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# The sequence HGLGHGHEQQHGLGHGH in the light chain of high molecular weight kininogen serves as a primary structural feature for zinc-dependent binding to an anionic surface

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**Abstract**

The histidine-glycine-rich region of the light chain of cleaved high molecular weight kininogen (HK) is thought to be responsible for binding to negatively charged surfaces and initiation of the intrinsic coagulation, fibrinolytic, and kinin-forming systems. However, the specifically required amino acid sequences have not been delineated. An IgG fraction of a monoclonal antibody (MAb) C11C1 to the HK light chain was shown to inhibit by 66% the coagulant activity and by 57% the binding of HK to the anionic surface of kaolin at a concentration of 1.5 $\mu$M and 27 $\mu$M, respectively. Proteolytic fragments of HK were produced by successive digestion with human plasma kallikrein and factor XIa (FXIa). Those polypeptides that bound tightly ($K_d$ = 0.77 nM) to a C11C1 affinity column were eluted at pH 3.0 and purified by membrane filtration. On 15% SDS polyacrylamide electrophoresis, the approximate $M_r$ was 7.3 kDa (range 6.2-8.1 kDa). Based on N-terminal sequencing, this polypeptide ($l_2$), which extends from the histidine residue 459 to a lysine at position 505, 509, 511, 512, 515, or 520, inhibits by 50% the coagulant activity expressed by HK at a concentration of 22 $\mu$M. The synthetic peptide HGLGHGH representing the N-terminal of the $l_2$ fragment was synthesized, tested, and found at 4 mM to inhibit the procoagulant activity of HK 50%. A synthetic heptadecapeptide, HGLGHGHEQQHGLGHGH (residues 459-475) included within the $l_2$ fragment, and with the ability to bind zinc, inhibited 50% of the HK coagulant activity at a concentration of 325 $\mu$M in the absence and presence of added $Zn^{2+}$ (30 $\mu$M). The specific binding of $^{125}$I-HK to a negatively charged surface (kaolin) was inhibited 50% by unlabeled HK (5 $\mu$M). HGLGHGH, at a concentration of 7.0 mM, inhibited the binding to kaolin by 50%. The heptadecapeptide inhibited the specific binding of $^{125}$I-HK to kaolin by 50%, at a concentration of 2.3 mM, in the absence of $Zn^{2+}$. In contrast, when $Zn^{2+}$ was added, the concentration to achieve 50% inhibition decreased to 630 $\mu$M, indicating that $Zn^{2+}$ was required to attain a favorable conformation for binding. Moreover, the $l_2$ fragment was found to inhibit 50% of the $^{125}$I-HK binding to kaolin at a concentration of 380 $\mu$M. These results suggest that residues contained within the $l_2$ fragment, notably HGLGHGHEQQHGLGHGH, serves as a primary structural feature for binding to a negatively charged surface.

**Keywords:** kininogen; peptides; surface binding

High molecular weight kininogen (HK) (Colman & Müller-Esterl, 1988) is synthesized in the liver as a single polypeptide chain and secreted into plasma where it complexes with prekallikrein (PK) (Mandle et al., 1976) and factor XI (FXI) (Thompson et al., 1977). Upon cleavage by human plasma kallikrein (Mori & Nagasawa, 1981), FXIa (Scott et al., 1985), or factor XIIa (Wiggins, 1983), HK liberates bradykinin, which mimics many inflammatory phenomena including induction of pain, vasodilation, and increased vascular permeability (Kato et al., 1981). Recent cloning of the cDNA for HK has allowed deduction of the amino acid sequence (Takagaki et al.,

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1985) and provided critical information for a detailed analysis of the structure-function relationship governing this multifunctional protein.

The mature form of HK contains 626 amino acids. On cleavage by kallikrein, the nonapeptide bradykinin is released. The cleaved protein contains an $NH_2$-terminal heavy chain (362 amino acids) bound to a COOH-terminal light chain (255 amino acids) by a single interchain disulfide bridge. Cleavage of HK is required for optimal expression of its procoagulant activity in the plasmatic environment (Scott et al., 1984). The light chain of HK contains the procoagulant activity (Mandle et al., 1976), which depends in part on its ability to associate with the zymogens, PK, through residues 556–595 of HK, or FXI, through residues 556–613 (Tait & Fujikawa, 1987). The coagulant activity of HK also depends on the binding of cleaved HK (Sugo et al., 1980) to anionic surfaces. This function is thought to be mediated through its histidine-glycine-rich region (residues 407–498). There are three homologous regions of about 30 amino acids, each within this histidine-glycine-rich region, and additional internal homologies can be observed within these regions. Thus, it is quite possible that the entire histidine-glycine-rich region evolved by gene duplication from units as small as the dipeptide, GH, or HG (Lottspeich et al., 1985).

We found that a monoclonal antibody (MAb) C11C1 to the light chain but not a MAb 2B5 to the heavy chain inhibited the ability of HK to bind to an anionic surface. We then used MAb C11C1 to immunopurify a hydrolytic fragment of HK that displayed similar inhibitory activities. We synthesized two peptide sequences contained within the hydrolytic fragment and two present in the N-terminal end of the histidine-glycine-rich region and tested the ability of each to inhibit the procoagulant activity of HK, the binding to FXI, the cleavage by kallikrein, and the association with an anionic surface (kaolin). The results provide new evidence that critical amino acid sequences of the histidine-glycine-rich region of HK are responsible for the interaction with negatively charged surfaces.

## Results

### *Effect of MAb C11C1 on the binding of $^{125}$I-factor XI to HK and on the binding of $^{125}$I-HK to kaolin*

To obtain data regarding the composition of the surface binding site of HK, we further characterized MAb C11C1, an antibody developed in our laboratory, which recognizes specifically the light chain of HK (Schmaier et al., 1987). We found that an IgG fraction of MAb C11C1 inhibits coagulant activity (Fig. 1, upper panel) and inhibits the binding of $^{125}$I-HK to kaolin by 57% at a final concentration of 27 $\mu$M (Fig. 1, lower panel). The effect was independent of $Zn^{2+}$ as it was similar in a buffer containing 1 mM EDTA. The action of MAb C11C1 is selective as there was no interference with $^{125}$I-FXI binding to HK (0.83 $\mu$M) as assessed by the ELISA assay and no changes in the rate of cleavage of HK by plasma kallikrein when these procedures were performed in the presence of MAb C11C1 (33 $\mu$M) (data not shown). In addition, MAb 2B5, which recognizes the heavy chain of HK (Schmaier et al., 1987), and which plays no role in surface binding, was unable to inhibit either the coagulant activity or the binding of HK to kaolin (Fig. 1).

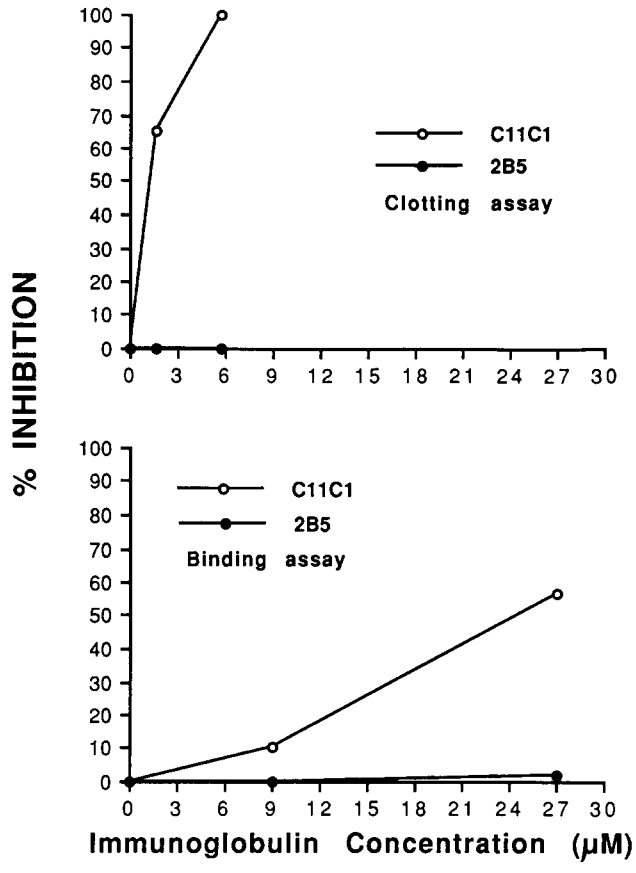


**Fig. 1.** Effect of MAb C11C1 and 2B5 upon the coagulant activity of HK (upper panel) and binding of $^{125}$I-HK to kaolin (lower panel). Details of the clotting assay and binding assay are described in the Materials and methods. MAb C11C1 is an antibody directed against the light chain of HK, and 2B5 is an antibody directed against the heavy chain of HK (Schmaier et al., 1987). Each value is the mean of duplicate assays.

### *Isolation and characterization of a polypeptide expressing the epitope recognized by MAb C11C1*

Because MAb C11C1 selectively inhibited the binding of HK to kaolin, it seemed appropriate to define the epitope in native HK that was recognized. When HK (Fig. 2, lane 1) was digested sequentially with plasma kallikrein and factor XIa, a series of polypeptides resulted that were an-

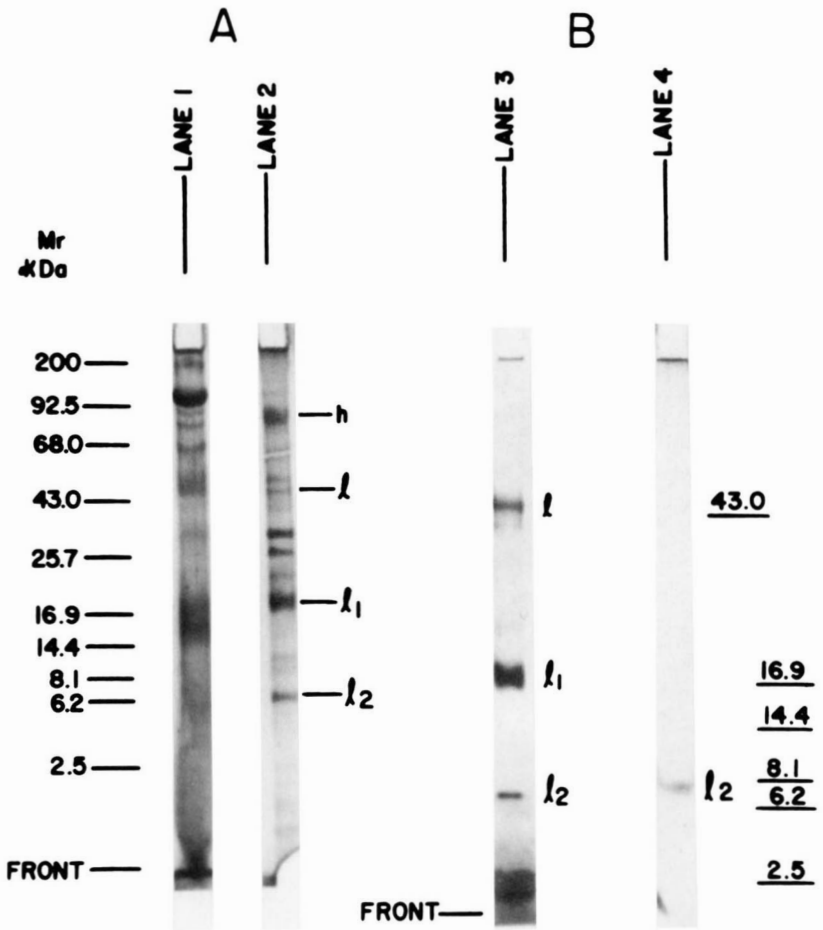


**Fig. 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the peptides purified using C11C1 affinity column. The 15% gels were stained with silver after fixation by glutaraldehyde. Lanes 1 and 2 were run for 2.5 h under reduced conditions. Lane 1 represents the partially cleaved starting material (120 kDa). Lane 2 shows the starting material after cleavage by human plasma kallikrein and FXIa. The molecular weight standards for lanes 1 and 2 are indicated on the left side. The third and fourth lanes were run for 3 h under nonreduced conditions. Lane 3 represents the different-sized peptides containing the epitope recognized by C11C1. Lane 4 represents the purified $l_2$ fragment. The molecular weight standards for lanes 3 and 4 are indicated on the right.

alyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2, lane 2). The largely intact heavy chain is seen along with a small amount of undigested light chain (l) as well as main fragments $l_1$ and $l_2$ and other minor fragments ranging from 30 kDa to 2.5 kDa. This hydrolysate was applied to a C11C1 affinity column (IgG immobilized to agarose) and extensively washed with 0.02 M Tris, pH 7.4, containing 0.5 M NaCl. The polypeptides remaining tightly adsorbed to the column ($K_d$ for HK = 0.77 nM) were eluted with 0.1 M citric acid, pH 3.0 (Fig. 2, lane 3). Three major components, l (45 kDa), $l_1$ (18 kDa), and $l_2$ (~7.3 kDa) emerged. The mixture of peptides containing the MAb C11C1 epitope was filtered through a Centricon-10 tube with a molecular weight exclusion of 10 kDa. As judged by 15% SDS gel electrophoresis, a single well-resolved polypeptide ($M_r$ ~ 7.3 kDa) passed through the filter (Fig. 2, lane 4). The filtrate was also subjected to HPLC analysis on a reverse-phase C4 column (280 × 4.6 min VYDAC®) equilibrated in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. Using a gradient of 0.8% acetonitrile over 51 min, the peptide emerged immediately following the injection fragment indicating it was not adsorbed. No other peptide was found over the next 50 min. The lack of adsorption suggested that it might be a basic peptide with highly hydrophilic composition.

When the peptide was subjected to amino acid analysis, it proved to be composed of predominantly His and Gly (data not shown). Moreover, the peptide did not adsorb to the Immobilon P-membrane normally used for

**Table 1.** *Sequence of the HK light chain fragment ($l_2$)*[a]

| Step | Amino acid sequence in pmol/cycle | |
|---|---|---|
| | Predominant amino acid | Next most abundant |
| 1 | His 2,319 | Arg 1,050 |
| 2 | Gly 6,649 | Ala 614 |
| 3 | Leu 1,507 | Lys 128 |
| 4 | Gly 1,530 | Lys 137 |
| 5 | His 394 | Asn 38 |
| 6 | Gly 830 | His 436 |
| 7 | – | |
| 8 | – | |
| 9–17 | – | |

[a] The major sequence determined was HGLGHGX. This sequence corresponds to residues 459–464. Therefore, the deduced sequence based on the molecular weight of this peptide (6.2–8.1 kDa) is HGLGHGHEQQHGLGHGHKFKLDDDLEHQGGHVLDHGHKHKHGHGHGKHKDKGKKDGKHDGWK, correspondent to 47–62 residues beginning with H-459 and ending with K-505, K-509, K-511, K-512, K-515, or K-520.

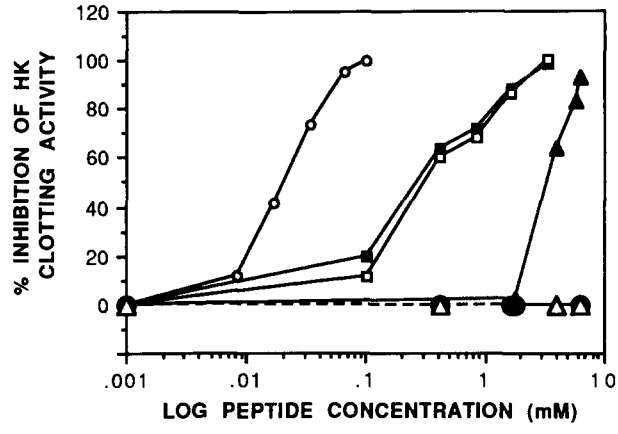


**Fig. 3.** Effect of the peptides derived from the histidine-rich region upon the coagulant activity of HK. The HK (0.06 μM) was incubated at 37 °C for 8 min in the presence of different concentrations of either unrelated control peptides RGYSLG (●—●) and HGLGGAKQAGDV (△—△) or the three histidine-rich-region-derived peptides HGLGHGH (▲—▲), HGLGHGHEQQHGLGHGH (□—□, no zinc; and ■—■, plus zinc), and $l_2$ fragment (○—○). Calcium (6 mM) was added to initiate the HK coagulant assay (see Materials and methods). Each value is the mean of duplicate assays.

sequencing. Therefore, it was covalently coupled to aminophenyl-glass fiber paper to obtain the N-terminal sequence. The first six amino acids were HGLGHG. Judging from the second most abundant amino acids from steps 2 through 6, the peptide is greater than 90% pure. This sequence is repeated twice in the sequence 459–465 and 468–475. Despite an adequate yield, no sequence was obtained from steps 7 through 17 (Table 1). Because the covalent coupling is to free COOH groups, the presence of a Glu in position 8 (amino acid 466) would prevent further sequencing as it would be part of the covalent attachment. If the sequence began at 468, then the sequencing would have continued until the first acidic amino acid D-480. Thus, the sequence must begin at H-459. Based on the $M_r$ estimated in lanes 2 and 3 (Fig. 2) and the N-terminal analysis, and the basic amino acid specificity of kallikrein and factor XI suggests that the peptide is from 6.2 to 8.1 kDa and most likely from the histidine residue 459 to lysine residues at 505, 509, 511, 512, 515, and 520. A mixture of these peptides is also possible.

### *Effect of the peptides derived from the histidine-rich region upon the coagulant activity of HK*

The $l_2$ fragment inhibits the coagulant activity 50% at a concentration of 22 μM (Fig. 3). To define which amino acid sequences contained in the $l_2$ fragment were important in inhibiting the HK coagulant activity, we synthesized and tested the heptapeptide HGLGHGH obtained from the N-terminal analysis (Table 1). At a concentration of 4 mM, the heptapeptide inhibited the coagulant activity of HK 50% (Fig. 3). Because the concentration of HGLGHGH was relatively high when compared with the $l_2$ fragment, we explored a larger peptide that contains the heptapeptide sequence repeated twice, linked together by a binding site for zinc HEQQH (HEXXH). This heptadecapeptide (HGLGHGHEQQHGLGHGH) at a concentration of 325 μM inhibited the coagulant activity of HK 50% (Fig. 3). Although this heptadecapeptide contains a zinc-binding domain, we did not observe any effect of the addition of $Zn^{2+}$ (30 μM) to the buffer (Fig. 3). However, plasma contains $Zn^{2+}$ at a concentration of 15–21 μM. The control peptides had no effect (Fig. 3).

### *Effect of HGLGHGH upon the binding of FXI to HK*

HK cofactor activity is due to its ability to interact with both surface and zymogen. To distinguish between these two possibilities, we first determined if the effect of the HGLGHGH peptide upon the HK coagulant activity was due to interference with the zymogen-binding domain, which is located near the carboxy-terminus on the HK light chain. Factor XI was chosen as its binding domain is larger and completely subsumes that for prekallikrein. $^{125}$I-FXI (5 nM) was preincubated for 30 min at 23 °C in the presence of different concentrations (1.8–13 mM) of either an unrelated control peptide (RGYSLG) or the histidine-rich region derived peptide (HGLGHGH) and then incubated in microplate wells that had been precoated with HK. Neither of the two peptides had any effect on the binding of radiolabeled FXI to HK. None of the tested peptides showed more than a 5% inhibition of the

binding of radiolabeled FXI to HK (data not shown). Therefore, the inhibition of the HK coagulant activity was not due to an effect on zymogen binding.

### *Effect of the peptides derived from the histidine-rich region upon the binding of $^{125}$I-HK to kaolin*

In addition to the binding to the zymogens FXI and prekallikrein, HK requires binding to negatively charged surfaces for the expression of its procoagulant activity. Therefore, we first investigated the effect of the histidine-rich-region-derived peptides and two unrelated control peptides (RGYSLG and HHLGGHKQAGDV) upon the binding of kallikrein-cleaved $^{125}$I-HK to kaolin. Unlabeled HK inhibits the $^{125}$I-HK binding to kaolin by 50% at a concentration of 5 $\mu$M (Fig. 4). When the heptapeptide was tested, at a concentration of 7.0 mM (Fig. 4), 50% of the $^{125}$I-HK binding to kaolin was blocked. However, when we studied the heptadecapeptide containing the heptapeptide sequence twice, a concentration of 2.3 mM was required to achieve 50% inhibition (Fig. 4). This peptide has a charge at pH 7.4 of +2 and contains 6 histidines among the 17 amino acid residues. To investigate the requirements for both charge and spacing of the histidines further, we tested two other peptides from the N-terminal end histidine-rich region of HK. The first peptide, residues 420–438, GKEQGHTRRHDWGHEKQRK, contains only 3 histidines out of 19 but has a charge of +4.5. At 3 mM, no inhibitory activity on the binding of $^{125}$I-HK to kaolin was observed. The second peptide, residues 439–458, HNLGHGHKHRRDQGHGHQRG, has a charge of +5 and 6 histidines of 20 amino acids. At 3 mM, no inhibition of labeled HK binding was found.

To test the possibility that zinc might affect the binding of the heptadecapeptide in a buffer (with no added zinc), we incubated HGLGHGHEQQHGLGHGH with $ZnCl_2$ (30 $\mu$M) at 56 °C and allowed it to slowly cool to 23 °C. Under these conditions, the zinc-heptadecapeptide inhibited the binding by 50% at a concentration of 630 $\mu$M (Fig. 4). Similar heating and cooling did not change the values of the heptadecapeptide for inhibition without $Zn^{2+}$. Because $Zn^{2+}$ appeared to have a role in the binding of the heptadecapeptide, we tested whether the reagents employed in the binding procedure contributed $Zn^{2+}$ (Table 2). Only the albumin-fibrinogen mixture contributed detectable $Zn^{2+}$, and the concentration (0.13 $\mu$M) was insignificant when compared to the added $Zn^{2+}$ (30 $\mu$M).

Finally, we found that the $I_2$ fragment inhibited the $^{125}$I-HK binding to kaolin 50% at a concentration of 380 $\mu$M (Fig. 4). These results together with the data on the inhibition of coagulant activity strongly suggest that amino acids contained within the $I_2$ fragment represent at least a significant portion of the binding site on HK to anionic surfaces, but the exact location of these residues in the primary sequence was not determined.

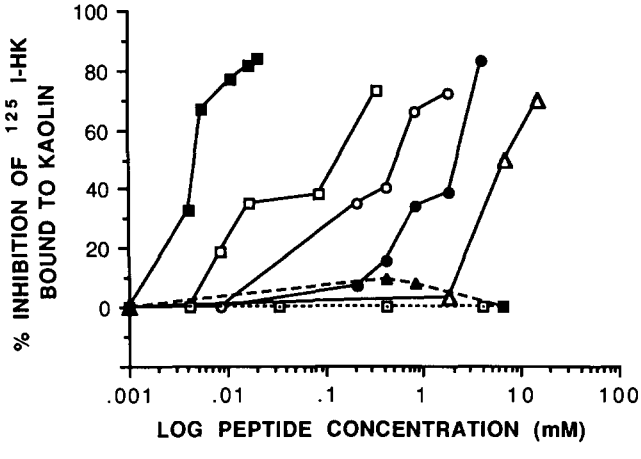


**Fig. 4.** Effect of the peptides derived from the histidine-rich region upon the binding of $^{125}$I-HK to kaolin. The figure shows the percent inhibition of the specific binding (see Materials and methods) of $^{125}$I-labeled and kallikrein-cleaved HK to kaolin in the presence of different concentrations of either two control peptides RGYSLG (▫—▫) and HHLGAKQAGDV (▲—▲), intact labeled HK (■—■), or the three histidine-rich-region-derived peptides HGLGHGH (△—△), HGLGHGHEQQHGLGHGH (●—●, no $Zn^{2+}$ added; and ○—○, $Zn^{2+}$ added), and $I_2$ fragment (▫—▫). Each experimental point represents the mean of duplicate determinations.

**Table 2.** *Concentration of zinc in the binding experiments of HK to kaolin*[a]

| Sample | $Zn^{2+}$ (mg/L) | $Zn^{2+}$ ($\mu$M) | % Recovery of added standard |
|---|---|---|---|
| HGLGHGH (7 mM) | <0.18 | <0.28 | 102 |
| HGLGHGHEQQHGLGHGH (3 mM) | <0.18 | <0.28 | 103 |
| Albumin (30 mg/mL) Fibrinogen (3 mg/mL) | <0.074 | 1.13 | 98 |
| Kaolin (1.25 mg/mL) | <0.018 | <0.28 | 104 |
| HK (0.66 $\mu$M) after dialysis | <0.018 | <0.28 | 99 |
| HK before dialysis | <0.018 | <0.28 | 99 |

[a] Each reagent above was dissolved at the assay concentration noted in the 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 buffer, which itself contained <0.018 mg/mL $Zn^{2+}$. $Zn^{2+}$ was determined as described in the Materials and methods.

## Discussion

The assignment of the surface-binding region of human HK to the histidine-glycine region has been based on indirect evidence. When bovine HK is digested with bovine kallikrein, a polypeptide was removed from the light chain corresponding to the histidine-glycine-rich domain of human HK (Han et al., 1976; Sugo et al., 1980; Ikari et al., 1981), which results in both the loss of coagulant activity and ability of cleaved bovine HK to bind to negatively charged surfaces. In human HK, unlike bovine HK, there is a substitution of Lys at position 402 for Arg that results in failure of human plasma kallikrein, which prefers arginine residues, to cleave the corresponding peptide from human HK. Therefore, no loss of activity occurs when human plasma kallikrein hydrolyzes human HK. Chemical modification studies by Ikari et al. (1981) and Retzios et al. (1987) indicate that histidine residues of bovine and human HK, respectively, are involved in the interaction of the molecule with negatively charged surfaces.

To directly define the requirements for interaction of human HK with negatively charged surfaces and to better understand whether the interaction involves the relative positive charge, specific linear or discontinuous amino acid sequences, we identified an MAb C11C1 that selectively inhibited the binding of $^{125}$I-HK to kaolin (Fig. 1) and then used the immobilized MAb to isolate the $l_2$ polypeptide, which exposed the epitope beginning with histidine residue 459 and ending with a lysine in the region 505–520 (Fig. 2). Because the carboxyterminal of this peptide was not determined, the size of the peptide is an estimate based on the 15% SDS gel and the specificity of hydrolytic enzymes used. That polypeptide inhibited both the coagulant activity (Fig. 3) and the ability of $^{125}$I-HK to bind to a negatively charged surface (Fig. 4). Of note, there is no overlap with the sequence 556–613 responsible for binding factor XI, nor does the polypeptide inhibit factor XI binding to HK. This $l_2$ fragment polypeptide may contain most if not all of the surface-binding region. Because that polypeptide contained as its N-terminal HGLGHG-X (Table 1), we synthesized and tested the heptapeptide HGLGHGH for its ability to block the HK procoagulant activity as well as the binding of $^{125}$I-HK to kaolin. Interestingly, HGLGHGH is a highly conserved sequence found in human as well as in bovine HK. In human HK, there are three homologous regions of 30 amino acids each within the histidine-glycine-rich region. Only in the second of these three homologous regions is the sequence HGLGHGH repeated twice (Lottspeich et al., 1985). The fact that this heptapeptide was able to inhibit the coagulant activity of HK (Fig. 3) as well as directly block the binding of HK to kaolin (Fig. 4) at concentrations two orders of magnitude higher than the $l_2$ fragment is not surprising, as such a small peptide may not assume the normal secondary and tertiary structure that exhibits the intact protein or the isolated C11C1 epitope. In addition, the heptapeptide may only block a portion of the polypeptide that interacts with the surface, or the surface-binding domain may contain critical elements that are not present in a linear sequence. Moreover, peptides such as RGDS, present in the natural domains of adhesive proteins that block binding of such proteins to cell receptors, also require millimolar concentrations to effectively compete with the whole protein (Bennett et al., 1988).

The sequence HGLGHGH in human HK, in addition to being repeated twice, forms part of a heptadecapeptide HGLGHGHEQQHGLGHGH. When the heptadecapeptide is tested by itself, its potency is twice that observed with the heptapeptide, HGLGHGH. However, when $Zn^{2+}$ is added, the concentration of the heptadecapeptide for inhibition of binding to kaolin decreases 3.7-fold. Moreover, in plasma where $Zn^{2+}$ is present at 20 $\mu$M (Braunwald, 1987), the peptide is equally inhibitory toward the coagulant activity of HK with or without added zinc. Our data that HK binding to kaolin is independent of $Zn^{2+}$, which agrees with Ikari et al. (1981) and suggests that the $Zn^{2+}$ dependence of the heptadecapeptide probably is due to facilitation of proper folding rather than to an effect on the direct interaction with kaolin. However, kaolin is an artificial anionic model surface. Retzios et al. (1987) suggested that the surface- and zinc-binding regions of HK both were mediated by histidine residues. The finding of Greengard et al. (1986) that factor XI binding to platelets required the presence of $Zn^{2+}$ was the first evidence that the surface and $Zn^{2+}$ binding domains of HK might play a physiologic role. Recent studies of HK binding to unstimulated and stimulated neutrophils (Gustafson et al., 1989), platelets (Gustafson et al., 1986), and endothelial cells (Iwaarden et al., 1988) indicate that $Zn^{2+}$ is required for efficient binding to the cell surface membrane, which contains multiple negative charges in the form of sialic acid residues. Whether the $Zn^{2+}$ is affecting only the HK or, in addition, putative cell receptors is unknown. $Zn^{2+}$ has been demonstrated to bind to HK (Shimada et al., 1987) and to facilitate the acceleration by HK of factor XII, PK, and FXI activation.

Because the heptadecapeptide requires higher concentrations to block the HK functions than that observed with the $l_2$ fragment, additional amino acids N-terminal or C-terminal to the HGLGHGHEQQHGLGHGH sequence may play a role in the binding surface domain of HK. The N-terminal region was ruled out as peptides 420–438 and 439–458 were inactive. Moreover, the charge is not the major determinant as both these peptides had a greater positive charge than the heptadecapeptide. Because peptide 439–458 had the same number of histidines as the heptadecapeptide, the spacing of the histidines

is probably important. The $I_2$ fragment thus contains most of the surface-binding domain since it inhibits the coagulant activity (in the presence of plasma concentrations of $Zn^{2+}$) 50% at 22 $\mu$M compared to the value of 5 $\mu$M for unlabeled HK to inhibit $^{125}$I-HK binding to kaolin. The heptadecapeptide and the $I_2$ fragment may be useful to probe the interaction of HK with human blood cells and other physiologic negatively charged surfaces, such as subendothelial matrix.

The deficiency of HK leads to in vitro coagulation impairment but not an in vivo hemostatic disorder (Colman et al., 1975). However, a large body of evidence supports the important role of HK and thus the contact system in body defenses (Colman, 1984). Synthetic peptides blocking or disrupting the interaction of HK with anionic surfaces may be useful to elucidate its primary or secondary role and as potential therapeutic agents in pathologic conditions in which activation of the contact system is known to occur.

## Materials and methods

Sodium dodecyl sulfate, methylene bis-acrylamide, and acrylamide were purchased from BioRad Laboratories (Richmond, California). Prestained molecular weight standards (200,000, 92,000, 68,000, 43,000, 25,700, and 18,400) were obtained from BRL (Gaithersburg, Maryland). Soybean trypsin inhibitor, human serum albumin (HSA), and dithiothreitol (DTT) were procured from Sigma Chemical Co. (St. Louis, Missouri). Kaolin (acid washed) was obtained from Fisher Scientific Co. (King of Prussia, Pennsylvania). Inosithin (mixed soybean phospholipids) was purchased from Associated Concentrates (New York), and microcentrifuge tubes (#699 and #690) were obtained from Sarstedt Co. (Princeton, New Jersey). Na$^{125}$I was procured from ICN Radiochemicals (Irvine, California). Iodogen was purchased from Pierce Chemical Co. (Rockford, Illinois). Coomassie brilliant blue R-250 was purchased from LKB Instruments (Rockville, Maryland). Normal pooled plasma (PNP), used as a reference standard, was purchased from George King Biomedicals, Inc. (Overland Park, Kansas). Plasmas deficient in FXI and HK were donated directly to us by a know FXI-deficient patient and M. Williams (Colman et al., 1975), respectively. Ninety-six-well tissue culture plates (no. 3075) were obtained from Falcon Plastics (Mt. Laurel, New Jersey). Disposable Immulon 7 Removawell® strips of 12 flat-bottom wells were procured from Dynatech Laboratories, Inc. (Chantilly, Virginia). Glu-Pro-Arg-*p*-nitroanilide (S-2366), Pro-Phe-Arg-*p*-nitroanilide (S-2302), Phe-Pro-Arg-*p*-nitroanilide (S-2238), and purified human fibrinogen were purchased from Helena Laboratories (Beaumont, Texas). Kodak X-Omat AR X-ray film was obtained from Eastman-Kodak Co. (Rochester, New York). Thrombin (53 nKat) was purchased from KabiVitrum (Helena Laboratories).

### *Purification of proteins*

High molecular weight kininogen (Kerbiriou & Griffin, 1979) and FXI (Sinha et al., 1985) were purified as previously described. HK was a single polypeptide chain of 120 kDa on reduced SDS polyacrylamide gels, and FXI had an $M_r$ of 160 kDa unreduced and 80 kDa reduced. The specific activity of HK was 12.5 U/mL and FXI was 250 U/mL. The concentrations of purified HK and FXI were determined by coagulant assays (Proctor & Rapaport, 1961) (assuming a normal plasma concentration of 0.67 $\mu$M for HK and 0.04 $\mu$M for FXI). Purified human plasma kallikrein and FXIa were kindly donated by Cheryl F. Scott from this institution.

### *Radiolabeling of FXI and HK*

FXI and HK were radiolabeled by a minor modification of the Iodogen method (Fraker & Speck, 1978) at pH 8.0 in the presence of 0.75 mol/L NaCl. The specific radioactivity of the peak tube was $1.0 \times 10^6$ cpm/$\mu$g for FXI and $1.3 \times 10^8$ cpm/$\mu$g for HK. Both proteins retained >90% of their coagulant activity compared with unlabeled protein.

### *Assay of HK coagulant activity*

The coagulant assay was performed as described elsewhere (Proctor & Rapaport, 1961) by a slight modification. In brief, 100 $\mu$L of total kininogen-deficient plasma (Williams plasma), 100 $\mu$L of 20 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl, 100 $\mu$L of kaolin (5 mg/mL in saline), and 100 $\mu$L at 0.2% inosithin in buffer were mixed. Normal plasma (10, 5, 2, or 1 $\mu$L) was added and incubated at 37 °C for 8 min; 100 $\mu$L of 30 mM $CaCl_2$ was added and coagulation time was measured. This procedure was used to generate a standard curve (log-log relationship). Samples for analysis were assayed, under the same conditions, using 5 or 10 $\mu$L of sample, and the data were expressed as percentage of normal pooled plasma. One unit (0.67 $\mu$M) is the amount of activity in 1 mL of normal pooled plasma.

### *Binding of $^{125}$I-FXI to HK-coated wells*

The binding of FXI to HK was studied using removable microplate wells by a slight modification (DeLa Cadena et al., 1988) of a previously reported assay (Akiyama et al., 1986). In brief, $^{125}$I-FXI was preincubated for 20 min at 23 °C in the presence of synthetic peptides, proteolytic fragments of HK, or MAbs to HK or appropriate controls for each, and then added into microplate

wells precoated with HK and incubated at 23 °C for 4 h. Then, remaining binding sites were blocked by the use of 0.02 M Tris chloride containing 0.15 M NaCl, 5 mg/mL BSA, pH 7.4. Additional microplate wells were coated with the above buffer instead of HK in order to obtain background counts arising from nonspecific binding of $^{125}$I-FXI to BSA. Finally, the wells were repeatedly washed, dried, and counted for radioactivity in a gamma counter.

### *$^{125}$I-binding of HK to kaolin*

The binding of HK to kaolin was studied as reported previously by this laboratory (Scott et al., 1984). In brief, $^{125}$I-HK (110 nM), which had been cleaved by kallikrein (Scott et al., 1984), was added into 0.4-ml Eppendorf tubes and additions made up to 20 μL with a buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4) containing 40 μM HSA and 8.8 μM fibrinogen. Then, 80 μL of the same buffer, containing 1.25 mg/mL of kaolin, were added. The tubes were then centrifuged in a microcentrifuge at 9,000 × *g* for 2 min at 23 °C. The supernatant was then removed and the pellets counted for radioactivity in a gamma counter. Because it has been shown that HK binds to stimulated and unstimulated platelets (Gustafson et al., 1986), neutrophils (Gustafson et al., 1989), and endothelial cells (Iwaarden et al., 1988), and that $Zn^{2+}$ is required for the efficient binding, we also tested the effect of $Zn^{2+}$ on this binding assay. Additional experiments were performed as described above, but in all instances the buffer contained either $Zn^{2+}$ (30 μM) or EDTA (1 mM). The kaolin was prewashed in 1 mM EDTA before its use in the binding experiments to eliminate bound $Zn^{2+}$. The nonspecific binding was determined in the presence of a 20-fold excess of unlabeled HK and substituted from the total binding to give the specific binding.

### *Concentrations of zinc in binding studies*

Atomic absorption spectroscopy (AA) was utilized to determine the concentration of zinc in the reagents of this study. The analysis was performed with a Perkin-Elmer model 3030 spectrometer using standard conditions for zinc (213.9 nm, air/$C_2H_2$ flame). A 1 g/L zinc stock standard solution (Fisher) was diluted with distilled, deionized water to produce working standards of 0.0909, 0.167, 0.500, 1.00, and 2.00 mg/L (1.39, 2.55, 7.65, 15.3, and 30.6 μM Zn, respectively). The instrument was calibrated with one standard and the remaining standards determined as unknowns, which showed a deviation of less than 2% from their calculated values. To 5.00 mL of each sample was added 1.00 mL of the 1.00 mg/L standard to check for physical and chemical interferences. The characteristic concentration (sensitivity) and detection limit were determined to be 0.0316 mg/L (0.483 μM) and 0.0180 mg/L (0.275 μM), respectively, for this analysis.

### *Preparation and analysis of kallikrein-cleaved HK and $^{125}$I-HK for binding studies*

$^{125}$I-HK and unlabeled HK in 0.15 M Tris acetate, pH 8.0, were incubated for 18 h with purified human plasma kallikrein (Scott et al., 1984) at 37 °C (1:250 molar ratio). Soybean trypsin inhibitor (sevenfold molar excess compared with kallikrein) was then added in order to stop the cleavage process. Polyacrylamide gel electrophoresis was carried out in the presence of SDS on 10% and 15% slab gels, according to the method of Laemmli (1970). $^{125}$I-HK that had been cleaved by kallikrein and Bethesda Research Laboratories molecular weight prestained standards (200 kDa to 12 kDa) were applied in parallel lanes. Following electrophoresis, the gels were fixed and the proteins stained with Coomassie brilliant blue R-250. After destaining, the gels were dried and subjected to autoradiography with Kodak X-Omat AR X-ray film. The apparent molecular weights were determined by plotting the migration of the standards versus log molecular weight and interpolating the unknowns.

### *Preparation of kallikrein and factor XIa-cleaved HK for isolation of polypeptides*

Purified HK (12–14 mg) was first incubated in the presence of purified plasma kallikrein (1:100 molar ratio) at 23 °C for 3 h. Then purified factor XIa was added to the reaction mixture (1:100 molar ratio) and incubated for 18 h at 23 °C. Under these conditions, the heavy chain of HK remained essentially intact as judged by the presence of a 68,000-Da band on SDS-PAGE electrophoresis under reduced conditions. In contrast, the light chain of HK is proteolyzed into different size peptides (Fig. 2).

### *Purification of MAb C11C1 and MAb 2B5*

The MAbs were produced and characterized as previously described (Schmaier et al., 1987). Culture supernatant fluid, C11C1 or ascites (2B5), was purified by a modified protein A affinity chromatography system (Affi-Gel Protein A, BioRad Corp., Richmond, California).

### *Preparation of an affinity column of immobilized MAb C11C1*

Approximately 12–14 mg of purified C11C1 antibody was coupled to 5 mL of Affi-Gel 10 according to the manufacturer's recommendations in a 0.1 M $NaHCO_3$, 0.2 M NaCl, pH 8.0 buffer.

*Amino acid composition*

Amino acid analyses were performed by Dr. Robert Harris at Virginia Commonwealth University, Medical College of Virginia Protein Sequence Facility. Amino acid composition was determined in two samples of protein following the sequencing experiment (see below). The glass fiber disk was macerated and immersed in 0.5 mL 6 N HCl. Following hydrolysis (22 h, 110 °C) in a sealed, evacuated glass tube, the sample was taken to dryness under vacuum (Speedvac) and dissolved in 200 μL, pH 2.2, loading buffer. Of this, 90 μL was subjected to compositional analysis.

*Covalent coupling of peptides for N-terminal amino acid sequence analysis*

Coupling of peptides was achieved by a method developed by Dr. Rudy Aebersold (pers. comm.) from The Biomedical Research Centre (Vancouver, British Columbia, Canada). Discs as well as reagents for coupling were kindly supplied to us by Dr. Aebersold. In brief: Sheets of aminophenyl-glass fiber paper were cut into circular disks of 1 cm diameter that were stored in capped plastic tubes. Peptide solution was applied in 50-μL aliquots and dried with a stream of cold air. Peptide was applied in high performance liquid chromatographic (HPLC) elution buffer (0.1% trifluoroacetic acid [TFA] in $H_2O/CH_3CN$). A fresh solution (20 mg/mL in $H_2O$ [w/v]) of *N*-ethyl-*N'*-3(dimethylaminopropyl)carbodiimide (EDC) was prepared immediately before use. Thirty microliters of coupling buffer (200 mM [3-aminopropyl]-triethoxysilane, pyridine, aminopropyltriethoxysilane 2-[4-morpholino]-ethanesoldonic acid [MES], pH 4.5), and 10 μL of EDC solution were applied to the dry disc. The coupling reaction was allowed to proceed for 60 min at 37 °C; then the disc was extensively washed with distilled $H_2O$ to remove excess EDC and any noncoupled peptide. The disc was then employed to obtain N-terminal sequence analysis using an Applied Scientific Biosynthesis Instrument by Dr. Robert Harris.

*Synthetic peptides*

Peptides HGLGHGH, GKEQGHTRRHDWGHEKQRK, HGLGHGHEQQHGLGHGH, and HNLGHGHKHRRDQGHGHQRG were synthesized by the solid-phase method (Erickson & Merrifield, 1976) using automatic instrumentation (Applied Biosynthesis Peptide Synthesizer Model 430A) and purified by HPLC (Waters) on C-18 columns. When each peptide was eluted with a linear acetonitrile gradient containing TFA, over 90% emerged in a single peak. The amino acid sequence was confirmed on a gas-phase sequencer (Applied Biosystems Model 470) with an on-line phenylthiohydantoin analyzer (Applied Biosystems Model 120). The first two of these syntheses and sequences were performed in the Macromolecular Analysis and Synthetic Laboratory of Temple University School of Medicine by Dr. John Holt (HGLGHGH) and Dr. Bradford Jameson (HGLGHGHEQQHGLGHGH). The third and fourth peptides were synthesized by Drs. Jahnen-Dechent and Müller-Esterl from the Institution for Physiological Chemistry and Pathophysiology, Johannes Gutenberg University, Mainz, Germany. Peptides RGYSLG and HHLGGHKQAGDV were purchased from Peninsula Laboratories, Inc. (Belmont, California) and were of similar purity.

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