Three noncontiguous peptides comprise binding sites on high-molecular-weight kininogen to neutrophils

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Khan, Mohammad M. H., Satya P. Kunapuli, Yingzhang Lin, Abraham Majluf-Cruz, Raul A. DeLa Cadena, Stuart L. Cooper, and Robert W. Colman. Three noncontiguous peptides comprise binding sites on high-molecular-weight kininogen to neutrophils. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H145–H150, 1998.—The binding of high-molecular-weight kininogen (HK) to neutrophils (polymorphonuclear leukocytes, PMN) is required for the stimulation of aggregation and degranulation by human plasma kallikrein as well as the displacement of fibrinogen from this cell surface. The putative receptor for HK is the leukocyte integrin Mac-1 (αMβ2), specifically and reversibly to 40,000–70,000 sites from cellular or plasma sources (12). HK binds saturably in the presence of HK to stimulate neutrophils, either chemotaxis (17), aggregation and degranulation (29). Plasma kallikrein requires neutrophils, stimulating chemotaxis (17), aggregation kallikrein serves as a naturally occurring agonist for protease that cleaves HK to yield bradykinin (26), a results in the formation of plasma kallikrein, a serine protease that cleaves HK to yield bradykinin (26), a product can inhibit binding of kininogen to PMN. Two contiguous peptides from D5 in the histidine-glycine-rich region, Gly442-Lys458 and Phe459-Lys478, each inhibit the binding of HK to PMN. This study has thus delineated three noncontiguous surface-oriented sequences on HK, which together comprise all or most of the binding site for human PMN.

The first three domains (D1–D3) are identical in HK and LK, and each is composed of the products of three exons. HK (23) and LK (16) both inhibit thrombin binding to and activation of platelets. D3, composed of exon 7–9 products, expresses two distinct sites, one inhibiting thrombin activation of platelets (16) and the other serving as a cell binding site to platelets (Fig. 1) (16). Exon 10 codes for domain 4 (D4), which contains bradykinin and is present in both kininogens as well as domains 5 (D5) and 6 (D6), present uniquely in HK, D5, rich in histidine, glycine, and lysine, is responsible for binding to anionic surfaces (Fig. 1) (7), whereas D6 contains the amino acid sequence responsible for complex formation with PK (27).

We have recently localized the sites on HK responsible for binding neutrophils to D3 and D5 (28). Fine mapping of kininogen binding sites on endothelial cells has recently been performed (13, 15). The aim of the present study is to map the binding regions on kininogens for neutrophils. We demonstrate that two short sequences within D3, contained within exon 7 and 9 products, respectively, and a single amino acid sequence within D5 form part or all of the HK binding site to neutrophils.

METHODS

Materials. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen) was obtained from Pierce Chemical. Na125I (100 mCi/ml) was obtained from Du Pont NEN (Billerica, MA). Hanks’ balanced salt solution (HBSS), free of calcium chloride, magnesium sulfate, and magnesium chloride, was obtained from Life Technologies. Histopaque-1083–1 was obtained from Sigma Diagnostics. All other reagents were of the highest purity available.

Isolation and purification of human neutrophils. Human neutrophils (polymorphonuclear leukocytes, PMN) were isolated and purified from whole blood by a modification (Majluf-Cruz, Khan, DeLa Cadena, and Colman, unpublished results) of a previously described method (3). Blood was drawn from normal volunteers after written informed consent on the morning of the experiment and collected in ACD-dextran (7.5%) to isolate PMN. After sedimentation of the whole blood for 30 min at 1 g, 15 ml of platelet-rich plasma were added to a 15-ml layer of Ficoll-Hypaque (1.119 g/ml), and the samples were centrifuged at 1,200 rpm for 45 min at 4°C. The supernatant was discarded, and the cell pellet at the bottom of the tube containing PMN was resuspended in 5 ml of a solution to lyse erythrocytes (0.15 M NH₄Cl and 0.01 M KHC₂O₃ in 100 ml of distilled water) and incubated for 3 min at 23°C. The tube containing the cell suspension was then gently vortexed and spun at 1,000 rpm for 5 min. This last step was repeated until the PMN pellet was free of erythrocytes. Then PMN were resuspended in a “blocking” buffer (to

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decrease nonspecific binding) containing either 1% BSA and 10 ml of avidin-conjugated alkaline phosphatase for fluorescent studies or 1% BSA in DMEM for cell binding studies employing radioactivity for 45 min at 4°C. After the blocking step, the PMN were then centrifuged at 1,000 rpm for 5 min and resuspended in binding buffer containing either 0.1% BSA in HBSS or 0.1% BSA in DMEM for fluorescent- and radioactive-based studies, respectively. For activated PMN, the cell pellets obtained after centrifugation of platelet-rich plasma with Ficoll-Hypaque were incubated directly with 5 mg/ml cytochalasin B for 3 min followed by the addition of 10⁻² M N-formyl-Met-Leu-Phe (FMLP) for 5 min at 37°C. After the activation step, the PMN were diluted 1:1 with either HBSS or DMEM at 4°C. The samples were then centrifuged at 1,000 rpm for 5 min, and the cell pellet was obtained and processed as described above.

Plasma proteins. Human purified HK cleaved by plasma kallikrein (specific clotting activity of 11.9 U/mg) was obtained from Enzyme Research Laboratories (South Bend, IN). Under nonreducing conditions, cleaved HK has two bands with molecular masses of 160 and 120 kDa on 10% polyacrylamide gels (Bio-Rad, Richmond, CA) with SDS, whereas under reducing conditions (3 mM dithiothreitol), it was primarily two bands with molecular masses of 62 and 45 kDa, respectively. HK was radiolabeled with Na¹²⁵I using Iodo-Gen (10). The specific radioactivity of the protein varied from 1 to 4.8 mCi/mg with >80% of the molecules of HK being iodinated. The radiolabeled protein (125I-HK) retained >95% of its procoagulant activity as well as its antigenic properties, as previously reported (12).

Bacterial expression and purification of HK D3 (Gly 235-Met357), D5 (Val384-Lys502), and D6 (Thr503-Ser626). The details of bacterial expression and purification of D3 (28) and D5 and D6 (9) have been described. (The NH₂ terminal and the COOH terminal are indicated by the three-letter code amino acid. The sequence of the peptide is designated in the one-letter code.) We have utilized similar methodology using HK cDNA (pHKG36) (18), kindly provided by Dr. S. Nakaniishi, as a template to express various D3 fragments in bacteria. The DNAs coding for various fragments were separately amplified by PCR using specific sets of primers and inserted in-frame into pGEX2T vector (Pharmacia, Piscataway, NJ) by the procedures described earlier (20, 28). This construct expresses the protein products as fusion proteins with glutathione S-transferase (GST) as the NH₂-terminal region. All of the sense primers contained a BamHI recognition site, and the antisense primers contained EcoRI recognition site. Products of exons 7–9 (Gly235-Gly292, Gly292-Gly328, and Gly329-Met357, respectively) of D3 were expressed using PCR with SPK-82A/B, SPK82C/D, and SPK-82E/F primers, respectively (Fig. 1) (19). All polypeptides were characterized by SDS-PAGE as previously described (19).

Peptide studies. Two peptides, Leu²³⁵-Ala²⁷⁷ (LNAENNA), derived from exon 7, one peptide, Glu²⁹²-Gly³²⁹, and Gly³²⁹-Met³⁵⁷, respectively) of D3 were expressed using PCR with SPK-82A/B, SPK82C/D, and SPK-82E/F primers, respectively (Fig. 1) (19). All polypeptides were characterized by SDS-PAGE as previously described (19).

Note that, in peptide Cys³³³-Cys³⁴₃, WE was substituted for the naturally occurring GC, the cysteine to introduce a disulfide bond and the glycine to mimic the turn in the D3 model (4). The peptides containing two cysteines were each air oxidized to the disulfide form. Three peptides, Gly⁴⁴₂-Lys⁴⁵₈ (GLGHGHEQHQHGLGHGHK), Phe⁴⁵⁹-Lys⁴₇₈ (FKLDDDDL-EHQGHGDDLHGK), and His⁴⁹₇-His⁴₉₈ (HKHGHHGKH-KNGKNGK), derived from D5 (Fig. 1), were kindly
RESULTS

Influence of concentration of HK on binding to PMN.
In preliminary studies, we used four different concentrations of {sup}125I-HK to establish an optimal concentration for competitive studies. Specific binding to activated PMN was found to saturate at 180 nM for {sup}125I-HK when it was determined. Accordingly, a concentration giving 60% of maximal binding (60 nM) for {sup}125I-HK was used.

Inhibition of {sup}125I-HK binding to PMN by exon 7–9 products of D3. We first used D3 to inhibit the binding of {sup}125I-HK to PMN. Unlabeled D3 in 100-fold molar excess (3 mM) inhibited the binding of {sup}125I-HK by 80.8 ± 1.5%. These results are similar to those reported from this laboratory (28) and confirm that one of the binding sites on HK is D3 on their common heavy chain. For further investigation of the cell binding epitopes of HK, three components of D3, exon 7–9 products, were used in 100-, 30-, 10-, 3-, and 1-fold molar excess to inhibit the binding of {sup}125I-HK (60 nM) to PMN. Binding of {sup}125I-HK (60 nM) to PMN (Fig. 2) was inhibited by exon 7 product as a function of concentration with 50% binding at 3,400 nM and by exon 9 product as a function of concentration with 50% binding at 1,650 nM. The exon 8 product showed only modest inhibition of binding. GST in 100-fold molar excess was used as control, since all recombinant polypeptides were fusion proteins with GST and showed no inhibition of binding of {sup}125I-HK to PMN. The inhibition by exon 7 and 9 products of HK binding (Fig. 2) was statistically significant. Exon 7 product inhibited less than D3 (P = 0.016), but exon 9 was not significantly different from exon 7.
Inhibition of $^{125}$I-HK binding to PMN by peptides derived from exons 7–9 of D3. Because exon 7 and 9 products of D3 showed concentration-dependent inhibition of binding to PMN (Fig. 2), we mapped the binding site on D3 by examining various peptides from exons 7–9 of D3 (Fig. 3). Leu<sup>271</sup>-Ala<sup>277</sup>, derived from exon 7, significantly inhibited ($P < 0.05$) binding of $^{125}$I-HK (60 nM) at 6,000, 1,800, and 600 nM but not at 180 or 60 nM. A significant difference ($P < 0.05$) was found between the 100- and 10-fold molar excess (6,000 and 600 nM, respectively). The 50% binding point was found at 3,470 nM. Scrambled peptide, NEANANL-NH$_2$ · TF A for Leu<sup>271</sup>-Ala<sup>277</sup> (LNAENNA) did not inhibit the binding of $^{125}$I-HK (60 nM) at 6,000 nM (Fig. 3). Ile<sup>268</sup>Ala<sup>277</sup> inhibited the binding of HK to PMN to the same extent as LNAENNA but was not more potent (data not shown). Cys<sup>333</sup>Cys<sup>352</sup>, derived from exon 9, inhibited the binding of $^{125}$I-HK significantly ($P < 0.05$) at 6,000, 1,800, and 600 nM but not at 180 or 60 nM. A significant difference ($P < 0.05$) was found between the 100- and 10-fold molar excess (6,000 and 600 nM, respectively). The 50% binding point was 520 nM. Glu<sup>307</sup>Glu<sup>323</sup>, derived from exon 8, showed no inhibition at 100-fold molar excess (Fig. 3).

**DISCUSSION**

This study deals with the binding of HK to a receptor on activated neutrophils. There may be a binding site on unactivated neutrophils, but it must be of very low copy number, below the level of detection of the assay.
we used. Standard neutrophils contain a considerable number of binding sites but were too variable. Therefore all neutrophils were maximally activated with FMLP for these studies. Because of the increase in binding sites, higher levels of 125I-HK were required than previously reported (11). The increase in the number of sites with neutrophil activation is consistent with the identification of Mac-1 as a major binding site for HK (27).

125I-HK was employed in these binding studies. As previously described (28), recombinant D3 inhibited the specific binding of HK to human neutrophils, and recombinant D5 displaced specifically bound HK. This inhibition was specific, since GST, the NH2-terminal portion of the fusion protein containing D3 or D5, failed to inhibit HK or LK binding, and D6, the PK binding site of HK, also did not alter HK binding. To further map the sites on D3, we first expressed recombinant exons 7–9 as fusion proteins with GST. Although we used the GST-kininogen constructs, we have previously shown (19) that, after thrombin cleavage and removal of GST by HPLC purification, the resulting exon products of kinogen behaved similarly to the GST fusion proteins in functional assays in inhibiting thrombin activation of platelets. We demonstrated that exon 7 (Gly235-Gln292) and exon 9 (Gly329-Met357) showed a concentration-dependent inhibition of binding of LK to neutrophils. In contrast, exon 8 showed no concentration dependence, and the inhibition was modest.

To further map each site, we chose specific peptides contained within each of the exon products. We have recently demonstrated that a heptapeptide Leu271-Ala277 within exon 7 product could inhibit thrombin activation of platelets with an IC50 of 65 mM (19). Study of a homology model of D3 constructed as previously described (9), based on the crystalline structure of egg white cystatin (2), indicated that this peptide formed a subdomain on the surface. We reasoned that this might serve as a binding site on neutrophils. The results (Fig. 3) show that this peptide is one of the most effective inhibitors of kinogen binding to neutrophils. The specificity is supported by the failure of randomly scrambled peptides to inhibit HK binding. The peptide Glu307-Glu323, on the first hairpin loop and the surface of the domain, failed to produce inhibition, consistent with the lack of a concentration-dependent inhibition of binding by exon 8 product.

Herwald et al. (15), using a monoclonal antibody to D3 that blocked biotinylated HK binding to endothelial cells, identified its epitope using synthetic peptides. A peptide contained in the exon 9 product, Leu231-Met357, inhibited HK binding with an IC50 of 60 mM, whereas Cys333-Lys345 (unoxidized) showed an IC50 of 113 µM. We synthesized a similar peptide, Cys333-Cys352, to test the hypothesis that the region which is in the second hairpin loop of D3 would be a binding site to neutrophils. The oxidized form of the peptide inhibited binding of HK to neutrophils in a concentration-dependent manner.

To further map the site on the unique light chain of HK, we examined various peptides from D5. We had previously described a 17-member peptide, His445-His475, that functioned as the epitope of a monoclonal antibody, C11C1 (25), which inhibited the coagulant activity of HK and blocked the binding to anionic surfaces (7). Using deletion mutagenesis, we have previously defined two binding sites to anionic surfaces, one similar to His445-His457 (histidine-glycine-rich region) and a second, His475-Lys502 (histidine-glycine-lysine-rich region), containing a consensus sequence for binding to heparin (9). We therefore studied three peptides derived from these two sites for their ability to block HK binding to neutrophils. Peptides Gly442-Lys458 and Phe459-Lys478, both rich in histidine and glycine, inhibited HK binding significantly in a concentration-dependent fashion (Fig. 4). The randomly scrambled peptide from the sequence Gly442-Lys458 did not inhibit HK binding, confirming the specificity of that peptide. In contrast, His475-His488, rich in histidine, glycine, and lysine, failed to inhibit HK binding at 100-fold molar excess. These results on neutrophils are different from those of Hasan et al. (13), who found that overlapping peptides encompassing a sequence His471-His488 had maximal inhibitory activity for binding of HK to endothelial cells and probably reflect differences in the identity of the receptor(s) between the two cells. It should be noted that D5 and selected peptides show modest inhibition and thus play a lesser role in binding to neutrophils than D3.

The use of three noncontiguous peptidyl sites for binding of a protein ligand to Mac-1 is not unique for HK. Such a situation has been reported for factor X (1). It should be noted that none of these three factor X peptides interfere with HK binding to endothelial cells (28). Thus HK binding to neutrophils resembles HK binding to endothelial cells only with regard to the binding site on exon 9 product. The binding site in D5 for endothelial cells contains a sequence with high concentration of basic residues that could bind heparin present on endothelial cell surfaces (5, 22) similar to that in antithrombin. In contrast, the binding sequences in neutrophils are rich in histidine and glycine and contain little lysine. The third, in exon 7 product, the heptapeptide Leu271-Ala277, which appears to be potent, was not described as an inhibitor of binding to endothelial cells. Results from our laboratory suggest that LNAENNA, which inhibited thrombin activation of platelets (19), inhibits thrombin binding to platelet GPIb but not HK binding to platelets. It appears that blood and vascular cells have different receptors, since the binding site on neutrophils, αMβ2, is not present on endothelial cells or platelets. It should be noted that although fibrinogen binding to platelets is Arg-Gly-Asp dependent, binding to neutrophils does not require Arg-Gly-Asp and thus uses different peptidyl motifs (11). However, the binding sites on HK show greater similarity, since the sites on D3 are limited to those exposed on the surface of the molecule, as supported by the homology model for D3 (4, 19). The three-dimensional structure of D3 is required for absolute confirmation of this supposition. Unfortunately, no model or tertiary structure is currently available for the site on
D5, and its structure awaits definition by NMR or X-ray crystallography.

The binding of HK is required for kallikrein stimulation of neutrophils as well as the displacement of fibrinogen from the neutrophil surface. Thus peptides that inhibit binding can be expected to decrease neutrophil activation as well as adhesion to perturbed endothelial cells. Therefore such peptides could serve as templates for design peptidomimetic drugs that could decrease unwanted inflammatory responses in blood exposed to artificial surfaces or to cytokines activated in response to sepsis or trauma.

We thank Rita Stewart for expert manuscript preparation. We appreciate the preparation of Fig. 1 by Dr. Robin Pixley.

This work was supported in part by National Heart, Lung, and Blood Institute Clinical Investigator Award HL-02681 (R. A. DeLa Cadena), a Grant-in-Aid from the American Heart Association, Southeastern Pennsylvania Affiliate (R. A. DeLa Cadena), a Fellowship Award of the Instituto Mexicano del Seguro Social, Mexico (A. Majluf-Cruz), National Heart, Lung, and Blood Institute Grant HL-41113 (S. L. Cooper), an Established Investigator Award from American Heart Association-Genevent (S. P. Kunapuli), and National Institutes of Health Program Project PO1-56914 (R. W. Colman).

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Received 29 January 1997; accepted in final form 16 March 1998.

REFERENCES


