhibits the surface-mediated coagulant activity of fac-

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1 For nomenclature of kininogens, cf. Colman, R. W., and Müller-Esterl, W. (1985) Thromb. Haemostasis 60, 340–341.

2 The abbreviations used are: H-kininogen, high molecular weight kininogen; ABTS, 2,2’-azino-bis(3-ethyl-2,3-dihydrobenzthiazoline)-6-sulfonate; ELISA, enzyme-linked immunosorbent assay; L-kininogen, low molecular weight kininogen; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.
tor XII, and its target epitope mapped to a sequence of 20 amino acid residues of the factor XII heavy chain (17, 18). Yet another monoclonal antibody directed toward a neoantigenic determinant of the factor XII heavy chain has enabled the analysis of the molecular mechanisms driving contact phase activation (19). Similar studies addressing structure-function relationships by virtue of monoclonal antibody probes were carried out with human factor XI (20). Taking advantage of the unique property of an antibody which selectively inhibits complex formation of H-kininogen and factor XI (21, 22), the kininogen-binding site of factor XI was roughly mapped to the NH₂-terminal portion (residues 1-102) of its heavy chain (23, 24).

Here we have set out to develop a set of monoclonal antibodies against human prekallikrein and to test them for their interference with functional properties of prekallikrein. Among the monoclonals directed against the prekallikrein heavy chain portion, a unique antibody (PK6) was selected which blocks the procoagulant activity of prekallikrein but not the factor XII-dependent activation of prekallikrein nor the proteolytic properties of activated kallikrein. We demonstrate that antibody PK6 selectively interferes with complex formation between prekallikrein and H-kininogen. Evidence is presented that the target epitope of PK6 is positioned topologically at or at least adjacent to the binding site of prekallikrein for H-kininogen. Therefore, antibody PK6 may provide a unique tool in studies of the hitherto unknown kininogen binding region of the prekallikrein molecule.

MATERIALS AND METHODS

RESULTS

Complex Formation of Prekallikrein and H-kininogen in the Presence of Antibodies—Failure to block prekallikrein activation or kallikrein activity by monoclonal antibody PK6 prompted us to study its capability for preventing complex formation between prekallikrein and H-kininogen. A competitive ELISA was set up using prekallikrein as the coating antigen, and biotinylated H-kininogen as the probe, and the biotin-avidin-peroxidase system as the reporter system. Binding of biotinylated H-kininogen was scrutinized in the presence of increasing amounts of antibody competitor. Specificity of the assay was demonstrated by the application of native H-kininogen which displaced biotinylated H-kininogen from the complex almost at equimolar concentrations whereas L-kininogen devoid of a prekallikrein-binding site failed to interfere with complex formation (data not shown). Among the monoclonal anti-heavy chain antibodies tested, PK6 (class C) was the only one capable of competing with H-kininogen for prekallikrein binding (Fig. 5). PK6 was even more effective in prekallikrein binding than native H-kininogen (factor of 16 at A₅₉₀nm = 0.75). Furthermore, Fab’ and F(ab’)₂ fragments of PK6 were equipotent in preventing kininogen binding to prekallikrein (not shown) thus excluding the possibility that displacement of kininogen was due to the Fc part of the intact immunoglobulin molecule. Polyclonal anti-prekallikrein IgG were equally effective in displacing H-kininogen (Fig. 5). Unlike PK6, monoclonal anti-heavy chain antibodies PK2 (class A), PK4 (class B) as well as anti-light chain antibodies PK14 (class D), and PK18 (class E) did not affect complex formation (exemplified for PK2 in Fig. 5).

3 Portions of this paper (including "Materials and Methods," part of "Results," Table I, and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
to those of the single components whereas precipitation lines corresponding to the single components were not detected. Incubation prior to electrophoresis of the complex with a 2.5-fold molar excess of PK6 resulted in the formation of two non-fusing precipitation lines at the relative positions of the single components, whereas the corresponding precipitation line of the complex was absent (Fig. 6, bottom panel). This finding indicated that dissociation of the heterodimeric non-covalent complex had occurred in the presence of PK6. Anti-heavy chain antibodies other than PK6 had no effect on complex dissociation (not shown).

Identification of the Antigenic Epitope of PK6—Interference of PK6 with prekallikrein-kininogen complex formation might suggest that PK6 binds to an epitope closely neighbored to or even incorporated in the H-kininogen-binding site of prekallikrein. To examine this possibility, a synthetic peptide of 31 amino acid residues (HK31) known to encompass the structural elements of the prekallikrein-binding site of H-kininogen was allowed to compete with PK6 for prekallikrein binding. This peptide combines with prekallikrein at a \( K_{\text{on}} \) similar to that of native H-kininogen (11). The competitive ELISA detailed above was employed, except that biotinylated HK31 instead of biotinylated H-kininogen was used as the probe. Specificity of the assay was demonstrated by application of increasing concentrations of nonderivatized peptide HK31 or native H-kininogen which efficiently competed for prekallikrein binding (Fig. 7) while an unrelated peptide of 24 residues was ineffective (not shown). Moreover, polyclonal anti-prekallikrein IgG were effective competitors of HK31 binding to prekallikrein whereas monoclonal antibodies PK2, PK4, PK14, and PK18 had no effect (exemplified for PK2 in Fig. 7). The most dramatic effect was observed with antibody PK6 which competed with biotinylated HK31 for prekallikrein binding in a concentration-dependent manner (Fig. 7). At the highest concentrations of PK6, binding of biotinylated HK31 was almost nullified. Conversely, HK31 was capable of displacing biotinylated PK6 from immobilized prekallikrein at a high molar excess of the peptide (not shown).

Cross-reactivity of Antibody PK6 with Factor XI—Because plasma prekallikrein and factor XI are homologous proteins of closely related structure (46) which compete for overlapping binding sites on the H-kininogen light chain (8, 11), we tested for the cross-reactivity of antibody PK6 with human factor XI (Fig. 8A). Titer plate-bound factor XI was readily detected by polyclonal antibodies against purified human factor XI. Application of monoclonal anti-prekallikrein antibody PK6 resulted in a strong signal indicating that PK6 effectively cross-reacted with factor XI. Among the other monoclonal anti-prekallikrein antibodies, only PK2 showed a weak cross-reactivity whereas antibodies PK4, PK14, and PK18 failed to bind to factor XI thus excluding the possibility that the factor XI preparation used for titer plate coating contained trace amounts of contaminating prekallikrein. Furthermore, affinity purified polyclonal IgG against prekallikrein barely recognized factor XI (Fig. 8A). To examine the possibility that PK6 interferes with H-kininogen binding to factor XI, a competitive ELISA was established with purified factor XI as the coating antigen and biotinylated H-kininogen as the probe (Fig. 8B). PK6 and native H-kininogen were almost equipotent in preventing binding of biotinylated H-kininogen to factor XI indicating that they might have similar affinities to factor XI. Similarly, affinity purified antibodies against factor XI were strong competitors of kininogen binding whereas monoclonal antibodies PK2, PK4, PK14, and PK18 were without effect (exemplified for PK2 in Fig. 8B).

Discussion

Prekallikrein is among the major plasma factors operative in the initiation and propagation of the contact activation system. The zymogen circulating in plasma as a heterodimer...
with H-kininogen (47) is activated on the contact phase by factor XII, (2). Specific cleavage of a single Arg-Ile bond (positions 371 and 372) results in the formation of a two-chain molecule consisting of a heavy chain of 371 residues derived from the NH2 terminus of the zymogen, and a light chain of 248 residues from the COOH terminus (48). The kallikrein heavy chain which combines with H-kininogen is composed of four tandemly arranged repeats of 90–91 residues (48). These repeated sequence segments are characterized by a well-conserved arrangement of 6 half-cysteine residues each, except for the fourth repeat which carries 2 extra half-cysteine residues, most probably engaged in the bridging of the heavy and light chains (46, 48). Extensive sequence similarity among prekallikrein and factor XI was reported (46). In particular, the four heavy chain tandem repeats are well conserved among the two proteins (46). To date, the heavy chain domain(s) involved in the complex formation of prekallikrein and H-kininogen has (have) not been identified.

As a first step to localize the target site of prekallikrein for H-kininogen, we have raised a panel of monoclonal antibodies against native prekallikrein and characterized their corresponding epitopes. Among 18 anti-prekallikrein antibodies tested, PK6 was chosen as the candidate antibody because it effectively blocked H-kininogen binding to prekallikrein, dissociated preformed complexes of the two proteins, and interfered with the procoagulant activity of prekallikrein. The corresponding Fab′ and F(ab′)2 fragments were also efficient competitors of H-kininogen binding to prekallikrein, thus excluding the possibility that interference of PK6 is due only to the Fc portion. Furthermore, reciprocal competition for prekallikrein binding of PK6 and peptide HK31 representing the “minimum” prekallikrein-binding site of H-kininogen was found. This result is consonant with the finding (50) that recombinant proteins of β-galactosidase fused to partial sequences of the H-kininogen light chain including the prekallikrein binding region are effective competitors of PK6. Significant cross-reactivity of PK6 with factor XI strongly implies that the structure of the antigenic epitope must be well conserved among prekallikrein and factor XI. Together these data suggest that PK6 interacts with a target epitope exposed by the prekallikrein (and factor XI) heavy chain with this structure being adjacent to or even incorporated in the H-kininogen-binding site (a summary of the interacting components is presented in Fig. 1). At present we cannot entirely rule out the possibility that PK6 binding induces a major conformational change in the prekallikrein molecule which results in blockage of H-kininogen binding. However, finding that peptide HK31 interferes with PK6 binding to prekallikrein argues against such a hypothesis.

Initial characterization of the antigenic epitope of PK6 revealed that this epitope is highly sensitive to structural changes in the prekallikrein molecule, e.g., due to reduction of the disulfide bonds and/or proteolytic fragmentation. Specifically, reduction of prekallikrein resulted in a complete loss of its capacity to bind to PK6 (and to H-kininogen as well). Hence, the antigenic site of PK6 is likely to represent a discontinuous rather than a continuous determinant (45).

The screening procedure described herein to detect binding site-directed antibodies relies largely on a ligand assay in which prekallikrein is immobilized on an inert matrix such as polystyrene, and biotinylated H-kininogen used as the reporter group. This ligand assay was found to be specific, reproducible, and sensitive. An analogous assay in which prekallikrein is replaced by factor XI was equally well suited to identify interfering antibodies. We could not establish a “reverse” ligand assay in which H-kininogen is surface-bound and biotinylated prekallikrein is used as the probe because biotinylation completely abolished the capacity of prekallikrein to bind kininogen (50). This problem is circumvented by an arrangement in which kininogen-bound prekallikrein is probed by a labeled anti-prekallikrein antibody. This modified assay has been successfully applied to the mapping of the prekallikrein-binding site harbored by the H-kininogen light chain (50).

According to Jerne’s (49) network theory anti-idiotypic antibodies raised against the paratope of first generation antibodies such as PK6 may be internal image(s) of the antigenic sequence, i.e., the H-kininogen binding region of prekallikrein, and therefore cross-react with the corresponding ligand, i.e., H-kininogen. Unfortunately, our attempts to demonstrate cross-reactivity of anti-idiotypes raised against PK6 with H-kininogen have failed so far. However, anti-idiotypic antibodies raised against a monoclonal antibody directed toward the kallikrein-binding region of H-kininogen (15) cross-react with prekallikrein and interfere with PK6 binding to prekallikrein (50), thus lending further support to our notion that the target site of PK6 overlaps the kininogen-binding site of prekallikrein. From the experimental results presented herein, we anticipate that PK6 provides a unique tool which will aid in the precise mapping of the H-kininogen-binding site of prekallikrein and/or factor XI. In this respect, the ability of PK6 to recognize smaller-sized fragments of prekallikrein is of major importance. We also envisage that PK6 may serve as a reagent to selectively block kininogen-mediated anchoring of prekallikrein (and factor XI) to the contact phase without touching the activatability of prekallikrein by factor XII, or the enzymatic activity of kallikrein.

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SUPPLEMENTARY MATERIAL

HEMP MOLECULAR WEIGHT KININOGEN BINDING SITE OF PREKALLIKREIN

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**Fig. 3.** Effect of monoclonal antibodies on the activated partial thromboplastin time of recombinant Fletcher plasma. Panel A: 20 µl of prekallikrein-deficient plasma (Fletcher) was mixed with an equivalent of 0.15 M NaCl, and incubated for 2 min at 37°C. Following addition of 180 µl kaolin reagent (Becton) and incubation at 25°C for 3 min, 100 µl of a solution of 25 µM was added, and the clotting time (R%) determined (calculated and broken horizontal line). Fletcher plasma was reconstituted with 1.25 µg of purified prekallikrein dissolved in 50 µl of 0.15 M NaCl, and 2 mg/ml Horseradish Peroxidase (HRP) in 0.1 M Tris-HCl, pH 7.4 (solid horizontal line). To test for the interference of antibodies with the reaction, 100 µl of 0.15 M NaCl, 10 µg/ml of monoclonal antibody 2E6 (A), 2E6 (B), 2E6 (C), and 2E6 (D) was added to 100 µl of 0.15 M NaCl incubated for 1 h at 37°C, and the clotting times measured as above. Note that the clotting time in the presence of polyclonal anti-prekallikrein IgG was extremely prolonged (7 1000 s), indicated by the open column. Panel B: The partial thromboplastin time of prekallikrein-deficient plasma (Fletcher) was measured in the presence of antikininogen-binding site antibodies 2E6 (A), 2E6 (B), 2E6 (C), and 2E6 (D). The partial thromboplastin times of Fletcher plasma (solid line) and prekallikrein-reconstituted Fletcher plasma (broken line) done in the same experiment are indicated.

**Fig. 4.** Antithrombotic activity of kaolin and activation of prekallikrein by h-factor XIIa in the presence of monoclonal antibodies. Panel A: Plasma kallikrein (0.1 µg) dissolved in 48 µl of 0.15 M NaCl, pH 8.3, including 1 mg/ml bovine serum albumin was preincubated with 2 µg of purified monoclonal antibodies 2E6 (A). 2E6 (B), 2E6 (C), and 2E6 (D) for 1 h at 37°C, and the kallikrein activity measured. To test the various possibilities, purified proteins supplemented with anti-kaolin antibodies were applied. Kallikrein was incubated with a 10-fold molar excess of mouse or polyclonal antibodies, and the antithrombotic activity assessed at 1, 3, 6, and 120 min of incubation time using a synthetic substrate. Experiments were performed using plasma kallikrein (0.1 µg/ml) and 1 µg/ml purified kaolin reagent. The data are expressed as the percentage of the maximal kallikrein activity. Panels A and B: Prekallikrein (5 µg/ml) were precipitated with 1.6 g/ml of protein A-Sepharose 4 Fastflow (Pharmacia) in 1/20 of the reaction volume. Each tube was incubated at 4°C for 30 min and the precipitate was collected by centrifugation. The activity of the supernatant was measured in the presence of 100 µM of kaolin reagent. Each point represents the mean ± SEM of triplicate determinations.