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Studies of binding of prekallikrein and Factor XI to high molecular weight kininogen and its light chain

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ABSTRACT **Prekallikrein and Factor XI have been reported to circulate as complexes with the coagulation cofactor high molecular weight (HMW)-kininogen. In this study we have shown that native HMW-kininogen possesses a strong binding site for prekallikrein and Factor XI with association constants of 3.4×10^7 M^{-1} and 4.2×10^8 M^{-1}, respectively. The diminished binding of prekallikrein relative to Factor XI may, in part, account for the ability of kallikrein to leave the surface and interact with other molecules of Hageman factor and HMW-kininogen. Prekallikrein and Factor XI appear to compete for binding to HMW-kininogen, suggesting a single (or closely overlapping) binding site(s). The purified light chain derived from kinin-free HMW-kininogen is shown to compete with native MHW-kininogen for binding to Hageman factor substrates and direct binding of the isolated light chain to prekallikrein and Factor XI is demonstrated. This binding of the light chain to prekallikrein and Factor XI appears to be essential to the function of HMW-kininogen as a coagulation cofactor and further digestion of the light chain with excess kallikrein destroys its coagulant activity.**

High molecular weight (HMW)-kininogen functions as a coagulation cofactor by forming complexes with the Hageman factor substrates, prekallikrein and Factor XI (1, 2). These complexes bind to certain negatively charged surfaces and then interact with surface-bound Hageman factor to initiate pathways of coagulation, fibrinolysis, and kinin formation. We have previously shown that digestion of human HMW-kininogen by plasma kallikrein does not diminish its ability to act as a coagulation cofactor (3, 4). Furthermore, when kinin-free HMW-kininogen was reduced, alkylated, and separated into heavy and light chains, all of the coagulant activity was associated with the light chain (4). In this study, we have compared the binding affinity of prekallikrein and Factor XI for HMW-kininogen and present evidence that they compete for binding to a site that is present on the light chain derived from kinin-free HMW-kininogen. In addition, further digestion of the isolated light chain by plasma kallikrein is shown to destroy its coagulant activity.

MATERIALS AND METHODS

QAE-Sephadex, SP-Sephadex, CM-Sepharose, concanavalin A-Sepharose, Sephadex G-200, and Sephadex G-25 (Pharmacia); bovine serum albumin (ICN); guanidine hydrochloride (Schwarz/Mann); kaolin, disodium EDTA, and Falcon polypropylene tubes (Fisher); diisopropyl fluorophosphate (Aldrich); hemostatic phosphatide (cephalin) (Nutritional Biochemicals); chloramine-T (Matheson, Coleman & Bell); and $Na^{125}I$ (New England Nuclear) were obtained as indicated. Factor XI- and prekallikrein-deficient plasma samples containing 0.38% citrate were fresh-frozen, platelet-poor plasma samples from severely deficient patients collected by SeraTec Biologicals (New Brunswick, NJ). Kinninogen-deficient plasma obtained from Ms. Williams was a gift from Robert W. Colman (Temple University Medical School, Philadelphia, PA).

Protein Purification. Prekallikrein was prepared from 4 liters of human plasma by the method of Mandle and Kaplan (5). Factor was prepared from human plasma by a modification of published procedures (5, 6). Plasma was desalted on Sephadex G-25 and fractionated by sequential chromatography on QAE-Sephadex A-50 and CM-Sepharose. The conditions and buffers were the same as those described in refs. 5 and 6. The Factor XI pool was dialyzed and fractionated on SP-Sephadex C-25 and concanavalin A-Sepharose as in ref. 7. HMW-kininogen was isolated from citrated human plasma as described by Thompson *et al.* (4). In order to isolate the heavy and light chains derived from kinin-free HMW-kininogen, purified HMW-kininogen was digested for 1 hr with 1% (wt/wt) plasma kallikrein. After reduction and alkylation (4) with dithiothreitol and iodoacetamide, respectively, the mixture was fractionated on a 2.5 × 100 cm column of Sephadex G-200 equilibrated with 6 M guanidine·HCl. The heavy and light chain peaks at 280 nm were clearly separated and both heavy and light chain-containing fractions were pooled, dialyzed against 0.1% ammonium bicarbonate, and lyophilized. After reconstitution in phosphate-buffered saline, the light chain fully retained the ability to correct HMW-kininogen-deficient plasma (4). The specific activities of prekallikrein, Factor XI, and HMW-kininogen were 40 units/mg, 165 units/mg, and 13 units/mg, respectively, if 1 unit is taken as the activity of each protein present in 1 ml of pooled normal human plasma as assessed by coagulant activity. The molecular weights of prekallikrein and Factor XI used in the calculation of association constants were 88,000 and 150,000, respectively.

Radiolabeling. Radiolabeling of prekallikrein and Factor XI was performed by using the chloramine-T method originally described by McConahey and Dixon (8). The specific activities for ^{125}I-labeled (^{125}I-) prekallikrein and ^{125}I-Factor XI were 0.31 $\mu Ci/\mu g$ and 0.23 $\mu Ci/\mu g$, respectively (1 Ci = 3.7×10^{10} becquerels). The functional activity of radiolabeled protein did not differ from that of the purified nonradiolabeled protein.

Preparation of Protein-Coated Test Tubes. HMW-kininogen (100 ng/ml), HMW-kininogen light chain (1 μg/ml), or HMW-kininogen heavy chain (1 μg/ml) containing 0.1 mM

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Abbreviations: HMW, high molecular weight; BSA/P_i/NaCl, 1% bovine serum albumin/phosphate-buffered saline/0.1 mM diisopropyl fluorophosphate at pH 7.4.

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diisopropyl fluorophosphate in phosphate-buffered saline was allowed to incubate in polypropylene tubes (12 × 75 mm) for 16 hr at 25°C. Nonadherent protein was removed by aspiration and the coated tubes were rinsed twice with 1% bovine serum albumin/phosphate-buffered saline/0.1 mM diisopropyl fluorophosphate at pH 7.4 (BSA/P_i/NaCl) to saturate any additional binding sites. The proteins were firmly adherent to the polypropylene tubes and could not be removed by heating the tubes in the presence of 1% sodium dodecyl sulfate or 8 M urea. A similar method for binding proteins to plastic tubes has been utilized (9) in which ^{125}I-fibrinogen was bound and used as an assay for plasmin. When the amount of kininogen bound to the tubes was determined by trace addition of ^{125}I-HMW-kininogen to unlabeled HMW-kininogen, 80% binding was obtained.

Binding of Hageman Factor Substrates to HMW-Kininogen-Coated Tubes. Fixed amounts of iodinated prekallikrein or Factor XI were added to a series of HWM-kininogen-coated tubes containing BSA/P_i/NaCl at 4°C. At fixed time intervals, nonbound protein was removed from the tubes by apsiration, and the tubes were washed twice with BSA/P_i/NaCl. The tubes were then assayed for residual radioactivity, which was compared to the amount of radioactivity initially added. Control tubes consisted of iodinated prekallikrein or Factor XI added to polypropylene tubes not previously coated with HMW-kininogen but washed extensively with BSA/P_i/NaCl. This pretreatment with BSA/P_i/NaCl was shown to prevent subsequent binding of HMW-kininogen by over 95%. Maximal binding of samples to HMW-kininogen-coated tubes occurred by 16 hr and was taken to represent equilibrium conditions; control tubes bound less than 5% of the counts bound by HMW-kininogen-coated tubes.

Scatchard Plots. Association constants for the binding of prekallikrein or Factor XI to HMW-kininogen were determined by using the method originally described by Scatchard *et al.* (10). Increasing amounts of iodinated prekallikrein or Factor XI were added to HMW-kininogen-coated tubes in the presence of BSA/P_i/NaCl for 16 hr at 4°C. After incubation, the contents were aspirated and the tubes were rinsed twice with BSA/P_i/NaCl. The tubes were then assayed for radioactivity and the data were expressed as the ratio of bound to free protein versus the amount of bound protein.

Coagulation Assays. The coagulant activities of prekallikrein, Factor XI, HMW-kininogen, and the light chain derived from digested HMW-kininogen were determined by a modification of the method of Proctor and Rapaport (11) for determining the partial thromboplastin time by using congenitally deficient plasma.

RESULTS

In the first series of experiments, the binding of iodinated prekallikrein or Factor XI to polypropylene tubes coated with HMW-kininogen was assessed. As shown in Fig. 1*A*, as the concentration of prekallikrein was increased, increased binding to HMW-kininogen was observed. The curve then flattened, suggesting a plateau; however, increased binding was again observed as the prekallikrein concentration was further increased. In Fig. 1*B* is shown a Scatchard plot of these data. A straight line can be drawn for those points representing binding at low prekallikrein concentrations. The points obtained at high prekallikrein concentrations, although not connected in the graph, were approximately horizontal with the abscissa. Fig. 2*A* demonstrates that a similar binding curve was obtained when ^{125}I-Factor XI was added to polypropylene tubes coated with HMW-kininogen, and a Scatchard plot of these data, shown Fig. 2*B*, was similar to that obtained with prekallikrein.

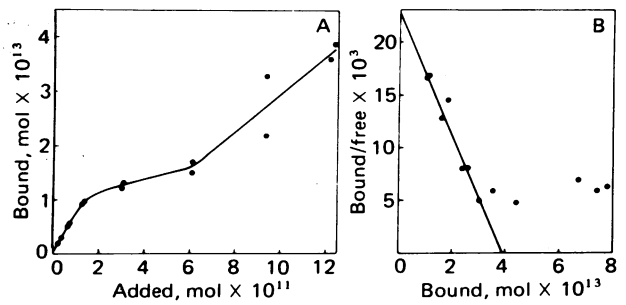

FIG. 1. (*A*) Plot of ^{125}I-prekallikrein bound to polypropylene tubes coated with HMW-kininogen vs. ^{125}I-prekallikrein added. (*B*) Scatchard plot of these data.

After several determinations, the binding constant for prekallikrein was calculated to be 3.4×10^7 M^{-1}. If it is assumed that 90% of plasma prekallikrein is bound, a calculated binding constant of 3.31×10^7 M^{-1} is obtained, assuming a prekallikrein concentration of 35 μg/ml and a HMW-kininogen concentration of 80 μg/ml. If 99% of the prekallikrein were bound, a binding constant of 4.2×10^8 M^{-1} would be anticipated. A similar theoretical calculation was made for Factor XI binding to HMW-kininogen by using a Factor XI concentration of 8 μg/ml (2). At 90–99% Factor XI binding, the calculated binding constant was 3.3×10^7 M^{-1} to 4.3×10^8 M^{-1}, whereas our experimental value was 4.6×10^8 M^{-1}. At equilibrium, there is therefore approximately 10% free prekallikrein but less than 1% free Factor XI.

Because only bimolecular complexes of HMW-kininogen and the Hageman factor substrates have been observed, we examined the ability of prekallikrein and Factor XI to compete for binding to HMW-kininogen. Fixed amounts of iodinated prekallikrein (0.1 μg) were added to HMW-kininogen-coated tubes containing increasing concentrations of Factor XI in BSA/P_i/NaCl and the tubes were incubated for 16 hr at 4°C. The reverse experiments were also performed in which fixed amounts of iodinated Factor XI (0.05 μg) were added to tubes containing increasing concentrations of prekallikrein in BSA/P_i/NaCl. The data were expressed as percentage of maximal binding in which maximal binding was taken to be the amount of iodinated protein bound to the HMW-kininogen-coated tube in the absence of nonradiolabeled competitor. Background binding, using radiolabeled prekallikrein or Factor XI added to uncoated polypropylene tubes in the presence of 1% bovine serum albumin, was subtracted from the data obtained when HMW-kininogen-coated tubes were used. As shown in Fig. 3, as the Factor XI concentration was increased, progressively less ^{125}I-prekallikrein was bound. Similarly, when the prekallikrein concentration was increased, progressively less ^{125}I-Factor XI was bound. Under the conditions of this experiment, a 100-fold excess of nonradiolabeled competitor diminished binding by

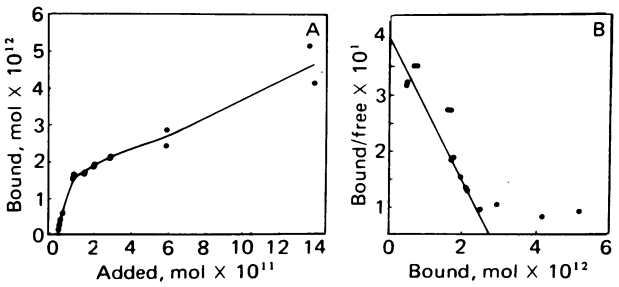

FIG. 2. (*A*) Plot of ^{125}I-Factor XI bound to polypropylene tubes coated with HMW-kininogen vs. ^{125}I-Factor XI added. (*B*) Scatchard plot of these data.

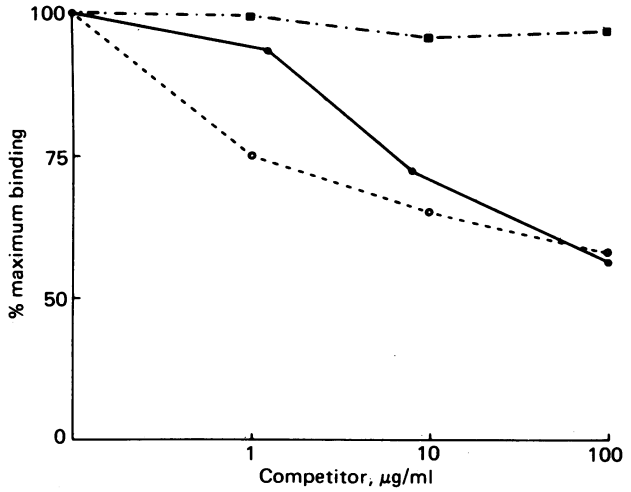

FIG. 3. Ability of prekallikrein and Factor XI to compete for binding to polypropylene tubes coated with HMW-kininogen. Increasing quantities of Factor XI were allowed to compete with ^{125}I-prekallikrein (●) or increasing quantities of prekallikrein were allowed to compete with ^{125}I-Factor XI (○). ■, Control showing the inability of IgG to compete with ^{125}I-prekallikrein for binding to HMW-kininogen coated tubes.

50%. As a control, binding of either ^{125}I-prekallikrein or ^{125}I-Factor XI to HMW-kininogen coated tubes was determined by using increasing concentrations of purified IgG instead of nonradiolabeled competitor at concentrations up to 100 μg/ml. No inhibition of binding was observed.

This result suggested that Factor XI and prekallikrein could compete for binding to a site on HMW-kininogen and we wished to determine next whether this site is present on the light chain derived from cleaved HMW-kininogen, because this is the coagulant part of the molecule. We therefore first coated polypropylene tubes with HMW-kininogen, and fixed amounts of iodinated prekallikrein (0.1 μg) or Factor XI (0.05 μg) were added in the presence of increasing concentrations of light chain in BSA/P_i/NaCl. After incubation for 16 hr at 4°C, the amount of binding to the HMW-kininogen-coated tubes was determined and the data were expressed as percentage of maximal

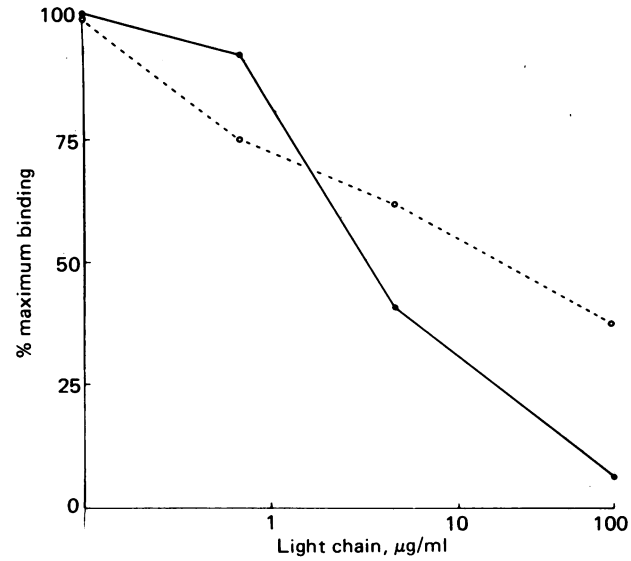

FIG. 4. Ability of increasing quantities of light chain to compete for binding of prekallikrein (●) or Factor XI (○) to polypropylene tubes coated with native HMW-kininogen.

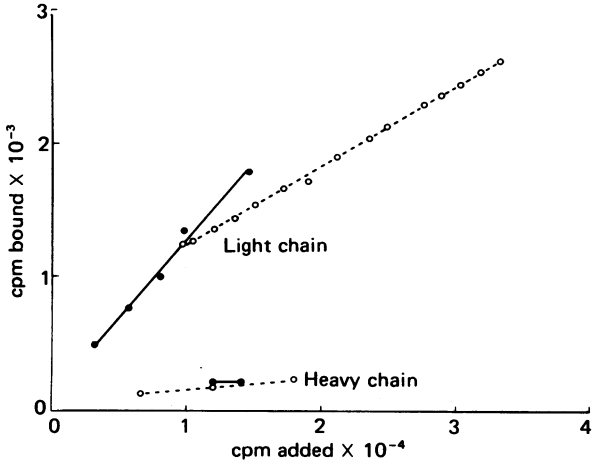

FIG. 5. Ability of prekallikrein (○) and Factor XI (●) to bind to polypropylene tubes coated with either heavy chain or light chain derived from kinin-free HMW-kininogen.

binding in which 100% was taken as the binding in the absence of additional light chain. As shown in Fig. 4, the addition of increasing quantities of light chain to either ^{125}I-prekallikrein or ^{125}I-Factor XI progressively diminished their binding to native HMW-kininogen. To examine binding in a direct fashion, polypropylene tubes were coated with either purified heavy or light chain, and increasing amounts of either iodinated prekallikrein or Factor XI in BSA/P_i/NaCl were added and the tubes were incubated for 16 hr at 4°C. The contents of the tubes were aspirated, the tubes were rinsed twice with BSA/P_i/NaCl, and the bound counts were determined. As shown in Fig. 5, as the quantity of either ^{125}I-prekallikrein or ^{125}I-Factor XI was increased, progressively increased binding to light chain-coated tubes was obtained, whereas no significant binding to heavy chain-coated tubes or noncoated control tubes (not shown) was observed.

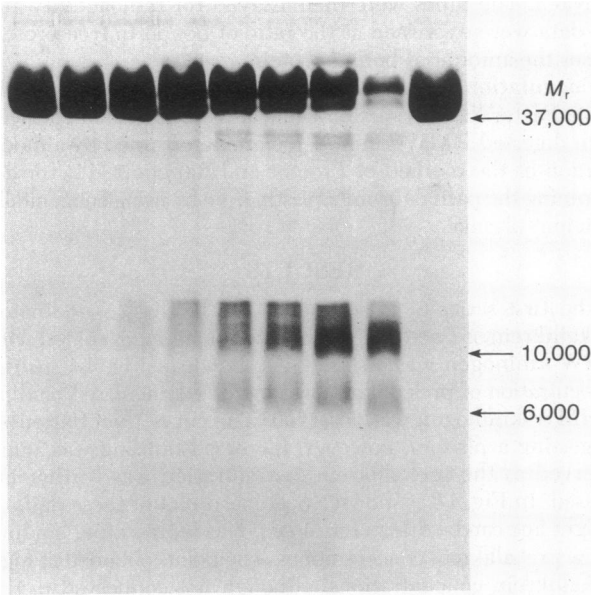

FIG. 6. Digestion of light chain derived from kinin-free HMW-kininogen by 10% (wt/wt) plasma kallikrein. A time course as assessed by slab sodium dodecyl sulfate gel electrophoresis in 15% acrylamide is shown consisting of time points at 0, 2, 4, 6, 8, 12, 18, and 24 hr. The final slot contains light chain incubated in buffer for 24 hr. M_rs: 37,000, light chain; 10,000 and 6,000, light chain fragments.

In Fig. 6 is shown a time course of digestion of purified human light chain by plasma kallikrein at a 1:10 ratio of kallikrein to light chain for a total of 24 hr. At intervals the samples were examined by sodium dodecyl sulfate gel electrophoresis in 15% polyacrylamide without reduction. The light chain was progressively digested to yield two major fragments. The smaller one had a molecular weight of 6000 and the larger one migrated at a molecular weight of approximately 10,000. The human light chain has a molecular weight of approximately 37,000 as assessed by sodium dodecyl sulfate gel electrophoresis; thus, release of a 14,000-dalton peptide would leave a residual fragment of 23,000 daltons. As the digestion proceeded, no such fragmentation was observed and each band seen undoubtedly represents a mixture of peptides of similar molecular weight. The progressively increasing doublet seen just beneath the light chain appears to represent autodigestion of plasma kallikrein (5). Progressive loss of coagulant activity accompanied light chain digestion such that only 10% of the original activity remained at 24 hr.

DISCUSSION

The function of HMW-kininogen as a coagulation factor appears to be related to its ability to complex with prekallikrein (1) and Factor XI (2). When the Hageman factor-dependent pathways are assessed functionally, HMW-kininogen markedly augments the activation of Hageman factor by kallikrein (12, 13) and it also augments the activation of both prekallikrein and Factor XI by activated Hageman factor (12–14). Wiggins *et al.* have reported that the binding of prekallikrein and Factor XI to surfaces is enhanced in the presence of HMW-kininogen (15) although direct binding to HMW-kininogen was not demonstrated. These data suggest that the affinity of HMW-kininogen for initiating surfaces exceeds that of either prekallikrein or Factor XI; thus, by virtue of complex formation, binding of the Hageman factor substrates to surfaces is enhanced. The size of the complexes formed in plasma suggests that binding to HMW-kininogen occurs as a 1:1 molar complex with either prekallikrein or factor XI (2).

We have utilized a nonactivating surface provided by polypropylene tubes to bind HMW-kininogen and examined the binding of prekallikrein and Factor XI to the surface-bound protein in the absence of any activation. The data shown in Figs. 1 and 2 demonstrate the existence of a strong binding site for either Factor XI or prekallikrein with binding constants of $3.4 \times 10^7\ M^{-1}$ and $4.2 \times 10^8\ M^{-1}$, respectively. Assessment of binding by gel filtration has suggested that at least 90% of each protein is formed as a complex with HMW-kininogen. When we calculated a theoretical binding constant by using the plasma concentration of each component, assuming 90 or 99% binding at equilibrium, good agreement with the experimental values was obtained. Colman and Scott have estimated that up to 10% of plasma prekallikrein may be free (16) and our experimental binding constant is in agreement with this observation. Factor XI, however, would be expected to exist 99% bound. Because no trimolecular complex is seen containing HMW-kininogen bound to both prekallikrein and Factor XI, we (2) previously suggested either that HMW-kininogen contains a single binding site which can interact with either prekallikrein or Factor XI, or that, if two sites are involved, they are sufficiently close that binding to one protein interferes with binding to the other. The data shown in Fig. 3 demonstrate that increasing concentrations of either Hageman factor substrate can compete with the binding of the other Hageman factor substrate. Their ability to compete with each other is quite similar and consistent with the fact that their binding constants are similar. In plasma, the concentration of HMW-kininogen is in excess of the sum of the prekallikrein and Factor XI concentrations so that, at equilibrium, each Hageman factor substrate is found almost entirely complexed.

Because the isolated light chain of HMW-kininogen functions as a coagulation cofactor, we questioned whether the light chain would be the portion of the HMW-kininogen molecule to bind prekallikrein and Factor XI. We have shown that purified light chain can compete with native HMW-kininogen for binding to either prekallikrein or Factor XI and that the light chain can be shown to bind directly to prekallikrein and Factor XI. In contrast, the isolated heavy chain possesses neither coagulant activity nor the ability to bind prekallikrein or Factor XI.

It appears likely that binding of HMW-kininogen or the light chain derived from digested HMW-kininogen is necessary for its function as a coagulation cofactor. However, the ability to bind prekallikrein and Factor XI may not be sufficient, and other effects of HMW-kininogen or its light chain with regard to binding to the surface and augmenting the interaction of Hageman factor with its substrates may also be operative. Because the presence of bovine fragment 1.2 as part of the bovine light chain appears to be important for coagulant activity (17, 18) (as is seen upon digestion of bovine HMW-kininogen with urinary kallikrein but not with plasma kallikrein), we attempted to further digest the isolated human light chain with plasma kallikrein and form fragments analogous to the bovine digestion products. No such fragmentation was seen. The light chain was progressively digested and coagulant activity was lost, but a mixture of small peptides resulted, indicating cleavage by plasma kallikrein at multiple points with similar susceptibility. These data, perhaps, explain those reported by Chan *et al.* (19) in which digestion by plasma kallikrein *did* destroy coagulant activity in human HMW-kininogen. Because plasmin is also a weak kininogenase, we digested HMW-kininogen with plasmin and examined the products by sodium dodecyl sulfate gel electrophoresis. The result was indistinguishable from that shown in Fig. 6. Nakayasu and Nagasawa (20) have also reported absence of a human counterpart of bovine fragment 1.2 upon digestion of human HMW-kininogen by either human plasma kallikrein or bovine plasma kallikrein. Thus, human HMW-kininogen appears to lack a susceptible bond for kallikrein cleavage other than those cleaved when bradykinin is released regardless of the source of plasma kallikrein. Our inability to release such a fragment upon digestion of the isolated human light chain is consistent with those observations. Yet further cleavage of the light chain is possible and is associated with loss of coagulant activity.

Once activation takes place, Factor XIa remains firmly bound to the surface, whereas considerable kallikrein is found in the fluid phase (15). Our data indicate that the binding affinity of Factor XI to HMW-kininogen is 10 times that of prekallikrein which may, in part, account for this observation. Kallikrein would then be available to digest unbound HMW-kininogen to generate bradykinin in the fluid phase and to digest other molecules of surface-bound Hageman factor at the surface–fluid interface.

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