

## Identification of a Major Heparin-binding Site in Kallistatin\*

Received for publication, June 30, 2000, and in revised form, September 10, 2000  
Published, JBC Papers in Press, October 2, 2000, DOI 10.1074/jbc.M005791200

Vincent C. Chen<sup>‡</sup>, Lee Chao<sup>‡</sup>, Daniel C. Pimenta<sup>§</sup>, Grant Bledsoe<sup>‡</sup>, Luiz Juliano<sup>§</sup>,  
and Julie Chao<sup>‡¶</sup>

From the <sup>‡</sup>Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425 and the <sup>§</sup>Departamento de Biofísica, Escola Paulista de Medicina, 100 Rua Tres de Maio, Sao Paulo 04044-020, Brazil

**Kallistatin is a heparin-binding serine proteinase inhibitor (serpin), which specifically inhibits human tissue kallikrein by forming a covalent complex. The inhibitory activity of kallistatin is blocked upon its binding to heparin. In this study we attempted to locate the heparin-binding site of kallistatin using synthetic peptides derived from its surface regions and by site-directed mutagenesis of basic residues in these surface regions. Two synthetic peptides, containing clusters of positive-charged residues, one derived from the F helix and the other from the region encompassing the H helix and C2 sheet of kallistatin, were used to assess their heparin binding activity. Competition assay analysis showed that the peptide derived from the H helix and C2 sheet displayed higher and specific heparin binding activity. The basic residues in both regions were substituted to generate three kallistatin double mutants K187A/K188A (mutations in the F helix) and K307A/R308A and K312A/K313A (mutations in the region between the H helix and C2 sheet), using a kallistatin P1Arg variant as a scaffold. Analysis of these mutants by heparin-affinity chromatography showed that the heparin binding capacity of the variant K187A/K188A was not altered, whereas the binding capacity of K307A/R308A and K312A/K313A mutants was markedly reduced. Titration analysis with heparin showed that the K312A/K313A mutant has the highest dissociation constant. Like kallistatin, the binding activity of K187A/K188A to tissue kallikrein was blocked by heparin, whereas K307A/R308A and K312A/K313A retained significant binding and inhibitory activities in the presence of heparin. These results indicate that the basic residues, particularly Lys<sup>312</sup>-Lys<sup>313</sup>, in the region between the H helix and C2 sheet of kallistatin, comprise a major heparin-binding site responsible for its heparin-suppressed tissue kallikrein binding.**

Kallistatin is a specific serine proteinase inhibitor (serpin) for human tissue kallikrein (1). It inhibits tissue kallikrein by forming a covalent enzyme-inhibitor complex (2). Our previous study showed that kallistatin also has binding activity toward chymotrypsin, but it behaves more like a substrate for chymotrypsin (1). In addition to acting as a proteinase inhibitor, kallistatin also has hypotensive and vasodilative effects inde-

pendent of its inhibitory activity toward tissue kallikrein (3, 4). Although the physiological functions of kallistatin have not been well defined, previous studies indicate that kallistatin acts as a multifunctional protein that exhibits unique functions at different tissues (4).

The reactive-center loop, particularly the P1 residue, determines the inhibitory specificity of a serpin. Substitutions of the P1 residue of kallistatin dramatically change the inhibitory specificity of kallistatin (1). High specificity and selectivity of kallistatin for tissue kallikrein is determined by its unique Phe at P1 position and probably also by other subsite-binding residues in the reactive-center loop (1). Other than the reactive-center loop, heparin-binding sites of a serpin can also regulate its inhibitory activity toward target serine proteinase. Kallistatin is one of the heparin-binding serpins, which include anti-thrombin, protein C inhibitor, plasminogen activator inhibitor, heparin cofactor, and protease nexin. For most of the heparin-binding serpins, heparin accelerates the inhibitory activity toward their target serine proteinases (5–9). The mechanisms have been explained by using a ternary complex and an allosteric model (5, 6, 10–13). Unlike most of these serpins, heparin suppresses the inhibitory activity of kallistatin toward tissue kallikrein, whereas it accelerates the interaction between kallistatin and chymotrypsin (1). The mechanism by which heparin inhibits the interaction between kallistatin and tissue kallikrein has not been explored.

The heparin-binding site of a serpin is pivotal not only in regulating the inhibitory specificity of a serpin but also in directing a serpin to its target tissues to perform its function (11, 14–16). It has been demonstrated that heparan sulfate proteoglycans located on the extracellular matrix and surface of vascular smooth muscle cells and endothelial cells can trigger the inhibition of proteinases by bringing serpins into close apposition with their target proteinases (14–16). As in the case of other heparin-binding serpins, the heparin binding activity of kallistatin may play an important role in its function on cellular surfaces. The heparin-binding sites of proteins usually contain clusters of basic amino acid residues, which provide positively charged regions for binding to the acidic groups of heparin by electrostatic interaction (17). Heparin-binding sites of antithrombin, plasminogen activator inhibitor, protease nexin, and heparin cofactor II have been located in the D helix (6, 11, 12, 18–20), whereas that of protein C inhibitor is located in the H helix (21). The location of the heparin-binding site in kallistatin, however, has not been identified.

In the present study, we attempted to identify the heparin-binding sites in kallistatin by two approaches: 1) to design synthetic peptides derived from surface regions of kallistatin that contain a high density of basic residues and to assess their heparin binding capacity; 2) to create kallistatin mutants by substituting basic residues with alanine in the putative hepa-

\* This work was supported by National Institutes of Health Grant HL 44083. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-9927; Fax: 843-792-4850; E-mail: chaoj@muscc.edu.

rin-binding regions and to analyze the heparin binding capacity of these mutants as well as the effects of heparin on the inhibitory activity of kallistatin toward tissue kallikrein. Identification of heparin-binding sites in kallistatin could provide significant insights into the structural and functional relationship of kallistatin, and pave the way for further studies of the regulation and the physiological function of kallistatin.

#### EXPERIMENTAL PROCEDURES

**Materials**—*Escherichia coli* strain TOP10, the pTrc-His B expression vector was purchased from Invitrogen (San Diego, CA); restriction enzymes, and isopropylthio- $\beta$ -galactoside were from Life Technologies, Inc. (Gaithersburg, MD); *Taq* polymerase was from PerkinElmer Life Sciences (Norwalk, CT); nickel-nitrilotriacetic acid-agarose was from Qiagen (Santa Clarita, CA); the POROS HE/1 column was from PerSeptive Biosystems (Cambridge, MA); Low molecular mass heparin (4500 Da) was purchased from Rhône-Poulenc (Aventis) and Upjohn (Kalamazoo, MI); D-Val-Leu-Arg-MCA (methylcoumarinamide) was from Enzyme System Products (Livermore, CA); human tissue kallikrein was purified as described previously (22); synthetic peptides were synthesized and provided by Dr. L. Juliano (from the Department de Biofisica, Escola Paulista de Medicina, Sao Paulo, Brazil).

**Molecular Modeling of Kallistatin**—The atomic coordinates of the intact serpins,  $\alpha$ 1-antitrypsin (1qlp), ovalbumin (1ova), antithrombin (1ant), and cleaved form of protein C inhibitor (1pai) were obtained from the Protein Data Bank at Rutgers University. A molecular model was created using the homology modeling module, Composer, in the SYBYL program (version 6.5, Tripos, Inc.). The topologically equivalent residues across these serpins were determined first based on sequence homology. A structural alignment of the serpins was then performed with the equivalent residues as a starting point. This alignment determined the structurally conserved regions (SCRs)<sup>1</sup> as well as the average framework of the SCRs. SCRs of kallistatin were generated by using fragments of the homologs to construct the backbone of the SCRs and a rule-based procedure to generate the side chains. The structurally variable regions were constructed by using fragments from known structures, which are compatible with the rest of the model, and then using sequence information to postulate which single fragment is best for use in the final model. The kallistatin model was then refined by side-chain torsion relieving and energy minimization. The backbone of the whole model was constrained and then energy minimized by steepest descent until the maximum derivative was less than 50 kcal/(mol·Å). The constraint was then removed, and additional minimization was performed until the maximum derivative was less than 5 kcal/(mol·Å) using steepest-descent algorithm. Finally, conjugate gradient minimization continued until the maximum derivative was less than 0.1 kcal/(mol·Å).

**Design of Synthetic Peptides**—Surface regions of kallistatin were determined by the kallistatin model created by the SYBYL program and a program, PEPTIDESTRUCTURE, in Wisconsin Package Version 10.0 (Genetics Computer Group (GCG), Madison, WI). The synthetic peptides were designed according to surface regions that encompass a high density of positively charged residues.

**Construction of Kallistatin Mutants Containing Mutations at Putative Heparin-binding Sites**—The kallistatin variant P1Arg, created as described previously (23), in the prokaryotic expression vector pTrc His B was used as a backbone to construct kallistatin mutants containing mutations at putative heparin-binding sites. The expression vector adds a hexahistidine sequence at the N terminus of the recombinant kallistatin for protein purification by metal-affinity chromatography. The kallistatin variants K307A/R308A and K312A/K313A were designed to contain alanine replacing the basic amino acid residues in the putative heparin-binding sites of the region between the H helix and C2 sheet and the other variant K187A/K188A in the F helix (Fig. 1). K307A/R308A and K312A/K313A were created by site-directed mutagenesis of kallistatin P1Arg cDNA using a sequence overlap-extension PCR method (24). The internal primers 5'-CAACTGTGTCGGCGCGCAATTTTACAAG-3'/5'-CTTGTAATAAATTCGCCGCCGCAACAAGTTG-3' were employed to replace Lys<sup>307</sup> and Arg<sup>308</sup> with Ala to create the kallistatin mutant, K307A/R308A, and 5'-GAATTTTTCGCGCGCTAGAGTTGC-3'/5'-GCAACTCTAGCGCGCGTAAAAATTC-3' were used to replace Lys<sup>312</sup> and Lys<sup>313</sup> with Ala to create the

mutant K312A/K313A. The mutated nucleotides are *underlined*. The outside primers were 5'-CGTCTATGAGGCTAAC-3' and 5'-CTATGGTTTCGTGGGGT-3' for both mutants. The mutant fragments synthesized by PCR were cleaved with *Xho*I and *Sal*I and replaced the counterparts of the pTrc His-based kallistatin P1Arg expression plasmids excised by the same restriction enzymes. The mutant K187A/K188A was created by the PCR method described previously. The primers 5'-TGATGGTGAGAGTTGCA-3' and 5'-TCCCTCGAGTTCCGCCGCGACGTGGTTCG-3', encompassing the desired alanine codon substitutions (*underlined*) for Lys<sup>187</sup>-Lys<sup>188</sup> and a *Xho*I site, were used to synthesize the mutant fragments of K187A/K188A by PCR. The PCR fragment was cleaved with *Sma*I/*Xho*I and cloned into the kallistatin P1Arg expression plasmid that had its counterpart excised by the same restriction enzymes. The mutations were confirmed by DNA sequencing.

**Expression and Purification of the Kallistatin Mutants**—The kallistatin mutants containing Ala substitutions at the putative heparin-binding sites were expressed in a large scale of cell culture, and then soluble cell lysates were isolated and purified by nickel-affinity (nickel-nitrilotriacetic acid, Qiagen) and heparin-affinity chromatography as described previously (23). Protein purity was assessed by SDS-PAGE and staining by Coomassie Blue. Concentrations of the mutants were determined by an enzyme-linked immunosorbent assay specific for human kallistatin (4).

**Peptide Competition by Kallikrein Binding Assay**—Synthetic peptides, dissolved in Me<sub>2</sub>SO at 5  $\mu$ M, were preincubated with 0.05, 0.1, and 0.2 unit/ml heparin in 17  $\mu$ l of reaction buffer, 20 mM sodium phosphate, pH 8.0, at 37 °C for 20 min. The same amount of Me<sub>2</sub>SO was added to the control groups containing no synthetic peptides. Subsequently, 1  $\mu$ l of the recombinant kallistatin (concentration, 0.4 mg/ml) was added into the reaction (final concentration, 0.4  $\mu$ M) for a 10-min incubation at 37 °C. Finally, 2  $\mu$ l of <sup>125</sup>I-labeled tissue kallikrein at 1  $\times$  10<sup>4</sup> cpm/ $\mu$ l was incubated with the mixture at 37 °C for 60 min. The reactions were stopped by SDS-loading buffer, resolved in 10% SDS-PAGE, and analyzed by autoradiography. A dose-dependent peptide competition assay was also performed to demonstrate the specific binding of the peptides to heparin. Different concentrations of the synthetic peptides were used to compete with 0.4  $\mu$ M kallistatin for 0.05 and 0.1 unit/ml of heparin in a 20- $\mu$ l reaction mixture. The synthetic peptides, F peptide at 2.5, 5.0, 7.5, 10 and 15  $\mu$ M, HC2 peptide at 1, 2, 3, 4 and 5  $\mu$ M, and C4 peptide at 5, 10 and 20  $\mu$ M, were used in the dose-dependent peptide competition assay.

**Fluorescence Titration of Kallistatin and the Mutants with Heparin**—A solution of kallistatin wild type (0.116  $\mu$ M) in 50 mM Tris-HCl, 100 mM NaCl, 0.2% (w/v) PEG 6000, pH 7.4, at 20 °C was titrated with small aliquots of a high concentration of heparin solution with minimal dilution (<10%). The protein fluorescence ( $\lambda_{\text{ex}} = 280$  nm,  $\lambda_{\text{em}} = 340$  nm, 10-nm slits) was measured in a 1-ml quartz cuvette, thermostabilized, and held in a Shimadzu RF-1501 spectrofluorometer under constant agitation. The dependence of the relative fluorescence change, *i.e.*  $\Delta F/F_0 = (F_0 - F_{\text{obs}})/F_0$ , where  $F_0$  and  $F_{\text{obs}}$  represent starting and observed fluorescence values, respectively, can be analyzed by nonlinear least-squares data fitting by the binding equation (Eq. 1) (25) using the Graft program (version 3.0, Erithacus Software Ltd.).

$$\frac{\Delta F}{F_0} = \frac{\Delta F_{\text{max}}}{F_0} \times \frac{[(P_0 + H_0 + K_d) - \sqrt{(P_0 + H_0 + K_d)^2 - 4P_0H_0}]}{2P_0} \quad (\text{Eq. 1})$$

In this equation,  $P_0$  is the total protein concentration,  $H_0$  represents the heparin concentration,  $K_d$  is the dissociation constant, and  $\Delta F_{\text{max}}/F_0$  is the maximum relative fluorescence change. For kallistatin P1Arg, K312A/K313A, and K307A/R308A mutants, the concentrations in the assay were 0.170, 0.141, and 0.160  $\mu$ M, respectively.

**Competitive Binding Assay**—Kallistatin wild type (0.116  $\mu$ M) was titrated with small aliquots of low molecular weight heparin in the same conditions as described above in the presence of different concentrations of synthetic peptides. The apparent  $K_d$  ( $K_{d\text{-app}}$ ) for the interaction of each peptide was determined by plotting the observed  $K_d$  ( $K_{d\text{-obs}}$ ) determined for the interaction of each peptide *versus* its concentration in the assay.

**Heparin Affinity Chromatography**—The relative affinity of kallistatin P1Arg and the kallistatin mutants for immobilized heparin was determined by heparin-affinity chromatography in a BioCAD SPRINT system. Samples of approximately 40–100  $\mu$ g in 2 ml of 20 mM sodium phosphate buffer, pH 7.0, 20 mM NaCl, were loaded onto an HE1/M column, which was saturated with the same buffer. The flow rate was controlled at 1 ml/min. The column was washed with the same buffer until the absorbance reached the base line. The mutant proteins were

<sup>1</sup> The abbreviations used are: SCR, structurally conserved regions; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; PEG, polyethylene glycol.

eluted with 50 ml of a NaCl gradient from 0 to 500 mM. The elution was monitored by absorbance at 280 nm. The salt gradient and elution profiles were plotted, and the concentrations of NaCl at elution peak were measured. Three different batches of each recombinant protein were analyzed for their heparin binding capacity.

**Heparin-suppressed Tissue Kallikrein Binding Assay**—Tissue kallikrein binding activity of the kallistatin mutants containing Ala substitutions in the putative heparin-binding sites was assessed by kallikrein-binding assay in the presence of different concentrations of heparin. Kallistatin P1Arg and mutants at concentration 0.6  $\mu$ M were preincubated with different concentrations of heparin, 0, 2.5, 5, 10, 20 and 30 units/ml, in 18.5  $\mu$ l of 20 mM sodium phosphate buffer, pH 8.0, at 37  $^{\circ}$ C for 10 min. Then 1.5  $\mu$ l of  $^{125}$ I-labeled tissue kallikrein at  $1 \times 10^4$  cpm/ $\mu$ l was added into the reactions, and the mixtures were incubated at 37  $^{\circ}$ C for 90 min. The samples were resolved on 10% SDS-PAGE under reducing condition and analyzed by autoradiography.

**Effect of Heparin on Tissue Kallikrein Inhibition Assay**—The effects of heparin on the inhibitory activity of the kallistatin mutants were analyzed by preincubating 0.2  $\mu$ M of the kallistatin mutants with 0, 2, 5, 10, 20, and 30 units/ml heparin in 45  $\mu$ l of 20 mM sodium phosphate buffer, pH 8.0, at 37  $^{\circ}$ C for 10 min. The reactions were then started by adding 5  $\mu$ l of 2 ng/ $\mu$ l tissue kallikrein (final concentration, 6 nM) into the reaction mixtures and incubating at 37  $^{\circ}$ C for 90 min. The residual tissue kallikrein activity was determined by adding 20  $\mu$ l of the reaction mixture in 2 ml of buffer containing 50 mM Tris-HCl, pH 8.0, and 30  $\mu$ M of Val-Leu-Arg-MCA. The rate of substrate hydrolysis was monitored at 380-nm excitation and 460-nm emission.

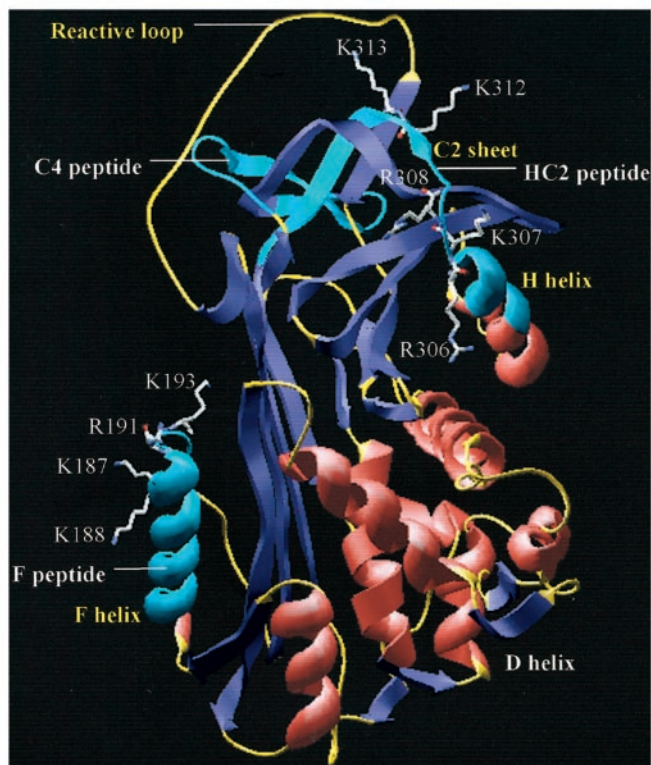
**Circular Dichroism (CD) Spectroscopy**—The conformation of the kallistatin mutants was estimated by CD spectroscopy. CD spectra were obtained using a Jasco J710 spectropolarimeter at a resolution of 0.2 nm, with a bandwidth of 2 nm. Ten spectra were averaged for each derivative. The recombinant kallistatins were diluted in 20 mM sodium phosphate, pH 7.0, 200 mM NaCl at a concentration of 0.4 mg/ml using a cell of 0.02-cm path length. Mean residue ellipticity was calculated according to the literature (26). Protein concentrations were derived from UV spectroscopy and confirmed by a specific enzyme-linked immunosorbent assay (4).

## RESULTS

**Molecular Modeling of Kallistatin**—The molecular structure of kallistatin was created by homology modeling as shown in Fig. 1A. Because the reactive-center loop is a highly variable region among serpins, there is no good template for modeling the reactive-center loop of kallistatin. Our previous study suggested that replacing the residues in the hinge region of the reactive loop with bulky residues may hinder a partial insertion of the reactive loop into A  $\beta$ -sheet and thus converting kallistatin from an inhibitor to a substrate for tissue kallikrein (data not shown). We assumed that the partial insertion of the hinge region is critical for the inhibitory conformation of kallistatin. Therefore, the atomic coordinates of antithrombin were chosen to model the reactive-center loop of kallistatin, P16-P5', because the reactive loop of antithrombin has the same number of residues as kallistatin and the hinge region of the reactive loop was partially inserted into the A  $\beta$ -sheet as predicted for serpins (27). The conformational energy of the kallistatin model is approximately  $-1300$  kcal $\cdot$ mol $^{-1}$ , indicating an acceptable conformation without serious steric clash. The F helix, H helix, and C2 sheet are indicated in Fig. 1. The length of the kallistatin molecule is about 72  $\text{\AA}$  as measured from the protruding reactive loop to the distal end of the molecule. The F helix is distant from the reactive loop, and the distance measured between the C $\alpha$  of the basic residues Lys<sup>187</sup>-Lys<sup>188</sup> in the F helix and the C $\alpha$  of the reactive center is about 46–48  $\text{\AA}$ . The loop between the H helix and C2 sheet crosses through the reactive loop and locates the basic residues Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup> and Lys<sup>312</sup>-Lys<sup>313</sup> in a proximate position to the reactive center with distances of 20–25 and 11–13  $\text{\AA}$ , respectively.

**Design of Synthetic Peptide**—Surface regions of kallistatin were determined by the program PEPTIDESTRUCTURE in Wisconsin Package Version 10.0 (Genetics Computer Group

### A



### B

1	MHLIDYLLLL	LVGLLALSHG	QLHVEHDGES	CSNSSHQQIL	ETGEGSPSLK	50
	<u>hA</u>		<u>sB6</u>	<u>hB</u>	<u>hC</u>	
51	IAPANADFAF	RFYYLIASET	PGKNIFFSPL	SISAAYAMLS	LGACSHRSRQ	100
	<u>hC1</u>	<u>hD</u>		<u>sA2</u>		
101	ILEGLGFNLT	ELSESDVHRG	FQHLHLTLNL	PGHGLETRVG	SALFLSHNLK	150
	<u>hE</u>	<u>sA1</u>	<u>F</u>	<u>hF</u>		
151	FLAKFLNDTM	AVYEAKLFHT	NFYDVTGTTQ	LINDHVKKPT	RSKLVDLVSE	200
	<u>sA3</u>	<u>C4</u>	<u>sC4</u>	<u>sC3</u>		
201	LKKDVLMLVL	NYIYFKALWE	KPPISSRTTP	KDPYVDENTT	VRVPMMLDQQ	250
	<u>sB1</u>	<u>sB2</u>	<u>sB3</u>	<u>hG</u>	<u>hH</u>	
251	EHHWYLDHRY	LPCSVLRMDY	KGDATVPFVL	PNQGMREIE	EVLTPPEMLR	300
	<u>HC2</u>	<u>sC2</u>	<u>sA6</u>	<u>hI</u>		
301	WNLLRKRNF	YKKGELHLPK	FSISGSVYLD	QILPRLGFTD	LFSKWADLSG	350
	<u>sA5</u>			<u>sC1</u>		
351	ITKQKLEAS	KSPHKATLDV	DEAGTEAAAA	TTFAIKFFSA	QTNRHILRPN	400
	<u>sB4</u>	<u>sB5</u>				
401	RPFLVVFST	STQSVLFLGK	VVDPTKP	427		

**FIG. 1. Synthetic peptides derived from the surface regions of kallistatin.** The synthetic peptides encompassing the putative heparin-binding sites were designed according to the surface regions of kallistatin determined by PEPTIDESTRUCTURE in the Wisconsin Package and by a kallistatin model created by COMPOSER in SYBYL. Two surface regions around the F helix and the region between the H helix and C2 sheet containing clusters of basic residues were selected to synthesize the peptides, designated as F peptide and HC2 peptide. Another surface region around the C4 sheet was selected as a negative control, designated as C4 peptide. A, the kallistatin structure was created by homology modeling. The F helix, H helix, and C2 sheet are labeled. The cyanic areas represent the regions of the synthetic peptides as indicated. The basic residues in both regions are shown as labeled. B, the secondary structural features of kallistatin are overlined in the amino acid sequence. The regions of the synthetic peptides are shaded. The basic residues in the putative heparin-binding regions are in boxes. The boldface characters indicate the names of the synthetic peptides.

(GCG)). A kallistatin model was created by homology modeling using COMPOSER, in SYBYL, to assist in the selection of the surface regions. Two surface regions, the F helix and the loop between the H helix and C2 sheet, contain clusters of positive-charged residues (Fig. 1B). To test whether these two highly

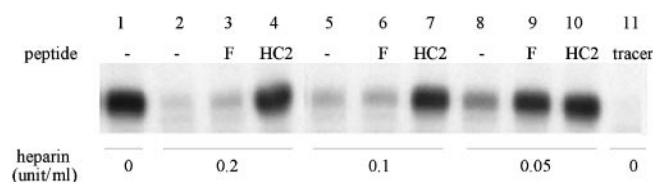


FIG. 2. Effects of the synthetic peptides on the heparin-inhibited complex formation between kallistatin and tissue kallikrein. F and HC2 peptides at  $5 \mu\text{M}$  were used to compete with  $0.04 \mu\text{M}$  kallistatin for 0.2, 0.1, and 0.05 unit/ml heparin in 18  $\mu\text{l}$  of reaction buffer, 20 mM sodium phosphate, pH 8.0, at  $37^\circ\text{C}$  for 20 min. A tissue kallikrein binding assay was conducted by adding  $2 \mu\text{l}$  of  $^{125}\text{I}$ -labeled tissue kallikrein at  $1 \times 10^4 \text{ cpm}/\mu\text{l}$  in each reaction at  $37^\circ\text{C}$  for 60 min. The tissue kallikrein-kallistatin complexes were analyzed by SDS-PAGE and autoradiography. The synthetic peptides and the concentrations of heparin added in each tissue kallikrein-binding assay are as indicated on each lane. -, without addition of peptide.

positive-charged regions are capable of binding with heparin, two synthetic peptides corresponding to both regions were synthesized: F peptide, VGTIQLINDHVKKETRGKIV-NH<sub>2</sub>, spanning amino acids 176–195, and HC2 peptide, RWNLLRKRNFYKKLELHLP-NH<sub>2</sub>, spanning amino acids 300–319. A scrambled peptide SCR, derived from HC2 peptide, was synthesized as RWNRLFRLKNEPKLHNLKL-NH<sub>2</sub>. The putative heparin-binding sites are *underlined*. A negative control C4 peptide derived from the surface region around the C4 sheet was synthesized as PFISSRTPKDFYVDENTTV-NH<sub>2</sub>, spanning amino acids 222–242. All synthetic peptides were dissolved in Me<sub>2</sub>SO for use.

**Peptide Competition by Kallikrein Binding Assay**—Heparin binding activity of the synthetic peptides was compared by peptide competition assay as shown in Fig. 2. Peptides with heparin binding activity can prevent heparin from binding to kallistatin by competing for the heparin-binding sites. Consequently, an effective competing peptide can free kallistatin from heparin binding and thus restore its tissue kallikrein binding activity. Kallistatin P1Arg forms a complex with tissue kallikrein in the absence of heparin (*lane 1*). The complex formation was inhibited in the presence of different concentrations of heparin (*lanes 2, 5, and 8*). The F peptide, representing the F helix region of kallistatin, did not reverse the inhibition of the complex formation by 0.2 and 0.1 unit/ml heparin (*lanes 3 and 6*). Only at a lower heparin concentration, 0.05 unit/ml, did the F peptide start to restore the tissue kallikrein binding activity of kallistatin (*lane 9*). The HC2 peptide, spanning the H helix and C2 sheet, however, restored the tissue kallikrein binding activity of kallistatin efficiently under 0.2, 0.1, and 0.05 unit/ml heparin (*lanes 4, 7, and 10*). Fig. 3 shows dose-dependent competition of the peptides with kallistatin for heparin. The HC2 and F peptides compete dose-dependently for heparin and reverse the inhibition of complex formation (Fig. 3, A and B). The HC2 peptide at  $1 \mu\text{M}$  starts to show competition for 0.2 unit/ml heparin, whereas the F peptide at  $5 \mu\text{M}$  competes for 0.1 unit/ml heparin. The control peptide C4, spanning over the C4 sheet, did not reduce the heparin inhibition of the tissue kallikrein-kallistatin binding at concentrations as high as  $20 \mu\text{M}$  (Fig. 3C). These results suggest that heparin-binding sites of kallistatin are likely located within the F helix and the region between the H helix and C2 sheet, and the latter region is probably the major heparin-binding site of kallistatin.

**Competitive Binding Assay**—The heparin binding activity of peptide HC2, which is relatively higher than that of peptide F2, was further assessed by competitive binding assay. Titration of kallistatin with heparin was performed in the presence of HC2 peptide at concentrations of  $0.984$  and  $5.920 \mu\text{M}$  or its scrambled peptide SCR at  $0.584$  and  $4.543 \mu\text{M}$ . Fig. 4 shows the equilibrium binding of kallistatin with heparin in the presence

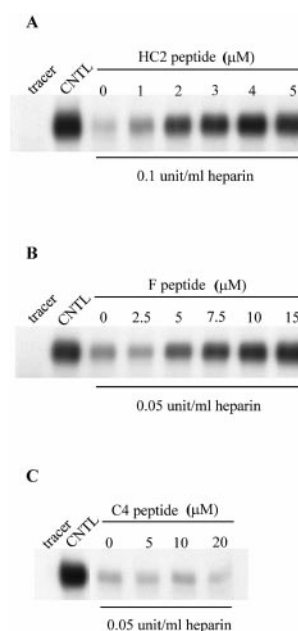


FIG. 3. Dose-dependent effects of the synthetic peptides on the heparin-inhibited complex formation between kallistatin and tissue kallikrein. Different concentrations of the synthetic peptides were used to compete with kallistatin for heparin in the tissue kallikrein-binding assay. *CNTL*, tissue kallikrein binding assay without addition of heparin and peptide. A, HC2 peptide at different concentrations, 1, 2, 3, 4, and  $5 \mu\text{M}$ , was added to compete with kallistatin for 0.1 unit/ml heparin. B, F peptide at concentrations of 2.5, 5, 7.5, 10 and  $15 \mu\text{M}$  was added to compete with kallistatin for 0.05 unit/ml heparin. C, C4 peptide at concentrations 5, 10, and  $20 \mu\text{M}$ , was added to compete with kallistatin for 0.05 unit/ml heparin.

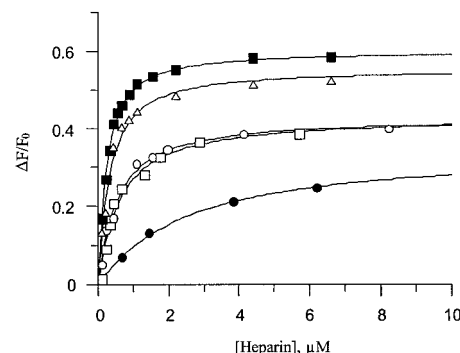


FIG. 4. Fluorescence titration of kallistatin for heparin binding in the presence of the synthetic peptides. Heparin was titrated in a solution of kallistatin at a concentration of  $0.116 \mu\text{M}$  (■) in the presence of  $0.984 \mu\text{M}$  HC2 peptide (●),  $5.920 \mu\text{M}$  HC2 peptide (●),  $0.584 \mu\text{M}$  SCR (△), and  $4.454 \mu\text{M}$  SCR (□).

of HC2 and SCR peptides. Table I shows that the shift in the  $K_{d\text{-obs}}$  value of kallistatin with heparin is more efficient in the presence of peptide HC2 as compared with SCR under a low peptide concentration.  $K_{d\text{-app}}$  for peptides HC2 and SCR were calculated as the  $x$  intercept for the plot of  $K_{d\text{-obs}}$  versus peptide concentration (Fig. 5). The obtained values were  $0.395 \mu\text{M}$  for HC2 peptide and  $2.574 \mu\text{M}$  for SCR peptide. These results indicate that peptide HC2 derived from the sequence between the H helix and C2 sheet can specifically bind to heparin not only by the positively charged residues but also by the sequence and structure of the peptide.

**Construction, Expression, and Purification of Kallistatin Mutants Containing Mutations at Putative Heparin-binding Sites**—The role of individual basic residues in the F helix and the region between the H helix and C2 sheet of kallistatin was further defined by site-directed mutagenesis. The kallistatin

TABLE I

Dissociation constant ( $K_{d-obs}$ ) determination for kallistatin in the presence of the synthetic peptides

$K_{d-obs}$  of kallistatin at 0.116  $\mu\text{M}$  was measured in the presence of peptide HC2 or SCR in 50 mM Tris-HCl, 100 mM NaCl, 0.2% (w/v) PEG 6000, pH 7.4, at 20 °C.

Peptide	Peptide concentration $\mu\text{M}$	$K_{d-obs}$
HC2	0.984	$0.448 \pm 0.010$
HC2	5.920	$2.459 \pm 0.105$
SCR	0.584	$0.266 \pm 0.040$
SCR	4.543	$0.551 \pm 0.050$

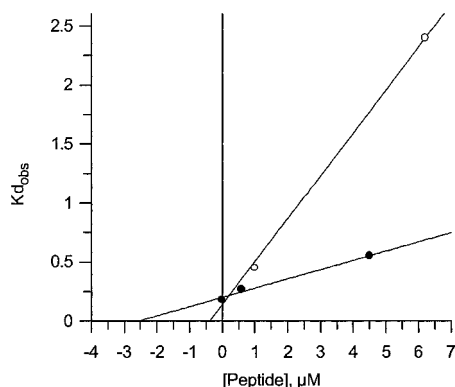


FIG. 5. Apparent dissociation constants ( $K_{d-app}$ ) determination for HC2 and SCR peptides. The x intercept for the plot of  $K_{d-obs}$  versus peptide concentration indicates  $K_{d-app}$  for peptides HC2 (○) and SCR (●).

variant P1Arg, which has the highest inhibitory activity toward human tissue kallikrein (1), was used as a model for mutagenesis. The basic residues Lys<sup>187</sup>-Lys<sup>188</sup> in the F helix, and Lys<sup>307</sup>-Arg<sup>308</sup> and Lys<sup>312</sup>-Lys<sup>313</sup> in the region between the H helix and C2 sheet were substituted with Ala, a neutral amino acid. Three kallistatin double mutants, K187A/K188A, K307A/R308A, and K312A/K313A, with double Ala substitutions for the basic residues, were engineered. The recombinant kallistatins were expressed and purified to apparent homogeneity (data not shown).

**Heparin Binding Activity of Kallistatin Mutants**—Heparin binding activity of kallistatin mutants was assessed by heparin-affinity chromatography. The heparin binding activity of the kallistatin variants with mutations in the putative heparin-binding sites was compared to kallistatin P1Arg. The results of the NaCl gradient elution pattern from a heparin-affinity column are shown in Fig. 6 and summarized in Table II. Kallistatin P1Arg, without mutation in the putative heparin-binding sites, was eluted at 340 mM NaCl. K187A/K188A, containing Ala substitutions for basic residues in the F helix, was eluted at 330 mM NaCl, indicating that the substitutions did not significantly interfere with its heparin binding activity. Both K307A/R308A and K312A/K313A, containing mutations in the region between the H helix and C2 sheet, showed reduced heparin binding activity as indicated by their elution was at 270 and 220 mM NaCl, respectively. These results suggest that the basic residues in the region between the H helix and C2 sheet are the heparin-binding sites of kallistatin.

**Dissociation Constants of Kallistatin and the Mutants**—The relative heparin binding activity of K312A/K313A and K307A/R308A mutants is lower than that of kallistatin P1Arg as measured by heparin-affinity chromatography. Definitive heparin binding activity was further determined by  $K_d$ . An accurate  $K_d$  determination can be obtained when the titration is performed at a protein concentration close to or lower than the

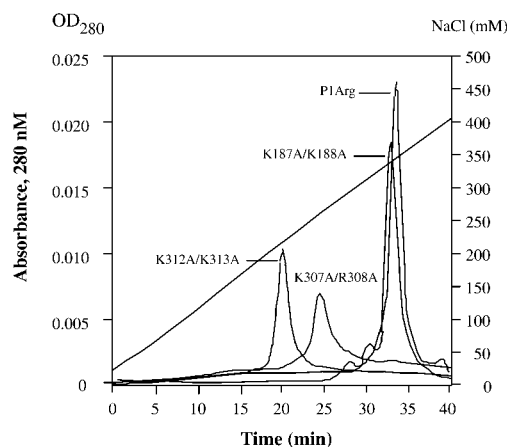


FIG. 6. Heparin binding capacity of the kallistatin mutants. The heparin binding activity of kallistatin P1Arg, K187A/K188A, K307A/R308A, and K312A/K313A was assessed by heparin-affinity chromatography. Approximately 40–100  $\mu\text{g}$  of kallistatins were loaded onto a heparin-affinity column (1.7-ml bed volume) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, 20 mM NaCl. The samples were eluted with 50 ml of NaCl gradient from 0 to 500 mM in phosphate buffer at a flow rate of 1.0 ml/min with detection at OD<sub>280</sub>. The concentration of NaCl was monitored by a conductivity meter in the BioCAD SPRINT system. The elution peaks for the kallistatin mutants are as indicated.

TABLE II

Relative heparin affinity of the kallistatin mutants analyzed by chromatography

	Elution by NaCl	Elution peak
	<i>mM</i>	
Kallistatin (P1Arg)	300–350	340
K187A/K188A	300–350	330
K307A/R308A	250–300	270
K312A/K313A	200–250	220

$K_d$  value (25). Fig. 7 shows the relative fluorescence change ( $\Delta F/F_0$ ) with increasing heparin concentrations for kallistatin wild type (Fig. 7A), P1Arg (Fig. 7B), K307A/R308A (Fig. 7C), and K312A/K313A (Fig. 7D). Table III summarizes the  $K_d$  values for kallistatin and the mutants, as well as their respective protein concentrations used for the measurement. Kallistatin wild type and kallistatin P1Arg have comparable  $K_d$  values. The  $K_d$  of K307A/R308A and K312A/K313A mutants are 2.5- and 16-fold higher than that of kallistatin P1Arg, respectively, indicating decreased heparin affinity of K307A/R308A and K312A/K313A mutants. The results demonstrate that Lys<sup>307</sup>, Arg<sup>308</sup>, Lys<sup>312</sup>, and Lys<sup>313</sup> are responsible for heparin binding activity of kallistatin, particularly Lys<sup>312</sup> and Lys<sup>313</sup> residues.

**Tissue Kallikrein Binding Activity of the Kallistatin Mutants in the Presence of Heparin**—A tissue kallikrein binding assay was performed in the presence of heparin to illustrate the effects of heparin on the tissue kallikrein binding activity of the kallistatin mutants as shown in Fig. 8. The complex formation between tissue kallikrein and kallistatin P1Arg was inhibited by heparin in a dose-dependent manner. The tissue kallikrein binding activity of K187A/K188A was also inhibited by heparin in the same manner. However, both K307A/R308A and K312A/K313A variants retained their relatively strong binding to tissue kallikrein as compared with P1Arg and K187A/K188A in the presence of heparin. K312A/K313A appears to be completely resistant to heparin inhibition at concentration as high as 30 units/ml. These results further confirm the location of the heparin-binding site and indicate that the basic residues in the heparin binding region of kallistatin are responsible for its

FIG. 7. Equilibrium binding of heparin to kallistatins monitored by fluorescence titration. A, Heparin was titrated into solutions of kallistatin wild type at concentration 0.116  $\mu\text{M}$ ; B, P1Arg at 0.170  $\mu\text{M}$ ; C, K307A/R308A at 0.160  $\mu\text{M}$ ; and D, K312A/K313A at 0.141  $\mu\text{M}$  in 50 mM Tris-HCl, 100 mM NaCl, 0.2% (w/v) PEG 6000, pH 7.4, at 20 °C. The relative change in kallistatin fluorescence was monitored by  $\lambda_{\text{ex}} = 280 \text{ nm}$ ,  $\lambda_{\text{em}} = 340 \text{ nm}$ , in 10-nm slits.

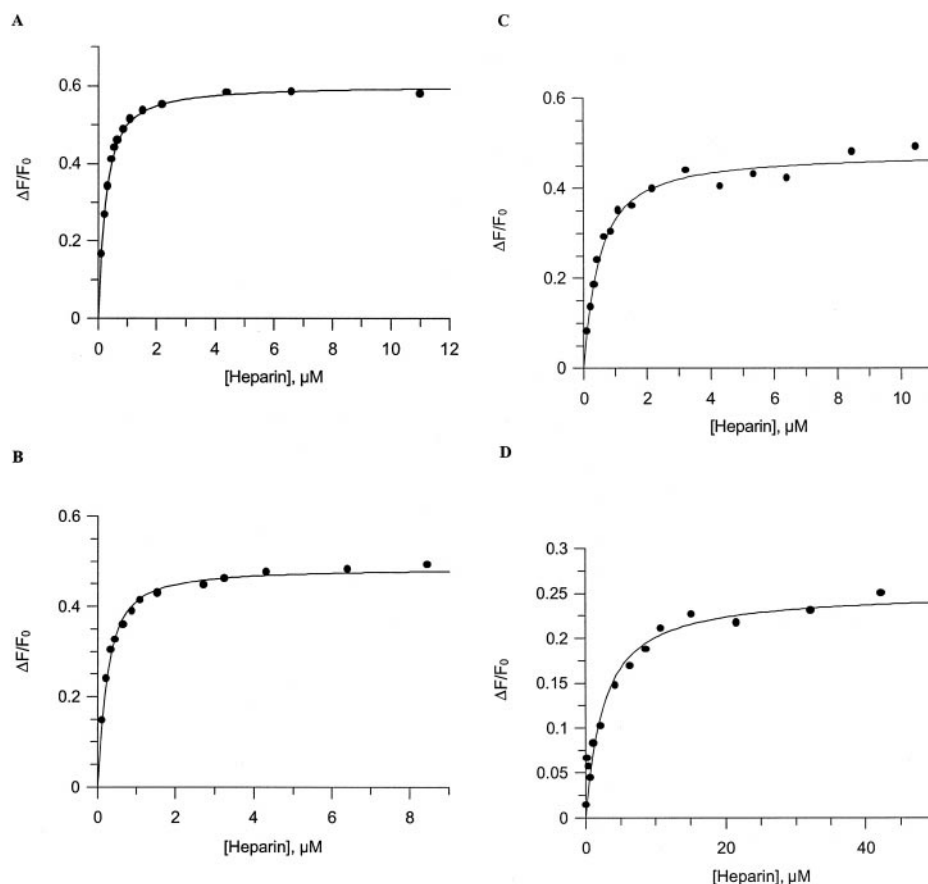


TABLE III

Dissociation constant ( $K_d$ ) determination for kallistatin and the mutants with heparin

$K_d$  was measured in 50 mM Tris-HCl, 100 mM NaCl, 0.2% (w/v) PEG 6000, pH 7.4, at 20 °C. The change in intrinsic fluorescence of kallistatins accompanying heparin binding was fit by the binding equation (Eq. 1) to obtain  $K_d$ .

Protein	Protein concentration	$K_d$
		$\mu\text{M}$
Kallistatin (wild type)	0.116	$0.185 \pm 0.008$
Kallistatin P1Arg	0.170	$0.158 \pm 0.009$
K307A/R308A	0.160	$0.402 \pm 0.030$
K312A/K313A	0.141	$2.549 \pm 0.050$

ability to suppress tissue kallikrein's activity.

**The Inhibitory Activity of the Kallistatin Mutants toward Tissue Kallikrein in the Presence of Heparin**—The inhibition of tissue kallikrein activity by kallistatin mutants can be quantified by measuring the residual amidolytic activities of tissue kallikrein following incubation with kallistatin. The results are shown in Fig. 9. After 90-min incubation, the enzymatic activity of tissue kallikrein was almost completely inhibited by all of the kallistatin mutants in the absence of heparin. Preincubation with heparin at 5 units/ml suppressed the inhibitory activity of kallistatin P1Arg and K187A/K188A and completely restored the tissue kallikrein activity. K307A/R308A and K312A/K313A mutants require a higher concentration of heparin to suppress their inhibitory activity toward tissue kallikrein. For K307A/R308A, preincubation with 5 units/ml heparin caused approximately 50% restoration of the tissue kallikrein activity. The tissue kallikrein activity was restored gradually to about 85% when 30 units/ml heparin was used. K312A/K313A, however, appeared to be more resistant to the inhibition by heparin. Even at 30 units/ml heparin, K312A/

K313A still inhibited about 70% of tissue kallikrein after the 90-min reaction. These results indicate that Lys<sup>312</sup>-Lys<sup>313</sup> acts as a major heparin-binding site responsible for the inhibitory effect of heparin on tissue kallikrein activity.

**Estimation of the Conformation of the Kallistatin Mutants**—The conformation of kallistatin mutants, P1Arg, K307A/R308A, and K312A/K313A, were analyzed by CD spectroscopy. The spectra showed that the wavelength-scanning profiles of K307A/R308A and K312A/K313A were identical to that of the P1Arg mutant (Fig. 10). The results indicate that the double Ala substitutions for the basic residues do not perturb the secondary structure of kallistatin and that these mutants retain the same structural integrity. The data demonstrate that the reduced heparin affinity is not due to a conformational change.

## DISCUSSION

In this study we identified the location of a major heparin-binding site in the kallistatin molecule by peptide competition assay and by site-directed mutagenesis and protein engineering. Two putative heparin-binding regions, the F helix and a region between the H helix and C2 sheet, were identified by a sequence analysis program and a molecular model of kallistatin. The HC2 peptide derived from the region between the H helix and C2 sheet displays a strong and specific heparin binding activity in competition assay. Mutagenesis at the basic residues of the kallistatin molecule by double Ala substitutions showed that K307A/R308A and K312A/K313A mutants, containing mutations in the region between the H helix and C2 sheet, had reduced heparin binding activity and were relatively resistant to the inhibition by heparin for tissue kallikrein binding. The K187A/K188A mutant, containing Ala substitutions in the F helix, retained the same heparin binding activity as kallistatin, and both of their inhibitory activities were suppressed by heparin. These results indicate that the major he-

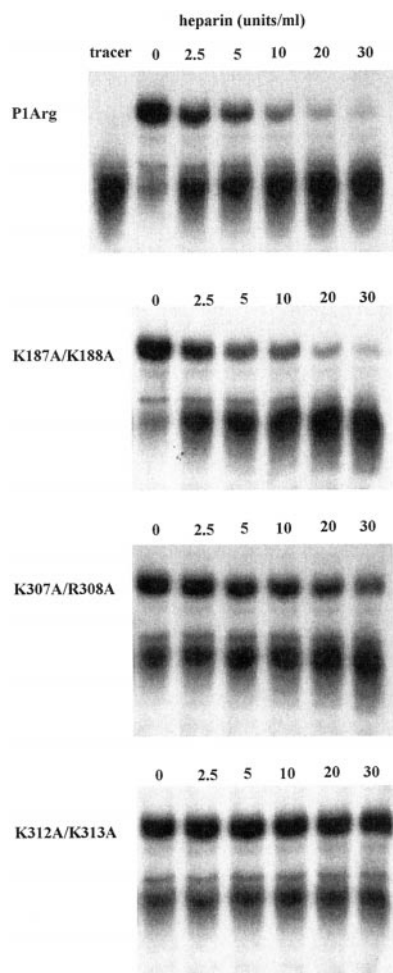


FIG. 8. Effects of heparin on the tissue kallikrein binding activity of the kallistatin mutants. Tissue kallikrein binding assays for the kallistatin mutants were performed in the presence of heparin. Different concentrations of heparin, 0, 2.5, 5, 10, 20, and 30 units/ml, were preincubated with  $0.6 \mu\text{M}$  kallistatin P1Arg, K187A/K188A, K307A/R308A, and K312A/K313A, respectively, in 20 mM sodium phosphate buffer, pH 8.0, at  $37^\circ\text{C}$  for 10 min. The reaction mixtures were then incubated with  $1.5 \times 10^4$  cpm of  $^{125}\text{I}$ -labeled tissue kallikrein for an additional 90 min. The results of the tissue kallikrein-binding assay for the individual mutants are shown as indicated.

parin-binding site responsible for the inhibitory effect of heparin is located in the region between the H helix and C2 sheet of kallistatin.

Heparin is a glycosaminoglycan that is a highly negatively charged molecule. The heparin-binding sites of proteins usually consist of consecutive basic amino acids that can bind to the acidic groups of heparin by electrostatic interaction (17). Two regions in kallistatin encompass clusters of basic amino acids: residues 174–186, DHVKKETRGKIVD in the F helix, and residues 303–316, NLLRKRNFYKKLEL corresponding to the region between the H helix and C2 sheet. In a peptide competition assay, the concentration of HC2 peptide required was less than 1/10 of that of the F peptide to show competition with kallistatin for heparin binding, suggesting the region between the H helix and C2 sheet has a strong heparin binding activity. The  $K_{d\text{-app}}$  values for peptide HC2 and its scrambled peptide SCR are separated by almost a factor of 10 (0.395 and  $2.574 \mu\text{M}$  for HC2 and SCR, respectively). This indicates that the specific heparin binding of HC2 peptide is contributed not only to the positively charged residues, but also to a specific structure and residue composition.

Although the peptide competition assay showed the heparin-binding capability of both regions, the results did not provide

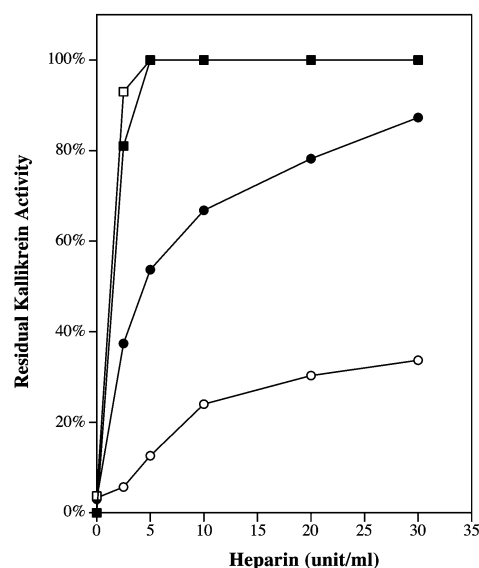


FIG. 9. Effects of heparin on the inhibitory activity of the kallistatin mutants toward tissue kallikrein. The kallistatin mutants, P1Arg (■), K187A/K188A (□), K307A/R308A (●), and K312A/K313A (○), at  $0.2 \mu\text{M}$  were preincubated with 0, 2, 5, 10, 20, and 30 units/ml heparin in 20 mM sodium phosphate buffer, pH 8.0, at  $37^\circ\text{C}$  for 10 min. The mixtures were then incubated with 6 nM tissue kallikrein at  $37^\circ\text{C}$  for 90 min. The remaining tissue kallikrein activity was measured by D-Val-Leu-Arg-MCA hydrolysis.

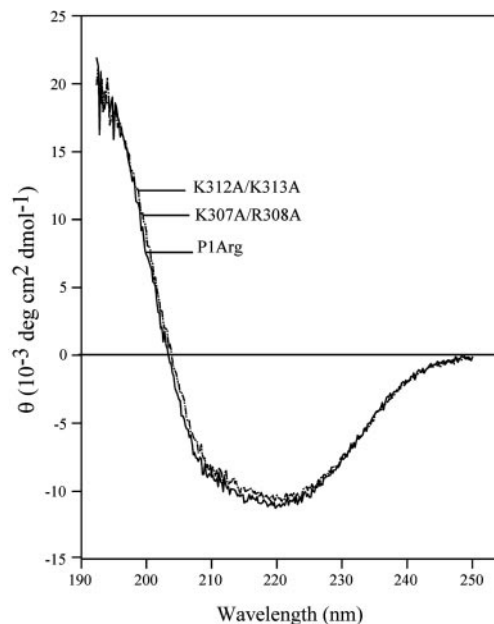


FIG. 10. CD spectra of kallistatin P1Arg, K307A/R308A, and K312A/K313A. The wavelength scanning applied on the CD analysis was from 190 to 250 nm.

direct relevance of those basic clusters to the heparin binding activity of kallistatin. There are three consecutive basic residues, Lys<sup>187</sup>-Lys<sup>188</sup>, Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup>, and Lys<sup>312</sup>-Lys<sup>313</sup>, distributed in both regions in kallistatin. To further address the importance of these basic clusters in heparin binding, double Ala substitution mutants, K187A/K188A, K307A/R308A, and K312A/K313A, were created to evaluate their heparin binding activity.

As assessed by heparin-affinity chromatography, the K187A/K188A mutant retained the same heparin-affinity capacity as P1Arg kallistatin. Moreover, the K187A/K188A mutant exhibited the same extent of heparin-suppressed effect on tissue kallikrein inhibition as kallistatin P1Arg. These results indi-

cate that Lys<sup>187</sup>-Lys<sup>188</sup> of kallistatin does not play a significant role in heparin binding. The lack of the heparin binding activity in the F helix can be explained by the scattering of three acidic residues, Asp<sup>184</sup>, Glu<sup>189</sup>, and Asp<sup>196</sup>, in this region that neutralizes the net positive charge of the F helix.

Consistent with the results of the competition assay, the relative heparin affinity of K307A/R308A and K312A/K313A mutants decreased as estimated by heparin-affinity chromatography, indicating that the basic residues, Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup> and Lys<sup>312</sup>-Lys<sup>313</sup>, are responsible for heparin binding. Although Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup> has higher positive-charge density than Lys<sup>312</sup>-Lys<sup>313</sup>, the significantly lower dissociation constant of the K312A/K313A mutant indicates that Lys<sup>312</sup>-Lys<sup>313</sup> are crucial heparin-binding residues of kallistatin.

Studies of the heparin-binding sites of serpins have shown that several basic residues in different regions coordinate for heparin binding but not all the heparin-binding sites are related to heparin-accelerated inhibition of proteinases (11–12, 21). Therefore, it is possible that regions other than the loop between the H helix and C2 sheet in kallistatin could also be involved in heparin binding. Nevertheless, the heparin-suppressed tissue kallikrein-binding assays showed resistance of both K307A/R308A and K312A/K313A to heparin suppression, indicating that the basic residues in the loop between the H helix and C2 sheet comprise not only a heparin-binding region but also are related to heparin-suppressed tissue kallikrein inhibition. Additionally, the CD spectra of the mutants showed that the double mutagenesis did not perturb the secondary structure of kallistatin, demonstrating that the reduced heparin affinity and resistance to heparin suppression are not due to a conformational change. Compared with Lys<sup>307</sup>-Arg<sup>308</sup>, Lys<sup>312</sup>-Lys<sup>313</sup> appeared to contribute more in heparin binding, because mutant K312A/K313A displayed less relative heparin affinity than mutant K307A/R308A. In agreement with the heparin affinity data, the inhibitory activity of K312A/K313A toward tissue kallikrein displayed a lower sensitivity to heparin suppression as compared with that of K307A/R308A toward tissue kallikrein in the heparin-suppressed inhibition assay. Taken together, our study indicates that Lys<sup>312</sup>-Lys<sup>313</sup> in kallistatin is a major heparin-binding site that is responsible for heparin-suppressed tissue kallikrein inhibition.

The heparin-binding sites of several serpins, such as antithrombin, heparin cofactor II, protease nexin I, and plasminogen activator inhibitor I, have been localized primarily in the D helix (6, 11, 12, 18–20). Protein C inhibitor, however, has a distinctive heparin-binding site located in the H helix (21). Kallistatin does not contain a high density of basic residues nor a conserved distribution of basic residues in the D helix as analyzed by amino acid sequence alignment with these serpins (Fig. 11). The heparin-binding site in kallistatin is in the region between the H helix and C2 sheet, which is adjacent to the H helix. In this region, kallistatin has a greater basic charge density than other serpins. Molecular modeling of kallistatin illustrates that Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup> is located at the C-terminal end of the H helix and Lys<sup>312</sup>-Lys<sup>313</sup> at the N-terminal end of the C2 sheet. The heparin-binding residues, Arg<sup>306</sup>-Lys<sup>307</sup>-Arg<sup>308</sup>, in kallistatin are conserved with the basic residues, 276–278, in the C-terminal boundary of the H helix in protein C inhibitor (Fig. 7). These conserved basic residues in protein C inhibitor are not related to heparin-accelerated proteinase inhibition, although they can bind heparin strongly (21). The heparin-binding residues Arg<sup>269</sup>-Lys<sup>270</sup>, responsible for heparin-accelerated proteinase inhibition, in the H helix in protein C inhibitor, however, is absent in kallistatin. Instead, kallistatin has the major heparin-binding site Lys<sup>312</sup>-Lys<sup>313</sup> at the N-terminal boundary of the C2 sheet, which is unique among

#### The D helix

	-----hd-----
HKS	L...SESDVHRGPFQHLHLTLNLP
PCI	S...SEKELHRGFGQLLQELNQR
ATIII	K...TSDQIHFFAKLNCRLYRKA
HCII	ASSKYEITTIHNLFRKLTHTLFRN
PN-1	G...VGKILKKINKAIVSKKKNK..
PAI-1	I...DDKGMAPALRHLYKELMGPW
ACT	T...SEAEIHQSPQHLRLTLNQSS
AT	I...PEAQIHGEGFQELRLTLNQPD

#### The region between the H helix and the C2 sheet

	---hH---	---s2C---
HKS	VLTPEMLMRWNNLLRKRNFYKLELHLPKF	
PCI	GLSEKTLRKLWLMFKKR...QLBLYLPKF	
ATIII	ELTPEVLQEWLDELEEM...MLVVHMPRF	
HCII	QLTPRVVERWQKSMNTR...TREVLLPKF	
PN-1	HISTKTIDSWMSIMVPK...RVQVILPKF	
PAI-1	ILSAQLISHWKGNMTRL...PRLVLPKF	
ACT	MLLPETLKRWRDLSLEFRE...TGELYLPKF	
AT	ELTHDIITKFLNEDRR...SASLHLPKL	

FIG. 11. Amino acid sequence alignment of the heparin-binding sites in serpins. The sequences of the D helix and the region between the H helix and C2 sheet of kallistatin (HKS), protein C inhibitor (PCI), antithrombin (ATIII), heparin cofactor II (HCII), protease nexin I (PN-I), and plasminogen activator I (PAI-I) are aligned with the sequences of antitrypsin (AT) and antichymotrypsin (ACT), which are non-heparin-binding serpins. Basic residues are double-underlined.

serpins (Fig. 11). The turn between the H helix and C2 sheet in kallistatin has a 3–4 residue insertion as compared with those in other serpins (Fig. 11). This insertion extends C2 sheet and bulges the loop outward according to the kallistatin model. Lys<sup>312</sup>-Lys<sup>313</sup> thus protrudes from the turn and is positioned toward the reactive-center loop of kallistatin. At this position, binding of heparin may easily affect the conformation of the reactive-center loop or docking of the loop into a reactive cleft of a serine proteinase.

Our results showed that the heparin affinity of kallistatin is not as high as other heparin-binding serpins. Nevertheless, the  $K_d$  value (0.185  $\mu\text{M}$ ) for kallistatin with heparin are only two times higher than the  $K_d$  (0.084  $\mu\text{M}$ ) described for heparin-antithrombin interaction in a solution with similar ionic strength (28), suggesting that kallistatin affinity for heparin is physiologically significant. Our previous study has shown that heparin affects the inhibitory activity of kallistatin differently, depending on the target proteinase (1). Heparin suppresses the inhibition toward plasma kallikrein and human and rat tissue kallikrein, whereas it accelerates the inhibition toward thrombin, activated protein C, and chymotrypsin by 5- to 50-fold (1). Similarly, heparin promotes the inhibitory activity of protein C inhibitor by about 30-fold toward thrombin and activated protein C, but inhibits the inhibitory activity toward human tissue kallikrein (21, 29).

The mechanisms of the heparin-accelerating proteinase inhibition have been explained by: (a) allosteric effects in which heparin induces conformational changes favorable for the binding of serpins with proteinases (10–13), and (b) a ternary complex model in which heparin acts as a template to bind serpin and proteinase and thus enhancing the association of both molecules (5, 6). Compared with kallistatin and protein C inhibitor, the serpins with heparin-binding sites in the D helix have greater inhibition enhancement (>1000-fold) upon heparin binding (6). It has been speculated (6) that the difference in rate enhancement can be accounted for by heparin binding through the H helix, where it positions the serpins in a less favorable orientation for reacting with the heparin-bound proteinases than when heparin binds through the D helix. The

location of kallistatin's heparin binding region may also explain the modest acceleration response to heparin by the same speculation, because the heparin binding region is adjacent to the H helix according to the molecular model of kallistatin.

The mechanism of the heparin-suppressed proteinase inhibition of kallistatin and protein C inhibitor is still not well defined. A study has speculated that the heparin-suppressed effect of protein C inhibitor is caused by an allosteric effect or steric hindrance upon heparin binding, although direct evidence is absent (30). While analyzing the reaction of kallistatin and tissue kallikrein in the presence of heparin by SDS-PAGE, we noticed that the cleaved form of kallistatin did not increase and the formation of kallistatin-kallikrein complex was still suppressed (data not shown). This suggests that heparin does not convert kallistatin to a substrate for tissue kallikrein. Therefore, the heparin suppression effect could be caused by slowing down or blockage of the association of kallistatin with kallikrein. The region between the H helix and C2 sheet in kallistatin stretches through the reactive-center loop that places the heparin-binding residues in proximity to the reactive-center loop as shown in the molecular model of kallistatin (Fig. 1). The proximity between the heparin-binding sites and the reactive-center loop suggests possible mechanisms for heparin-suppressed proteinase inhibition. First, binding of heparin to these sites may generate steric hindrance that obstructs the insertion of the reactive-center loop into the reactive cleft of tissue kallikrein. Second, heparin binding may induce a conformational change of the reactive-center loop that reduces the inhibitory activity of kallistatin. Third, because the electrostatic interactions between the basic residues in the heparin-binding sites and the acidic residues around the reactive cleft of tissue kallikrein may efficiently position the reactive-center loop of kallistatin into the reactive cleft, heparin could mask the basic residues and thus interfere with the enzyme-inhibitor interaction.

In summary, we have shown that the region between the H helix and C2 sheet in kallistatin is a major heparin-binding domain related to heparin-suppressed tissue kallikrein inhibition. The basic amino acids Lys<sup>312</sup>-Lys<sup>313</sup> in this region are the heparin-binding residues that are significantly resistant to the heparin-suppressed effect. The identification of the heparin-

binding sites in kallistatin is critical for further investigation of the physiological function and regulation of kallistatin.

*Acknowledgment*—We thank Dr. Erika Büllesback for technical support on circular dichroism spectroscopy work.

#### REFERENCES

- Chen, V. C., Chao, L., and Chao, J. (2001) *J. Biol. Chem.* **276**, (in press)
- Zhou, G. X., Chao, L., and Chao, J. (1992) *J. Biol. Chem.* **267**, 25873–25880
- Chen, L. M., Chao, L., and Chao, J. (1997) *Hum. Gene Ther.* **8**, 341–341
- Chao, J., Stallone, J. N., Liang, Y. M., Chen L. M., Wang, D. Z., and Chao, L. (1997) *J. Clin. Invest.* **100**, 11–17
- Griffith, M. J. (1982) *J. Biol. Chem.* **257**, 7360–7365
- Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) *J. Biol. Chem.* **267**, 8795–8801
- Pratt, C. W., and Church, F. C. (1992) *J. Biol. Chem.* **267**, 8789–8794
- Tollefsen, D. M., Pestka, C. A., and Monafu, W. J. (1983) *J. Biol. Chem.* **258**, 6713–6716
- Van Deerlin, V. M. D., and Tollefsen, D. M. (1991) *J. Biol. Chem.* **266**, 20223–20231
- Owen, B. A., and Owen, W. G. (1990) *Biochemistry* **29**, 9412–9417
- Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14683–14688
- Ersdal-Badju, E., Lu, A., Zuo, Y., Picard, V., and Bock, S. C. (1997) *J. Biol. Chem.* **272**, 19393–19400
- Evans, D. L., Marshall, C. J., Christey, P. B., and Carrell, R. W. (1992) *Biochemistry* **31**, 12629–12642
- Cooper, S. T., Neese, L. L., Dieuccio, M. N., Liles, D. K., Hoffman, M., and Church, F. C. (1996) *Clin. Appl. Thrombosis/Hemostasis* **2**, 185–191
- Priglinger, U., Geiger, M., Bielek, E., Vanyek, E., and Binder, B. R. (1994) *J. Biol. Chem.* **269**, 14705–14710
- Shirk, R. A., Church, F. C., and Wagner, W. D. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 1138–1146
- Cardin, A. D., and Weintraub, H. J. R. (1989) *Arteriosclerosis* **9**, 21–32
- Ehrlich, H. J., Gebbink, R. K., Keijer, J., and Pannekoek, H. (1992) *J. Biol. Chem.* **267**, 11606–11611
- Stone, S. R., Brown-Luedi, M. L., Rovelli, G., Guidolin, A., McGlynn, E., and Monard, D. (1994) *Biochemistry* **33**, 7731–7735
- Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., and Church, F. C. (1991) *J. Biol. Chem.* **266**, 8129–8135
- Shirk, R. A., Elisen, M. G. L. M., Meijers, J. C. M., and Church, F. C. (1994) *J. Biol. Chem.* **269**, 28690–28695
- Shimamoto, K., Chao, J., and Margolius, H. S. (1980) *J. Clin. Endocrinol. Metab.* **51**, 840–848
- Chen, V. C., Chao, L., and Chao, J. (2000) *Biochim. Biophys. Acta* **1479**, 237–246
- Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1989) *Nucleic Acids Res.* **17**, 723–733
- Olson, S. T., Björk, I., and Shore, J. D. (1993) *Methods Enzymol.* **222**, 525–559
- Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1972) *Methods Enzymol.* **27**, 675–735
- Wei, A., Rubin, H., Cooperman, B. S., and Christianson, D. W. (1994) *Nature Struct. Biol.* **1**, 251–258
- Belzar, K. J., Dafforn, T. R., Petitou, M., Carrell, R. W., and Huntington, J. A. (2000) *J. Biol. Chem.* **275**, 8733–8741
- Ecke, S., Geiger, M., Resch, I., Jerabek, I., Stingl, L., Maier, M., and Binder, B. R. (1992) *J. Biol. Chem.* **267**, 7048–7052
- Sonja, E., Geiger, M., and Binder, B. R. (1997) *Eur. J. Biochem.* **248**, 475–480