High Molecular Weight Kininogen Binds to Platelets by Its Heavy and Light Chains and When Bound Has Altered Susceptibility to Kallikrein Cleavage

By Frank J. Meloni, Ellen J. Gustafson, and Alvin H. Schmaier

The unstimulated platelet surface contains a specific and saturable binding site for high molecular weight kininogen (HK) and low molecular weight kininogen (LK). Investigations were performed with purified heavy and light chains of HK to determine which portion(s) of the HK molecule binds to the platelet surface. Purified 64-Kd heavy chain of HK and 56-Kd light chain of HK, independently, inhibited 125I-HK binding to unstimulated platelets with a 50% inhibitory concentration ([IC50] of 84 nmol/L (apparent Ki, 30 nmol/L) and 30 nmol/L (apparent Ki, 11 nM), respectively. The ability of each of the purified chains of HK to independently inhibit 125I-HK binding was not due to cleavage, reduction, and alkylation of the protein, because two-chain HK, produced by treating HK the same way as purifying the separate chains, inhibited binding similarly to intact HK. Further, purified HK alone inhibited 125I-HK binding to platelets (Ki, 17 ± 1 nmol/L, n = 7). The 64-Kd heavy chain of HK was a competitive inhibitor on a reciprocal plot of 125I-HK-platelet binding with an apparent Ki of 28 ± 6 nmol/L (n = 4). Independently, purified 56-Kd light chain of HK was also found to be a competitive inhibitor of 125I-HK–platelet binding, with an apparent Ki of 11 ± 3 nmol/L (mean ± SEM, n = 4). These indirect studies indicated that HK binds to platelets by two portions of the molecule, one on the heavy chain and another on the light chain. Studies with 125I-light chain of HK showed that it specifically bound directly to platelets in the presence of zinc, since it was blocked by HK, light chain of HK, or EDTA, but not by LK, C1s, C1 inhibitor, plasmin, factor XII, or fibrinogen. Purified light chain of HK did not inhibit direct 125I-LK binding to platelets. HK was found to bind to platelets in an unmodified form. HK bound to platelets was cleaved by plasma or urinary kallikrein at a slower rate than the same concentration of soluble HK or HK bound and subsequently eluted from the platelet surface. Cleavage of platelet-bound HK correlated with bradykinin liberation. These studies indicate that HK has two domains on its molecule that bind to platelets. Further, platelet-bound HK is protected from kallikreins’ proteolysis. This latter finding suggests that cell binding may modify the rate of bradykinin liberation from HK.

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Materials. Na125I (50 mCi/mmol) and [14C]-5-hydroxytryptamine (51.5 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Iodogen (chloroamide 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril) was obtained from Pierce Chemical, Rockford, IL. N-butylphthalate was obtained from Fisher Scientific, King of Prussia, PA. Apiezon A oil was obtained from Biddle Instruments, Bluebell, PA. High and low molecular mass standards for sodium dioctyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
were purchased from BioRad, Richmond, CA. All other materials were obtained from Sigma Chemical, St Louis, MO, and were the best grade commercially available.

**Plasma and platelets.** Pooled normal plasmas (lots N10 and 313D) were purchased from George King Biomedical, Overland Park, KS. Normal donors were young women and men (age, 21 to 45 years) who were receiving no medication and who gave written informed consent. Fresh blood was collected and platelet-rich plasma and platelet-poor plasma were prepared as previously described. Total kininogen-deficient plasma (plasma deficient in both HK and LK) was donated by M. Williams, Philadelphia, PA. HK-deficient plasma, Fitzgerald trait, was a generous gift of Dr A.G. Sechi, Henry Ford Hospital, Detroit, MI.

**Proteins.** HK was purified using a modified method of Kerbiriou and Griffin as previously reported. These preparations of HK, when reduced with 2% β-mercaptoethanol and applied to a 7.5% SDS-PAGE, migrated mostly as a single band at 120 Kd with greater than 98% purity and with a specific activity of 12 to 20 U/mg. Purified HK was radiolabeled with Na$^{131}$I using iodogen by the method of Fraker and Speck as previously described. The specific radioactivity of the protein varied from 3 to 16 μCi/μg, with a mean value of 10 μCi/μg and 60% of the molecules were iodinated (range, 14% to 85%). Radiiodine incorporation into protein was determined by the ratio of atoms of $^{131}$I incorporated into the protein as determined by assaying radioactivity in a gamma counter to the molecules of recovered $^{131}$HK as determined by antigen concentration performed by radial immunodiffusion. The radiolabeled protein was greater than 95% trichloroacetic acid-precipitable and retained greater than 95% of its procoagulant activity, as well as its antigenic properties.

The 64-Kd heavy and 56-Kd light chains of HK were prepared by the procedure of Kerbiriou and Griffin from a partially cleaved preparation of purified HK (see "HK," Fig 1). The HK preparation used for the purification of these chains was partially cleaved during its purification from plasma, resulting in a preparation of HK that was partially kinin-free. Since this preparation of HK was not good enough for radiolabeling for binding studies, it was used to purify the kinin-free chains of HK. Human urinary kallikrein (generously provided by Dr Harry Margolius, Medical University of South Carolina, Charleston) was incubated with HK at approximately a 1/100 molar ratio (33 μg HKU in 33 μL was added into 10 mg HK in 2 mL of 10 mmol/L Tris, 0.15 mol/L NaCl, pH 7.8) for 16 hours at 37°C to completely cleave the HK for the separation of its heavy and light chains. The fully cleaved HK was then reduced with 50 mmol/L DTT for 1 hour, followed by alkylation in the dark with 120 mmol/L iodoacetamide for 30 minutes, followed by dialysis against 0.01 mol/L sodium acetate, 0.04 mol/L NaCl, pH 5.4. This material was applied to an SP Sephadex column in 0.01 mol/L sodium acetate, 0.08 mol/L NaCl, pH 5.4. The heavy chain of HK was not adsorbed to the cation exchanger, while the light chain was eluted with a linear NaCl gradient from 0.08 mol/L to 0.5 mol/L. On an 8% SDS-PAGE, the heavy chain of HK was a single band at 64 Kd, and the light chain of HK was a single band at 56 Kd (Fig 1). The purified 64-Kd heavy chain had no procoagulant activity; the purified 56-Kd light chain had a specific activity of 8 U/mg. On immunoblot using monoclonal antibodies directed to the heavy and light chains of kininogens, the purified light chain of HK was only detected by the light-chain antibody. Purified light chain of HK was radiolabeled with Na$^{125}$I using iodogen as previously reported for HK. The specific radioactivity of the protein was 5.8 μCi/μg and 15% of the molecules were iodinated. Radiolabeled light chain of HK was greater than 95% trichloroacetic acid-precipitable and retained greater than 90% of its procoagulant activity.

LK was purified as previously described. LK was characterized as having a single immunoprecipitin arc to antisera directed toward total human plasma kininogen antigen, but no procoagulant ability to correct the clotting defect of total kininogen-deficient plasma. Further, on immunoblot, it was only detected by monoclonal antibodies directed against the heavy chain of the kininogens. On reduced SDS-PAGE, purified LK migrated as a single band at 66 Kd (Fig 1). LK was also radiolabeled with Na$^{125}$I using iodogen by the method of Fraker and Speck as previously reported. The specific radioactivity of the $^{125}$ILK was 7 μCi/μg and greater than 21% of the molecules were iodinated. The radiolabeled LK was greater than 95% trichloroacetic acid-precipitable and retained its antigenic properties.

![Fig 1. Purified subunits of HK and LK.](image-url)
HK Binds to Platelets by Two Domains

Human plasma kallikrein was purified by the procedure of Scott et al. Recombinant human urinary kallikrein for platelet-bound HK cleavage studies was generously provided by Dr F.-K. Lin, AMGen, Thousand Oaks, CA. Factor XIII was kindly provided by Charles Greenburg, Duke University Medical Center, Durham, NC. CI inhibitor was purified as previously reported. C1s was a generous gift of Thomas Busby, American Red Cross, Rockville, MD. Plasmin was generously provided by Richard Hart, American Diagnostica, Greenwich, CT. Human fibrinogen was purchased from Kabo (Stockholm, Sweden). Basic peptides GHKKHER, from an amino acid sequence 444 to 449 of the light chain of HK, and HGLGKQG from an amino acid sequence 459 to 465 and 469 to 475 of the light chain of HK, were prepared in the Temple University Pepthide Synthesis Laboratory (Philadelphia, PA) using an Applied Biosystems peptide synthesizer (Model 430A; Foster City, CA) using t-Boc amino acids and Pam resins.

Functional and immunochaemochemical assays. HK procoagulant activity was measured by a one-stage, kaolin-activated clotting assay using total kininogen-deficient plasma as substrate. Samples were compared with a daily standard curve of pooled normal human plasma diluted 1:10 to 1:1,000 with 0.01 mol/L Tris and 0.15 mol/L NaCl at pH 7.4. One unit was defined as that amount of HK procoagulant activity in 1 mL of pooled normal plasma. HK antigen was also assayed by electroimmunoassay using monospecific antisera to the light chain of HK as described previously. LK antigen was measured by radial immunodiffusion using antisera directed toward total human kallikrein antigen. The amount of LK was determined by comparison with Fitzgerald plasma, which has been previously characterized for its concentration of LK. The concentration of the purified light and heavy chains of HK were also determined by the protein assay of Bradford using bovine serum albumin (BSA) as a standard, as well as radial immunodiffusion using antisera that detects total kinogen. Immunoblotting was performed by a modified procedure of Towbin et al with monoclonal antibodies to kallikrein as previously reported.

Preparation of washed platelets. Platelet-rich plasma was obtained from citrated human blood collected into polypepropylene tubes and gel filtered on a Sepharose 2B column, equilibrated in calcium-free Tyrode’s buffer (0.135 mol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO3, 0.36 mmol/L Na2HPO4, pH 7.35) with Hepes (14.7 mmol/L), BSA (1 mg/mL), and dextrose (3.5 mg/mL). This buffer contained 1 mmol/L Mg2+ and is called Hepes buffer. Platelets were counted electronically (Coulter Electronics, Hi-aleah, FL).

Binding experiments. In all binding experiments, platelets were washed 3 times with calcium-free Tyrode’s buffer (0.135 mol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO3, 0.36 mmol/L Na2HPO4, pH 7.35) with Hepes (14.7 mmol/L), BSA (1 mg/mL), and dextrose (3.5 mg/mL). This buffer contained 1 mmol/L Mg2+ and is called Hepes buffer. Platelets were centrifuged at 9,600 g for 15 minutes before centrifugation through oil. A 15-minute incubation of the radioligand with platelets with or without competitors was performed, because in previous studies it was learned that the 125I-HK and 125I-LK–platelet binding interaction was fully reversible at this time. Competitive inhibition of binding studies by HK, cleaved, andalkylated HK, and LK on 125I-HK binding to platelets were also performed. In these experiments, 125I-HK (8.3 nmol/L) was incubated with platelets in Hepes buffer containing 50 μmol/L Zn2+ and increasing concentrations of the competitor in 0 to 100-fold molar excess.

A second approach was used to confirm the results of the experiments of competitive inhibition of binding. Concentration-dependent binding experiments were performed with 125I-HK in the absence or presence of unlabeled HK, the 64-Kd heavy chain of HK, or the 56-Kd light chain of HK. In these experiments, increasing concentrations of 125I-HK (0.83 to 7.5 nmol/L) were added to an incubation mixture with platelets in Hepes buffer containing 50 μmol/L Zn2+ and increasing concentrations of the competitor in 0 to 100-fold molar excess.

Direct binding of 125I-LK to platelets was measured using 125I-LK (10 nmol/L) added to cell suspensions in Hepes buffer containing 50 μmol/L Zn2+ as previously reported. Direct 125I-HK concentration-dependent binding experiments were performed as previously reported. The direct binding of 125I-light chain of HK to platelets was determined using 5 nmol/L radiolabeled light chain added to platelets in Hepes buffer containing 50 μmol/L Zn2+. In all instances of direct binding experiments, nonspecific binding was determined by the addition of a 50-fold molar excess of the unlabeled competitor. In all instances, specific binding of the radioligand to platelets was determined by subtracting nonspecific binding (binding in the presence of a 50-fold molar excess of unlabeled ligand) from total binding according to the procedure of Schreiber et al.

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Expression and calculations of binding experiments. Calculation of bound HK was based on the specific activities of the radiolabeled ligand. Inhibition of binding of 125I-HK to platelets by HK, purified heavy or light chain of HK, or LK was analyzed by a computer program developed by Canellas and Karu to determine the 50% inhibitory concentration (IC50). The apparent Ki of the competitor was determined by the technique of Müller as previously reported. Using the formula Ka = Kd/[1 + ([I] - [T])], where [I] equals the molar concentration of the IC50 of the competitor and [T] is the molar concentration of the added 125I-HK, although this technique was developed for the calculation of antigen/antibody interactions, previous studies from this laboratory indicated that it could reliably evaluate the affinity of radiolabeled kinogen binding to various cells when used to evaluate binding performed under equilibrium conditions. For these experiments, equilibrium binding conditions are defined as specific, containing the pellet were amputated and counted in a rack gamma counter (LKB Instruments, Rockville, MD) to determine bound counts. Unbound or free counts were determined by subtracting bound counts from total counts.
reversible, and saturable binding. Inhibition of concentration-dependent binding experiments was analyzed by double reciprocal plots of 1/ specifically bound versus 1/free. The lines for the reciprocal plot were graphed after being analyzed by a weighted linear regression analysis, which was determined by using the formula of \( n = 2/(SEM)^2 \), where \( n \) is the number of determinations of added radioligand at one concentration and the SEM is determined from all the experiments of the specifically bound radioligand at that concentration. The apparent \( K_i \) of unlabeled HK or its purified chains to inhibit \( ^{125}\text{I}-\text{HK} \)-platelet binding was determined by a secondary plot of the slope of the double reciprocal plot versus inhibitor concentration. The apparent \( K_i \) was also independently calculated by a computer program for competitive inhibition kinetics using a Model 4P Computer, Tandy Corporation, Fort Worth, TX. Calculation of the Kd of direct \( ^{125}\text{I}-\text{HK} \) binding to platelets was performed by the method of Scatchard. Briefly, a linear regression was determined from the ratio of the bound radioligand/free radioligand versus bound, after nonspecific binding was subtracted from both.

**Characterization of platelet-bound HK.** The electrophoretic mobility of platelet-bound \( ^{125}\text{I}-\text{HK} \) was determined by SDS-PAGE followed by autoradiography. In these experiments, the platelet pellets were pretreated with 1 mmol/L leupeptin to prevent cleavage by calpain when solubilized with SDS.5 These solubilized platelets were then reduced with \( \beta \)-mercaptoethanol (5% vol/vol), followed by boiling for 5 minutes before application to the gel. Additionally, the ability of purified plasma kallikrein (4 nmol/L) or recombinant human urinary kallikrein (15 nmol/L) to cleave platelet-bound \( ^{125}\text{I}-\text{HK} \) was assessed. In these experiments, 2 mL of platelets (2.5 x 10^9 platelets/mL) in Hepes buffer containing \( Zn^{2+} \) (50 \( \mu \)mol/L) were made 8.3 nmol/L with \( ^{125}\text{I}-\text{HK} \) after incubation for 15 minutes. Platelets were separated from their suspension media by centrifugation through an oil gradient and the platelet pellet was washed once by centrifugation and then resuspended in one fifth of the volume with Hepes buffer (1.25 x 10^9 platelets/mL, final concentration). Since approximately 3.5% of the added radioligand was bound to the platelet pellet, the final concentration of platelet-bound \( ^{125}\text{I}-\text{HK} \) in the resuspended platelets was 1.5 nmol/L. Purified kallikreins were then added at the concentrations given above to the resuspended platelet pellets and at various times from the addition of the kallikrein (0 to 120 minutes), 50- \( \mu \)L aliquots were removed. Preliminary experiments showed that the initial exposure of \( ^{125}\text{I}-\text{HK} \) to the oil gradient did not alter the kininogen’s susceptibility to cleavage by the kallikreins (data not shown). The platelet aliquots were then centrifuged through another oil gradient, and the platelet pellets, after washing once by resuspension and centrifugation, were solubilized in SDS-PAGE sample buffer. Aliquots of the supernatant after the second centrifugation were also saved at each time point to represent platelet-bound \( ^{125}\text{I}-\text{HK} \) that eluted from the platelet surface during the incubation period. Last, a concentration of \( ^{125}\text{I}-\text{HK} \) identical to that found in the platelet suspension was incubated in buffer with the same concentration of the kallikreins, simultaneously with the cleavage experiments for platelet-bound radioligand. The autoradiograms of the dried SDS-PAGE from these experiments were scanned with a Hoefer Model GS 300 scanner (San Francisco, CA). The scans were then analyzed with Hoefer’s GS 360, Version 2.2 software.

**Characterization of bradykinin liberation from kallikrein-cleaved HK.** Purified HK (9 nmol/L) in 0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.8, was incubated with purified plasma kallikrein (45 nmol/L) or purified recombinant human urinary kallikrein (170 nmol/L) for 16 hours at 37°C. At the end of the incubation, the reaction was stopped by the addition of SDS-PAGE sample buffer. Aliquots of the intact and cleaved HKs were electroblotted onto nitrocellulose (0.1 \( \mu \)m pore size) by the procedure of Towbin et al.38 After blocking the nitrocellulose with Blotto, one immunoblot was performed using a primary goat antibody directed to human heavy and light chains of kininogen, followed by a radiolabeled rabbit anti-goat antibody (Pel Freez, Rogers, AR). A second immunoblot on the intact and cleaved kininogens was performed using a murine monoclonal antibody to bradykinin as the primary antibody followed by a radiolabeled sheep anti-mouse antibody (Pel Freez). The monoclonal antibody to bradykinin was generously provided by Dr. Julie Chao, Medical University of South Carolina, Charleston. This antibody has the property to recognize the bradykinin sequence in intact kininogens on Western blots.

**RESULTS**

**Determination of the chain of HK that binds to platelets.** Initial experiments determined which of the purified chains of HK inhibited \( ^{125}\text{I}-\text{HK} \)-platelet binding to platelets. When purified 56-Kd light chain of HK or 64-Kd heavy chain of HK were added in 25-fold or greater molar excess to \( ^{125}\text{I}-\text{HK} \), each chain independently inhibited the binding of \( ^{125}\text{I}-\text{HK} \) to unstimulated platelets by 87% or greater and 74%, respectively. The ability of each of the purified chains of HK to inhibit \( ^{125}\text{I}-\text{HK} \)-platelet binding were further evaluated in competitive inhibition of binding experiments (Fig 2A). Increasing concentrations of the 64-Kd heavy chain of HK and purified 56-Kd light chain of HK, independently, were able to inhibit the binding of \( ^{125}\text{I}-\text{HK} \) to unstimulated platelets. The \( IC_{50} \) of the purified heavy chain was 85 nmol/L (apparent \( K_i \), 30 nmol/L), while the \( IC_{50} \) of purified light chain on \( ^{125}\text{I}-\text{HK} \)-binding to platelets was 30 nmol/L (apparent \( K_i \), 11 nmol/L). The ability of the purified heavy or light chains of HK to inhibit \( ^{125}\text{I}-\text{HK} \)-platelet binding did not result from the reduction and alkylation of the cleaved protein used to prepare the purified subunits. An equal molar mixture of two-chain HK produced by human urinary kallikrein cleavage, reduction, and alkylation inhibited \( ^{125}\text{I}-\text{HK} \)-binding to platelets with an apparent \( K_i \) of 16 ± 4 nmol/L (n = 6), a value similar to the apparent \( K_i \) of 14 ± 2 nmol/L (n = 6) produced by unmodified HK (Fig 2B). Furthermore, since LK and HK are identical in structure from the N-terminal portion of the molecule through the first 12 amino acid residues on the N-terminal portion of the light chain, we wanted to confirm that the heavy chain of LK contained a domain that bound to the platelet surface. Increasing concentrations of LK inhibited \( ^{125}\text{I}-\text{HK} \)-binding to platelets with an apparent \( K_i \) of 17 ± 1 nmol/L, n = 7 (mean ± SEM) (Fig 2B).

To confirm by an independent method that each chain of HK inhibited HK binding to unstimulated platelets, we performed concentration-dependent binding studies in the absence or presence of various competitors (intact HK, purified heavy chain of HK, and purified light chain of HK) (Fig 3). To validate the use of this approach, initial binding experiments of \( ^{125}\text{I}-\text{HK} \) were performed with HK as the competitor and the data were analyzed by reciprocal plots (Fig 3A). Increasing concentrations of purified HK (10 to 25 nmol/L) competitively inhibited, as indicated by the common intercept on the ordinate, the ability of \( ^{125}\text{I}-\text{HK} \) to bind to unstimulated platelets. On a secondary plot of the slope of the reciprocal plot versus the inhibitor concentra-
HK BINDS TO PLATELETS BY TWO DOMAINS

500 - 400 - 300 - 200 - 100 -

A

% OF TOTAL BOUND

0 25 50 75 100

log COMPETITOR (nM)

1237

B

% OF TOTAL BOUND

0 25 50 75 100

log COMPETITOR (nM)

Fig 2. Competitive inhibition of 125I-HK binding to platelets. (A) Gel-filtered platelets in Hepes buffer were incubated at 37°C in the presence of Zn²⁺ (50 μmol/L), Ca²⁺ (2 mmol/L), 125I-HK (4.16 nmol/L), and increasing amounts of (0 to 10-fold molar excess) of purified heavy (■) or light (○) chain of HK. At 15 minutes, 125I-HK-platelet binding was determined as indicated in the Methods. The data shown in the figure are the mean of three or more experiments. (B) Platelets in Hepes buffer were incubated in the presence of Zn²⁺ (50 μmol/L), 125I-HK (8.3 nmol/L), and increasing amounts (0 to 100-fold molar excess) of purified LK (○), HK (■), or cleaved, reduced, and alkylated HK (□). The data expressed are the mean of five or more individual experiments.

Concentration-dependent binding experiments were performed next, with the purified chains of HK as competitors.

Purified 64-Kd heavy chain of HK was found to be a competitive inhibitor by reciprocal plot of 125I-HK–platelet binding (Fig 3B). Increasing concentrations of purified 64-Kd heavy chain of HK (10 to 37.5 nmol/L) inhibited

Fig 3. Reciprocal plot of inhibition of 125I-HK-platelet binding. (A) 125I-HK in a concentration of 0.83 to 4.16 nmol/L was added to gel-filtered platelets in Hepes buffer containing Zn²⁺ (50 μmol/L) and Ca²⁺ (2 mmol/L) in the absence (○) or presence of 10 nmol/L (●), 15 nmol/L (□), or 25 nmol/L (■) purified HK. At 15 minutes, 125I-HK-platelet binding was determined as indicated in the Methods. The plotted data represent the mean of six individual experiments fitted by weighted linear regression."# (B) 125I-HK in a concentration of 0.83 to 7.5 nmol/L was incubated with platelets in Hepes buffer, Zn²⁺ (50 μmol/L) and Ca²⁺ (2 mmol/L) in the absence (○) or presence of 10 nmol/L (●), 15 nmol/L (□), or 25 nmol/L (■) purified 56-Kd light chain of HK. The plotted data represent the mean of six experiments calculated by weighted linear regression analysis. (C) 125I-HK in a concentration of 0.83 to 7.5 nmol/L was added to platelets in Hepes buffer, Zn²⁺ (50 μmol/L) and Ca²⁺ (2 mmol/L) in the absence (○) or presence of 10 nmol/L (●), 15 nmol/L (□), or 25 nmol/L (■) purified 56-Kd light chain of HK. The plotted data represent the mean of six experiments calculated by weighted linear regression analysis.

Concentration-dependent binding experiments were performed next, with the purified chains of HK as competitors.

... (data not shown), the apparent Ki of HK on 125I-HK binding was 7 nmol/L. When these data were independently analyzed by a computer program for competitive inhibition, the apparent Ki of HK on 125I-HK binding was found to be similar at 19 ± 5 nmol/L, n = 3 (mean ± SEM). These values were similar to the Kd (15 ± 4 nmol/L, n = 6) determined by recent direct 125I-HK–platelet binding experiments and analyzed by a Scatchard plot (data not shown), as well as to the Ki obtained with HK in the experiments of competitive inhibition of binding (14 ± 2 nmol/L, n = 6) (Fig 2B).

...
was 12 nmol/L when analyzed on a secondary plot of the shown), the apparent Ki of purified 64-Kd heavy chain of binding region on the kininogens has been independently analyzed by a computer program, and an apparent Ki of 11 ± 3 nmol/L, n=4 (mean ± SEM), was found. When these data were independently analyzed by a computer program, an apparent Ki of 12 nmol/L was also found to produce competitive inhibition of 125I-HK binding to platelets. The apparent Ki of purified 56-Kd light chain of HK (10 to 25 nmol/L) was also found to be specific, because only unlabeled light chain and intact HK were able to inhibit 125I-light chain binding (Table 1). LK,Cls,C1 inhibitor, plasmin, factor XIII, and fibrinogen were not able to influence light chain binding to unstimulated platelets (Table 1). Further, there was an absolute zinc requirement for 125I-light chain of HK binding to platelets, since its omission greatly reduced binding, and 10 mmol/L EDTA also inhibited light chain binding. The ability of HK or light chain of HK to bind to platelets was not due to the highly basic, histidine-rich region on this part of the protein, because two peptides, GHKHER and HGLGHGH, derived from this region of the light chain of HK did not inhibit 125I-light chain binding (Table 1). Finally, although LK and HK reciprocally inhibited each other from binding to platelets, LK and the light chain of HK did not mutually exclude each other from binding (Table 1). LK did not inhibit 125I-light chain of HK from binding to platelets, and the light chain of HK did not inhibit 125I-LK from binding (Table 1).}

**Characterization of form and function of platelet-bound HK.** Studies were performed to characterize HK bound to platelets. 125I-HK was found to bind to platelets in an unmodified form (Fig 5). The electrophoretic mobility of 125I-HK recovered from platelets was identical to the starting 125I-HK, as well as the unbound portion. Since HK bound to platelets in an unmodified form, further investigations were performed to determine if platelet-bound HK was a substrate for plasma and tissue kallikreins (Figs 6 and 7). When 1.5 nmol/L soluble 125I-HK was incubated with 4 nmol/L plasma kallikrein, a concentration that represents the activation of less than 1% of plasma prekallikrein, the radioligand was rapidly cleaved (within 1 minute) into a

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*Table 1. Specificity of the Binding of the Plasma Kininogens and the Light Chain of HK to Platelets*

Gel-filtered platelets (2 x 10^9/ml) were incubated at 37°C for 15 minutes with 8 nmol/L 125I-HK, 5 nmol/L 125I-light chain of HK, or 10 nmol/L 125I-LK in Hepes buffer containing 50 μmol/L Zn²⁺ in the absence or presence of a 50-fold molar excess (unless stated otherwise) of competing protein or peptide. The data are the means of two individual experiments for each ligand. The percent inhibition was calculated by the formula one minus the ratio of binding of the radioligand in the presence of the competitor over binding of the radioligand in the absence of the competitor less nonspecific binding from both times 100. Nonspecific binding for each radioligand was defined as the amount of binding seen in the presence of a 50-fold molar excess of unlabeled ligand and by definition represents 100% inhibition.
HK BINDS TO PLATELETS BY TWO DOMAINS

Fig 5. Characterization of platelet-bound 125I-HK. 125I-HK (SM) was incubated with washed platelets in Hepes buffer in the presence of 50 μmol/L Zn++. The platelet-bound 125I-HK (BOUND) after treatment with 1 mmol/L leupeptin, the unbound 125I-HK (UNB), and the 125I-HK starting material (SM) were reduced with 5% β-mercaptoethanol and boiled for 10 minutes and then applied to an 8% SDS-PAGE. The figure is a photograph of an autoradiogram. The numbers to the left of the gel represent molecular mass standards in kilodaltons.

64-Kd heavy chain and a 56-Kd light chain (seen as two thick bands between the 66- and 43-Kd markers), resulting in less than 6% of the total HK remaining intact at 120 Kd (Fig 6A). Alternatively, when 1.5 nmol/L platelet-bound 125I-HK was incubated with the same concentration of plasma kallikrein, the rate of cleavage of the platelet-bound HK was retarded when compared with the fluid-phase incubation. Approximately 30% of the radioligand remained intact at 120 Kd after 1 minute (Fig 6C). Full cleavage of the 120-Kd 125I-HK was completed in the fluid phase by 10 minutes, but even at 120 minutes, some of the platelet-bound 125I-HK remained uncleaved. In this experiment, a control platelet-bound 125I-HK incubated without plasma kallikrein for 2 hours at 37°C also became cleaved. The extent of cleavage was similar to that of platelet-bound 125I-HK incubated with plasma kallikrein for 1 minute. The protection from cleavage only occurred with platelet-bound HK, because any 125I-HK that eluted from the platelets during the incubation was cleaved almost as rapidly as the kininogen incubated in solution with 4 nmol/L plasma kallikrein (Fig 6B).

Similar findings occurred when recombinant human urinary kallikrein was used as the kininogenase (Fig 7). In a soluble cleavage reaction (Fig 7A), 1.5 nmol/L intact 120-Kd 125I-HK was rapidly cleaved by human urinary kallikrein (15 nmol/L) into 64- and 56-Kd bands (which appear as one thick band in Fig 7). A 46-Kd band faintly appeared at 10 minutes, becoming more prominent at 120 minutes. At 10 minutes, only 5% of the radioligand remained intact. After 10 minutes' incubation, 1.5 nmol/L platelet-bound 125I-HK was less cleaved, since 32% of the radioactivity remained at 120 Kd (Fig 7C). Even at 120 minutes' incubation, 19% of the platelet-bound 125I-HK was intact (120 Kd) and seemed to be resistant to cleavage by human urinary kallikrein. In a control incubation for 2 hours at 37°C, 125I-HK bound to platelets and not treated with kallikrein did not become cleaved (Fig 7C). The protection of platelet-bound HK from kallikrein cleavage only occurred with the radiolabeled HK that remained platelet-bound. 125I-HK that eluted from the platelets was rapidly cleaved, within 10 minutes, by human urinary UNB SM

Fig 6. Plasma kallikrein cleavage of platelet-bound 125I-HK. 125I-HK (8.3 nmol/L) was added to 2 mL of platelets (2.5 × 10⁶ platelets/mL) in Hepes buffer containing Zn++ (50 μmol/L) and incubated for 15 minutes. After centrifugation through an oil gradient, approximately 3.5% of the radioligand bound to the platelet pellet. The platelet pellet was resuspended to a total volume of 0.4 mL before incubation with plasma kallikrein. (A) Fluid phase represents an incubation for 0 to 120 minutes at 37°C of 1.5 nmol/L 125I-HK with purified plasma kallikrein (4 nmol/L) in buffer. SM represents the starting 125I-HK. (B) Bound/eluted represents 125I-HK incubated with 4 nmol/L plasma kallikrein that eluted off the platelets during the 1- to 120-minute incubation. (C) Platelet-bound represents a time course of 4 nmol/L plasma kallikrein cleavage of 1.5 nmol/L platelet-bound 125I-HK. CN represents a control incubation of 125I-HK on or eluted off platelets for 120 minutes without kallikrein. All samples were reduced with 5% β-mercaptoethanol and boiling before application to the SDS-PAGE. The figure is a photograph of an autoradiogram. The numbers to the left of the figure represent molecular mass markers in kilodaltons. The numbers at the bottom of each panel represent the incubation time in minutes of the radiolabeled HK with plasma kallikrein.
kallikrein, since only 7% of the protein remained at 120 Kd (Fig 7B).

Although previous reports have shown that cleavage of HK in the patterns seen in Figs 6 and 7 with plasma and urinary kallikrein correlated with bradykinin liberation, additional studies were performed to confirm that the cleavage of radiolabeled HK was associated with bradykinin liberation (Fig 8). As shown in Fig 8, both plasma and urinary kallikrein cleaved HK from an intact band at 120 Kd to a thick band that extends from 64 to 56 Kd. Another faint band at 46 Kd is also seen in the cleavage experiment with plasma kallikrein. On immunoblot of these same kallikrein-treated specimens, mostly cleaved kininogen is seen, although a small quantity of intact 120-Kd HK was detected using an antibody that recognizes both the heavy and light chains of HK (Fig 8B). However, when an antibody to bradykinin, which recognizes on Western blot kinin in intact kininogen, was used for the immunoblot, the starting material only was detected (Fig 8C). In Fig 8, no intact or kallikrein-cleaved HKs were seen on the immunoblot with the anti-bradykinin antibody, although faint bands of intact HK at 120 Kd were present on the autoradiogram in support of the findings seen in Fig 8B. These findings indicated that the cleaved kininogen seen in Figs 6 and 7 must be bradykinin-free. Since platelet-bound HK is protected from kallikrein cleavage, these data indicate that the binding of HK to platelets modulates the rate by which bradykinin can be liberated from this pool of kininogen.

**DISCUSSION**

HK and LK both serve as the parent proteins for bradykinin. The light chain of HK contains binding regions for artificial surfaces and prekallikrein and factor XI, which give HK its cofactor role in the activation of the plasma zymogens, factor XII, prekallikrein, and factor XI. The heavy chain of HK serves as an inhibitor of cysteine proteases, including platelet calpain. Expression of HK on platelets or other cell membranes of the vasculature may allow for the integration of these three different activities of HK.

Since platelets circulate in a resting state, we chose to focus our investigations on determining which region(s) on HK binds to unstimulated platelets using the purified heavy and light chains of HK. Initial investigations showed that LK and the heavy and light chains of HK independently produced competitive inhibition of 125I-HK–platelet binding (Fig 2). Since LK directly binds to platelets, it was expected that the heavy chain of HK could inhibit 125I-HK binding to platelets. To independently confirm these findings and to determine the mechanism and inhibitory constants of the purified heavy and light chains on 125I-HK binding to platelets, concentration-dependent binding experiments were performed in the presence of various competitors and analyzed by reciprocal plots (Fig 3). The validity of this experimental approach was confirmed by determining the Ki of HK on 125I-HK binding to platelets. The Ki of 7 to 19 nmol/L found in recent, direct 125I-HK binding to platelets, concentration-dependent binding experiments were performed in the presence of various competitors and analyzed by reciprocal plots (Fig 3). The validity of this experimental approach was confirmed by determining the Ki of HK on 125I-HK binding to platelets. The Ki of 7 to 19 nmol/L produced by HK on 125I-HK binding (Figs 2A and 3A) is similar to the Kd of 15 nmol/L found in recent, direct 125I-HK binding to unstimulated platelets. It is important to note that the present values reported by us for the Kd and Ki for direct 125I-HK binding to platelets and HK’s competitive inhibition of binding of 125I-HK to platelets, respectively, are higher than that we had previously published. The values reported here represent a recalculation of that published data and the performance of additional experiments. These present values serve to correct those we previously published for 125I-HK binding to unstimulated platelets.

Using the methods of competitive inhibition and concentration-dependent binding experiments in the presence of chains of HK, we were able to determine that, in addition to the purified heavy chain of HK, the purified light chain of HK is also able to independently function as an inhibitor of 125I-HK binding to platelets (Figs 2 and 3). These indirect binding experiments indicate that HK binds to platelets by two domains on its molecule. Since the light chain of HK is known to interact with artificial, negatively charged sur-
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Fig 8. Kallikrein cleavage of HK results in bradykinin liberation. HK (9 μmol/L) in 0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.8, was cleaved with purified plasma kallikrein (45 nmol/L) or purified recombinant human urinary kallikrein (HUK; 170 nmol/L) and applied to an SDS-PAGE. SM represents intact HK starting material, and PK represents plasma kallikrein cleaved HK. A portion of this material was stained with Coomassie blue and photographed (A). Another aliquot was electrophoresed onto nitrocellulose and immunoblotted with an antibody to the heavy and light chain of kininogen (B) or bradykinin (C). The figure is a photograph of the stained SDS-PAGE or the autoradiogram of the immunoblot. The numbers to the left of the photographs represent molecular mass standards in kilodaltons.

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faces, it has been assumed without any specific data that the light chain should contain a domain that binds to cell surfaces. Confirmation of this assumption that the light chain of HK does contain a region that can bind to platelets is shown by the present direct 125I-light chain of HK-platelet binding studies (Fig 4). 125I-light chain binding to platelets was specific, since only unlabeled light chain and HK, but not LK, inhibited binding (Table 1). The zinc requirement for light chain binding to platelets may be necessary to form a bridge between the platelet surface and the anionic charged region on domain 5 of the light chain of HK. However, two histidine-rich peptides from domain 5, one of which binds zinc, did not block 125I-HK binding to platelets. Therefore, the zinc requirement for light chain binding to platelets may be for the expression of the putative kininogen receptor, similar to the postulated reason for the requirement of zinc ion for LK binding to platelets. Although LK inhibits HK binding and HK inhibits LK binding to platelets, the finding that purified 56-Kd light chain of HK did not inhibit 125I-LK binding to platelets and LK did not inhibit 125I-light chain binding (Table 1) indicates that the region of LK that binds to platelets is not the 12 amino acids on the N-terminal portion of the light chain. This finding also suggests that both binding domains on the light and heavy chains of HK independently interact with different regions of the platelet surface. It is possible that platelets and perhaps other cells have two receptors for the kininogens, one for the heavy chain domain and another for the light chain of HK. However, 125I-HK and 125I-LK binding to platelets have been characterized as having a single saturable site with similar affinities and number of binding sites (Table 2). This apparent paradox could be explained alternatively by one platelet receptor which has contiguous sites for binding each domain on HK. To clarify these alternative interpretations, further studies are necessary to determine the physiochemical platelet receptor(s) for the plasma kininogens.

Table 2. Kininogen Expression on Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Binding Exp</th>
<th>Ki or Kd (nmol/L)</th>
<th>No. of Sites</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125I-HK</td>
<td>DB</td>
<td>15 ± 4</td>
<td>911 ± 239</td>
<td>8</td>
</tr>
<tr>
<td>125I-LK</td>
<td>DB</td>
<td>27 ± 2</td>
<td>647 ± 147</td>
<td></td>
</tr>
<tr>
<td>HC INH HK</td>
<td>CI</td>
<td>30</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HC INH HK</td>
<td>CK</td>
<td>28 ± 6</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>LC INH HK</td>
<td>CI</td>
<td>17</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>LC INH HK</td>
<td>CK</td>
<td>11 ± 3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>17 ± 1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>14 ± 2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>19 ± 5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>12 ± 1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>27 ± 9</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Granulocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125I-HK</td>
<td>DB</td>
<td>10 ± 1.3</td>
<td>4.8 × 10⁴</td>
<td>5</td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>10 ± 7</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125I-HK</td>
<td>DB</td>
<td>52 ± 13</td>
<td>9.3 × 10⁴</td>
<td>6</td>
</tr>
<tr>
<td>HK INH HK</td>
<td>CI</td>
<td>20 ± 5</td>
<td>—</td>
<td>6</td>
</tr>
</tbody>
</table>

Abbreviations: HK, high molecular weight kininogen; LK, low molecular weight kininogen; HC, 64-Kd heavy chain of high molecular weight kininogen; LC, 56-Kd light chain of high molecular weight kininogen; INH, inhibits radiolabeled, DB, direct concentration-dependent binding experiments analyzed by the method of Scatchard; CI, competitive inhibition of binding experiments, the IC₅₀ calculated using the procedure of Müller; DB, concentration-dependent binding experiments performed in the presence of the competitor indicated and analyzed by reciprocal plots.
The \( K_i \) (11 to 17 nmol/L) for the light chain and the \( K_i \) (17 to 30 nmol/L) for the heavy chain inhibition of \(^{125}\text{I}-\text{HK} \) binding to platelets are similar to the \( K_d \) of direct \(^{125}\text{I}-\text{HK} \) binding (15 nmol/L) and the \( K_i \) (7 to 19 nmol/L) produced by HK inhibition of \(^{125}\text{I}-\text{HK} \) binding. In previous binding studies with HK to endothelial cells and granulocytes, we showed that the \( K_i \) calculated from the \( I_C_{50} \) and using the formula of Müller was similar to the \( K_d \) determined by direct binding experiments when binding was performed under equilibrium conditions. The similarity of these binding constants for all forms of the kininogens suggests that when intact HK is bound to platelets, the two portions of the molecule cannot each bind in an optimal manner. This type of noncooperative interaction with an apparent loss of entropy on binding has been noted previously in subunit binding of heavy meromyosin to actin and in factor V binding to phospholipid vesicles. Further, it is of interest that the affinity of both HK and \( \text{LK} \) for unstimulated platelets is similar to the affinity of HK binding to granulocytes and endothelial cells (Table 2). Since the affinity for kininogens' expression on cells within the vasculature is, on average, approximately 20 nmol/L and the plasma concentrations of HK and \( \text{LK} \) are 600 nmol/L and 2,400 nmol/L, respectively, all the intravascular binding sites for the kininogens must be saturated in vivo. If there is a single platelet receptor that is competed for equally by the plasma kininogens, one could postulate a fourfold greater relative occupancy for \( \text{LK} \) over HK on the platelet surface. Although the number of sites for the membrane expression of HK varies with the cell type, the density of distribution of HK molecules on each of these cells are similar when adjusted for the surface area of the specific cell type. This finding suggests that there is the potential for the homogenous expression of the kininogens on cells within the vasculature.

The finding that HK binds to platelets as an intact protein (Fig 5) suggests that it may be processed under certain conditions on the platelet surface. One form of processing of membrane-expressed HK would be to cleave it to liberate bradykinin. Release of bradykinin from platelet-bound HK may be physiologically important at sites of platelet plug formation on injured endothelium, because bradykinin is a potent stimulator of endothelial cell function. HK also binds as an intact protein to endothelial cells, although it appears to be cleaved by enzymes associated with endothelial cells. The finding that platelet-bound HK was protected from both plasma and tissue kallikrein cleavage suggests that the membrane expression of HK can serve to modulate the rate of liberation of bradykinin (Figs 6 to 8). The protection of platelet-bound HK from kallikrein cleavage is physiologically relevant, because it was shown to occur at kallikrein concentrations less than or equal to 1% of potential plasma kallikrein. The higher concentration of human urinary kallikrein (15 nmol/L) needed to achieve similar degrees of HK cleavage as plasma kallikrein (4 nmol/L) is characteristic of HK being the preferred substrate of plasma kallikrein, rather than tissue kallikrein. These findings indicate a new and important finding for the membrane expression of the kininogens. Modulation of the rate of liberation of bradykinin from cell-bound kininogens may be important in the autocrine regulation of blood pressure. Although the rate of bradykinin liberation from platelet membrane-bound HK is slower than fluid-phase cleavage, bradykinin freed at the cell surface may also be protected from kininas. Further studies are needed to determine whether bradykinin liberated from membrane-bound kininogen can be more influential on the metabolism of cells than an equal quantity liberated in plasma.

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High molecular weight kininogen binds to platelets by its heavy and light chains and when bound has altered susceptibility to kallikrein cleavage

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